Plant Science Research 34 (1&2) : 1-09, 2012

Plant Science Research

ISSN 0972-8546

Analysis of genetic variability and phylogenetic relationships among the species of *Vigna* Savi (Fabaceae) using molecular markers

P. K. Acharya[1], A. K. Mukherjee[2] and P. C. Panda[1Ψ]

[1] Taxonomy & Conservation Division, Regional Plant Resource Centre, Bhubaneswar 751 015, Odisha, India

[2] Central Institute for Cotton Research, Nagpur 440010, Maharashtra, India

ARTICLE INFO

Article history:
Received : 14 November 2012
Received in revised form : 01 December 2012
Accepted : 01 December 2012

Keywords:
genetic diversity
phylogeny
Vigna species
RAPD
ISSR

ABSTRACT

Genetic diversity and species relationships among 9 species of *Vigna* (*V. aconitifolia, V. adenantha, V. mungo, V. pilosa, V. radiata, V. sublobata, V. trilobata, V. umbellata and V. unguiculata*) with 29 accessions collected from Odisha, Andhra Pradesh and Assam were analysed using 11 RAPD and 16 ISSR markers. A total of 2426 fragments were amplified with both the primer sets, of which 2368 bands were of polymorphic nature. Eighteen unique bands and 58 monomorphic bands were also detected. In the present study, clustering of species in the dendrograms constructed using RAPD, ISSR and combined data was not in complete agreement with the intra-generic classification of Verdcourt (1970) as modified by Marechal *et al.* (1978) but certain species followed the pattern of species placement stated there in. *Vigna sublobata*, considered as a variety of *V. radiata* by many is established here as a distinct species. The phylogenetic tree generated from combined RAPD and ISSR data revealed segregation of taxa into two distinct clusters, one with *Vigna radiata-Vigna mungo* belonging to the sect. Ceratotropis of the subgenus Ceratotropis along with *V. unguiculata* of the sect. Vigna of subgenus Vigna; and the other with members of sect. Leptospron of subgenus Sigmoidotropis and sect. Angulares and sect. Aconitifoliae of the subgenus Ceratotropis. The necessity to reinvestigate the sectional classification of the sub-genus Ceratotropis of genus *Vigna* has been suggested.

1. Introduction

The legume tribe Phaseoleae (*sensu* Polhill *et al.,* 1981) is the largest Papilionoideae tribe with about 84 genera and 1500 species and economically the most important group containing genera like *Phaseolus, Vigna, Glycine, Cajanus, Clitoria, Macrotyloma, Lablab, Pachyrhizus, Pueraria* and *Psophocarpus*. The genus *Vigna* is comprised of 82 species distributed among 7 subgenera namely, Vigna, Haydonia, Plectotropis, Ceratotropis, Lasiospron, Sigmoidotropis and Macrorhyncha (Verdcourt, 1970; Maréchal *et al*., 1978). The subgenus Ceratotropis consists of 16-17 species including *V. mungo, V. radiata, V. trilobata, V. aconitifolia, V. umbellata*, which are distributed across Asia and constitute an economically important group of cultivated and wild species with rich diversity in India (Arora, 1985; Babu *et al*., 1985). Based on cross compatibility studies and seedling characteristics, Tateishi (1996) recognised three isolated genepools in Asian *Vigna i. e. angularis-umbellata* (azuki bean group), *radiata-mungo* (mungbean group) and *aconitifoila-trilobata* (mothbean group), which was supported by isozyme and molecular studies (Jaaska and Jaaska, 1990; Kaga *et al*., 1996. Tomooka *et al*. (2000) proposed a revised list of taxa in the subgenus Ceratotropis and suggested three groups, giving them taxonomic rank as section *Angulares* (azuki bean group), *Radiateae* (mungbean group) and *Aconitifoliae* (mothbean group) and an undetermined section. Subgenus *Vigna* is by far the most species-rich subgenus, most of which are endemic to Africa including *V. unguiculata* and *V. subterranea*. *Vigna. adenantha* comes under the subgenus Sigmoidotropis, which is of Neotropical origin and is considered primitive.

The genetic diversity and molecular phylogeny of different species of *Vigna* has been studied by a number of workers in many parts of the world but with more emphasis on

Ψ *Corresponding author; Email:*pcpanda2001@yahoo.co.in

cultivated species like mung bean, azuki bean, cowpea and moth bean (Kaga *et al*., 1996; Tomooka *et al*., 2002; Goel *et al*., 2002; Souframanien and Gopalakrishna, 2004; Bisht *et al*., 2005; Seehalak *et al*., 2006; Simon *et al*., 2007; Vir *et al*., 2010; Tantasawat *et al*., 2010; Javadi *et al*., 2011). Only a few of them have analysed the genetic diversity and phylogeny of wild *Vigna* species along with accessions of cultivated crops. In the present study the genetic diversity and phylogenetic relationship among 9 species and 29 accessions of *Vigna* have been studied using 11 RAPD and 16 ISSR primers.

2. Materials and methods

2.1 Plant materials

The viable seed samples of 29 accessions belonging to 9 species of *Vigna* (*V. aconitifolia, V. adenantha, V. mungo, V. pilosa, V. radiata, V. sublobata, V. trilobata, V. umbellata and V. unguiculata*) were collected from forests and institutions of Odisha, Andhra Pradesh and Assam. The accession number, locality of collection and abbreviation used for each of the taxon is shown in Table 1. The seed materials were geminated in pro-trays under greenhouse conditions at Regional Plant Resource Centre, Bhubaneswar and the tender leaves were used for DNA extraction for molecular analyses. The herbarium specimens have been deposited in the herbarium of RPRC, Bhubaneswar Odisha (India).

2.2 Isolation, purification and quantification of genomic DNA

Genomic DNA was extracted from the leaf tissues using the modified CTAB (cetyl-trimethyl-ammonium-bromide) protocol (Doyle and Doyle, 1990) with little modification. Two grams of leaf tissues from young seedlings were ground with extraction buffer composed of 100 mM sodium acetate (pH 4.8), 500 mM NaCl, 20 mM EDTA (pH 8.0); 100 mM Tris (pH 8.0); 2% Polyvinyl pyrollidone (PVP) and 2% CTAB. The precipitated DNA was washed twice with 70% ethanol, stored in micro centrifuge tube and dried in DNA-mini vacuum dryer (DNA Mini, Germany). The dried DNA was dissolved in excess amount of $T_{10}E_1$ buffer (Tris-Cl 10mM, EDTA 1mM pH 8).

The RNA was removed by the standard technique of RNase-A treatment, incubation, washing with chloroform: isoamyl alcohol (24:1) and centrifugation. The dried DNA

Table 1
Details of the plant samples used for the study

Sl. No.	Species	No of accessions studied	Place(s) of collection	Code used
1	*Vigna trilobata*	4	Bhubaneswar Khurda Kandhamal	Vt BBS1 &2 Vt KUR Vt KML
2	*Vigna sublobata*	5	Khurda Nayagarh Kalahandi Kandhamal	Vs KUR Vs NGD Vs KND Vs KML1&2
3	*Vigna umbellata*	2	Kandhamal Nayagarh	Vu KML Vu NGD
4	*Vigna radiata*	4	Ganjam Bhubaneswar Nayagarh	Vrad GN Vrad BBS Vrad NGD 1&2
5	*Vigna mungo*	7	Ganjam Silchar, Assam Bhubaneswar Nayagarh	Vmun GN1&2 Vmun ASM Vmun BBS Vmun NGD1,2&3
6	*Vigna pilosa*	1	Khurda	Vpil
7	*Vigna unguiculata*	2	Nayagarh	Vung LONG Vung SHORT
8	*Vigna aconitifolia*	2	Anantapur, Andhra Pradesh	Va1, Va2
9	*Vigna adenantha*	2	Bhubaneswar	Vad1, Vad2

was dissolved in minimum amount of $T_{10}E_1$ buffer (pH-8.0). The quality and quantity of DNA was measured by UV-VIS Spectrophotometer (Bio Photometer, Eppendorf, Germany). For final checking of the quality as well as quantity of DNA, the DNA was loaded in 0.8% agarose gel stained with Ethidium Bromide (0.5µg/ml) and one side diluted uncut ë DNA as standard and electrophoresed. After quantification, the DNA was diluted with $T_{10}E_1$ buffer to a working concentration of 100 ng/ml. Again the DNA was loaded in 0.8% agarose gel stained with Ethidium Bromide (0.5µg/ml) and electrophoresed to see the uniformity of concentration in all the samples. Finally, it was diluted to 25ng/µl with $T_{10}E_1$ buffer and the DNA samples were stored at -20ºC in a deep freezer for PCR analysis.

2.3 Random amplified polymorphic DNA (RAPD) analysis

Prior to polymerase chain reaction (PCR), random decamer primers (Operon Technologies, Alameda, USA) were dissolved in double sterilized $T_{10}E_1$ buffer (pH 8.0) to the working concentration of 15 ng/ml. Twenty best selected primers as per the reproducibility and amplification pattern from A, D, N, P, S, T and AF series (Operon Technologies, Alameda, CA.) were selected for RAPD analysis (Table 2). The RAPD analysis was performed as per the methodology described by Williams *et al.* (1990). Each amplification reaction mixture of 25 ml volume contained 2.5 ml of 10X assay buffer (100 mM Tris-Cl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.1% gelatin), 200 mM of each dNTPs (dATP, dCTP, dGTP and dTTP) (MBI Fermantas, Lithuania) 15 ng of primer, 0.5 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India) and 25 ng of template DNA. The amplification reaction was carried out in GeneAmp PCR System 9700 (Applied Biosystems, Germany). The amplification was performed in three steps PCR. Initial denaturation of the template DNA was carried out at 94 ^{0}C for 5 min for one cycle. The second step was carried out for 42 cycles and each cycle consisting of three temperature steps i.e. one min at 92 ^{0}C for denaturation of template, one min at 37^{0}C for primer annealing followed by two min at 72^{0}C for primer extension. The final step consisted of only one cycle i.e. 7 min at 72 ^{0}C for complete polymerization. The soak temperature was 4^{0}C. After the completion of the PCR 2.5 ml of 6X loading dye (MBI Fermentas, Lithuania) was added to the amplified products and were stored at –20^{0}C till further use.

2.4 Inter simple sequence repeat (ISSR) analysis

For the present investigation twenty (20) anchored and non-anchored microsatellites were used as primers. These simple sequence repeats were synthesized and procured from Genei (Bangalore Genei Pvt. Ltd, Bangalore, India). The details of their sequence, base pairs and codes used are given in the Table 3. The ISSR analysis was performed as per the methodology given by Zietkiewicz *et al.* (1994). Each amplification reaction mixture of 25 ml contained 20ng of template DNA, 2.5ml of 10X assay buffer (100mM Tris-HCl pH 8.3, 0.5M KCl and 0.01% gelatin), 1.5mM $MgCl_2$, 200mm each of dNTPs, 44ng of primer and

Table 2
Details of RAPD primers used and bands amplified

Primer Name	Sequence	Total no. of Bands	No. of Polymorphic Bands	No. of Monomorphic Bands	No. of Unique Bands	% of Polymorphic Bands (PPB)	Resolving Power	Primer Index
OPA 02	5'TGCCGAGCTG3'	48	48	0	2	100	3.3	2.00
OPD 20	5'ACCCGGTCAC3'	86	86	0	0	100	5.93	2.95
OPN 02	5'ACCAGGGGCA3'	66	66	0	0	100	4.55	2.32
OPA 03	5'AGTCAGCCAC3'	82	82	0	3	100	5.65	2.93
OPN 06	5'GAGACGCACA3'	80	80	0	1	100	5.51	3.29
OPN 11	5'TCGCCGCAAA3'	66	66	0	2	100	4.55	3.02
OPN 12	5'CACAGACACC3'	119	119	0	0	100	8.20	4.38
OPN 16	5'AAGCGACCTG3'	63	63	0	3	100	4.34	2.58
OPA 10	5'GTGATCGCAG3'	59	59	0	1	100	4.06	1.14
OPA 18	5'AGGTGACCGT3'	91	91	0	1	100	6.27	3.82
OPN 04	5'GACCGACCCA3'	34	5	29	0	14.70588	2.34	0.28
TOTAL		794	765	29	13	96.34761	54.75	28.74

Table 3
Details of ISSR primers used and bands amplified

Primer Name	Sequence	Total no. of Bands	No. of Polymorphic Bands	No. of Monomorphic Bands	No. of Unique Bands	% of Polymorphic Bands (PPB)	Resolving Power	Primer Index
AGG6	5'AGGAGGAGGAGGAGGAGG3'	119	119	0	1	100	8.20	2.60
AK 10	5'CTCTCTCTCTCTCTCTA3'	80	80	0	0	100	5.51	3.42
AK 7	5'GTGGTGGTGGTGGTG3'	68	68	0	0	100	4.68	1.47
CCA 5	5'CCACCACCACCACCA3'	89	89	0	0	100	6.13	1.82
GA 10	5'GAGAGAGAGAGAGAGAGAGA3'	121	121	0	0	100	8.34	3.42
GA9T	5'GAGAGAGAGAGAGAGAGAT3'	64	64	0	1	100	4.41	2.26
GA9TA	5'GAGAGAGAGAGAGAGAGATA3'	165	165	0	0	100	11.37	5.51
Oligo 1 a	5'AGAGAGAGAGAGAGAGG3'	87	87	0	1	100	6.00	2.56
Oligo 1 b	5'AGAGAGAGAGAGAGAGC3'	118	118	0	2	100	8.13	2.37
Oligo 2 a	5'AGAGAGAGAGAGAGAG3'	74	74	0	0	100	5.10	2.00
Oligo 2 b	5'GAGAGAGAGAGAGAGAG3'	59	59	0	0	100	4.06	1.92
Oligo 3 b	5'GACAGACAGACAGACA3'	65	65	0	0	100	4.48	2.70
Oligo 5b	5'GACAGACAGACAGACAG3'	184	184	0	0	100	12.68	3.58
Oligo 8A	5'CTCTCTCTCTCTCTCTCTG3'	64	64	0	0	100	4.41	2.80
Oligo 9A	5'GCTCTCTCTCTCTCTCT3'	135	135	0	0	100	9.31	4.49
TGA 9	5'TGAGAGAGAGAGAGAGAGA3'	140	111	29	0	79.28571	9.65	3.53
TOTAL		1632	1603	29	5	98.22304	112.55	46.52

0.5 U Taq DNA polymerase. The amplification was carried out in a PCR system. (GeneAmp PCR System 9700, Applied Biosystems). The first cycle consisted of denaturation of template DNA at 94^0C for 5 min, primer annealing at specific temperature for particular primer (as indicated in the Table 2) for 1 min and primer extension at 72 ^{0}C for 2 min. In the subsequent 42 cycles the period of denaturation was reduced to 1 min while the primer annealing and primer extension time was maintained same as in the first cycle. The last cycle consisted of only primer extension at 72^0C for 7 min. the amplified products were resolved in 2% agarose gel stained with ethidium bromide.

The PCR products for RAPD were separated in 1.4 % agarose gel while those of the ISSR products were resolved in 2 % agarose gel. After electrophoresis, the gel was visualized under the UV-transilluminator (BioRad, USA) and photographed. The gel was also documented in Gel Doc 2000 (BioRad, USA) for scoring the bands.

2.5 Scoring and analysis of data

The data was scored as '1' for the presence and '0' for the absence of the band for each primer genotype combination for RAPD and ISSR (Gherardi *et al.*, 1998). Resolving power of the RAPD was calculated as per Prevost and Wilkinson (1999). Resolving power is: Rp=ΣIB (IB=Band informativeness= 1-[2x(0.5-P)], P is the proportion of the species containing the band. Jaccard's coefficient of similarity (Jaccard, 1908) was measured and a phylogram based on similarity coefficients generated by Unweighted Pair Group Method using Arithmetic averages (UPGMA) (Sneath and Sokal, 1973), and the SAHN (Sequential Agglomerative Hierarchial and Nested) clustering was obtained. The entire analysis was performed using the statistical package NTSYS-pc 2.02e (Rohlf, 2000).

3. Results

3.1 Assessment of genomic relationship by RAPD analysis

Eleven RAPD primers used in the experiment led to amplification of 794 bands. Of these 765 fragments were polymorphic in nature. The pattern of banding is shown in Fig. 1. The details of primers used and bands amplified are given in Table 2. The highest numbers of fragments (119) were amplified by the primer OPN 12 and lowest (34) by the

primers OPN 4. Maximum total number of 42 bands were amplified in *Vigna adnantha* (Vad1) and minimum (17) in *Vigna aconitifolia* (Va1).

The tree generated based on RAPD data placed *Vigna pilosa* in a separate position with only 19% similarity with the rest of the taxa (Fig. 2). The other 28 species and accessions were divided into 2 distinct clusters, the smaller being the two accessions of *Vigna aconitifolia* (Vac1 & Vac2). The larger cluster was bifurcated into two groups of 14 and 12 accessions with a similarity coefficient of 0.24. The clade with 12 taxa contained accessions of *Vigna sublobata, Vigna umbellata, Vigna trilobata* and *Vigna adenantha* and the other had species like *Vigna radiata, Vigna mungo* and *Vigna unguiculata* and other accessions. With the former group, further division to *Vigna sublobata-Vigna umbellata* group and *Vigna trilobata-Vigna adenantha* group, could be noticed and both these clusters shared about 29% genetic similarity. Similarly, in the late cluster of 14 taxa, 2 accessions of *Vigna unguiculata* were the first to come out of the clade and had a similarity of about 28%. Besides, the rest of the accessions segregated to *Vigna radiata* and *Vigna mungo* accessions and both the species had 43% common genomic characters. However, a single accession of *Vigna trilobata* got mingled up with the accessions of *Vigna radiata*.

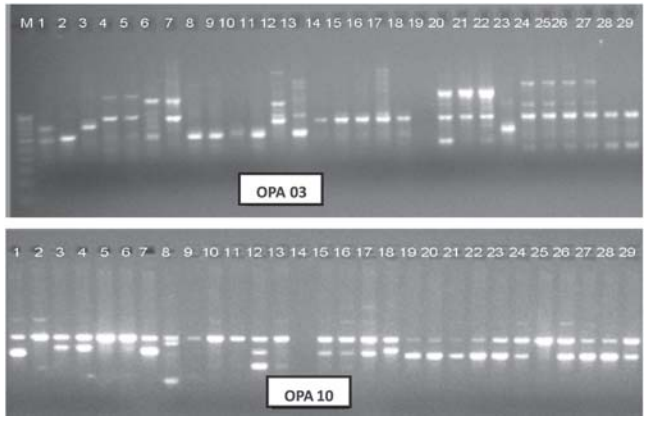

Fig. 1. RAPD banding pattern in different species of *Vigna* with use of the primers OPA03 (LKA 2) and OPA10 (LKA 5). M-marker.

Similarity coefficient calculated on the basis of RAPD data indicated that the accessions of *Vigna radiata* GN and BBS were closely related with a similarity coefficient of 0.933. *Vigna pilosa* and *Vigna sublobata*-KUR showed only 8% similarity and thus distantly located in the dendrogram. All species of the genus *Vigna* accessions within them had an average similarity of 0.434.

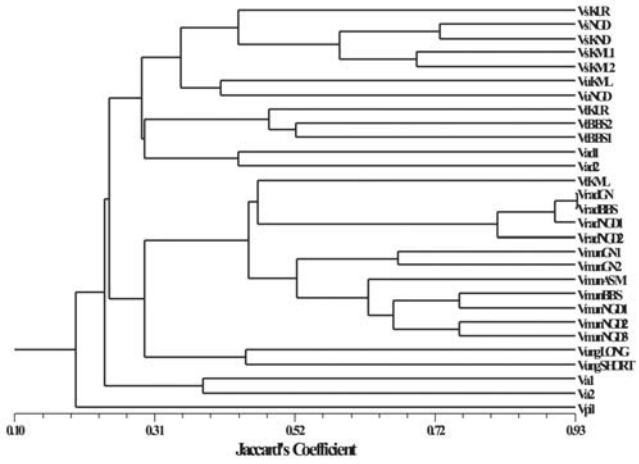

Fig. 2. Dendrogram showing genetic relationship among different species of *Vigna* as revealed from RAPD analysis.

3.2 Genome analysis using ISSR markers

The pattern of banding with the use of 16 selected ISSR primers is presented in Fig. 3. In this case, out of a total of 1632 bands amplified, 1598 bands were polymorphic and 29 monomorhic in nature; only 5 were private ones (Table 3). A maximum of 184 bands were amplified with the primer (GACA)4G and the lowest 59, in case of primer G(AG)8. The highest resolving power (12.689) and Primer Index (5.517) were recorded for the primer (GACA)4G and (GA)9 TA respectively. In *Vigna radiata*-BBS a total of 70 bands were amplified, which was higher among all the species and accessions.

It was noticed that the two subspecies of *Vigna unguiculata* got separated in the cladogram (Fig. 4) in the first place from all other species with a similarity of 32%. The rest 8 species formed two major groups, one with *Vigna radiate, Vigna mungo* and their genotypes and other six species in the second cluster. The smaller cluster had 4 accession of *Vigna radiata* and 7 accessions of *Vigna mungo*, which were closely located justifying their entity as biological species. Most of the accessions of *Vigna radiata* and *V. mungo* from a particular geographical area tended to come together. Within the large group containing 16 accessions of 6 species of *Vigna*, two genotypes of *Vigna aconitifolia* were out-grouped in the dendrogram at a similarity of 37%. After exclusion of *Vigna aconitifolia*, the rest of the taxa were separated into 2 clusters of 7 accessions each; one containing *Vigna sublobata, Vigna umbellata* and *Vigna pilosa* and the other with *Vigna adenantha, Vigna trilobata* and lone genotype of *Vigna umbellata*. *Vigna adenantha* got separated from *Vigna trilobata* group at a similarity level of about 42%. All accessions of *Vigna sublobata* and *Vigna trilobata* formed tight clusters within their respective sub-clusters in the dendrogram.

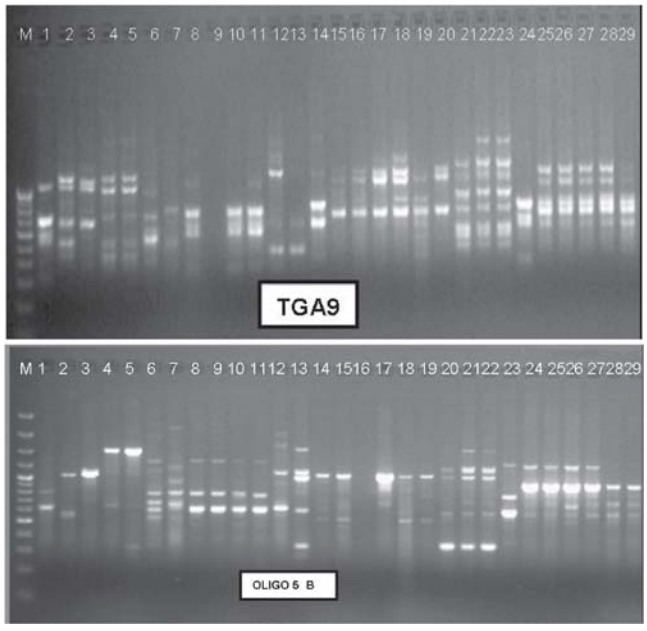

Fig. 3. ISSR banding pattern in species of *Vigna* with the primers TGA9 and Oligo 5 B. M- marker.

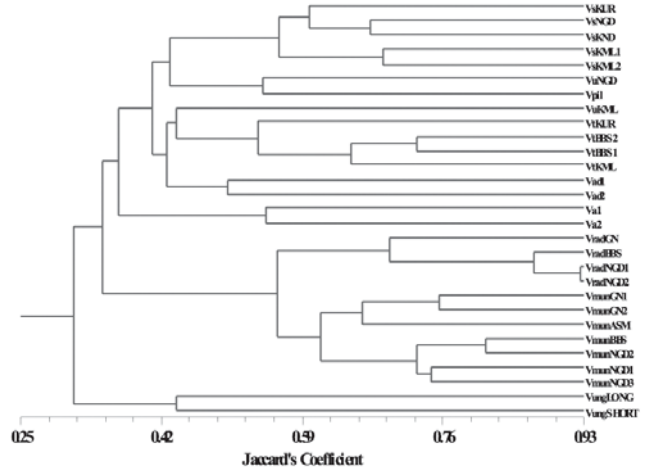

Fig.4. Phylogenetic tree showing genetic relationship among different species of *Vigna* based on ISSR data.

3.3 Analysis of phylogeny using RAPD and ISSR combined markers

A total of 2426 bands were produced with the use of 27 ISSR and RAPD primers, of which 2368 bands were of polymorphic nature. Eighteen unique and 58 monomorphic bands were also detected. *Vigna adenantha* (Vad1) resolved highest number of fragments (109), while *Vigna aconitifolia* (Vac1) produced the least number (63) of bands.

The tree generated from combined RAPD and ISSR markers data (Fig.5) revealed similar type of relationship among the species and accessions as found with ISSR analysis with little deviation. Here, two distinct clusters were observed, one with 16 accessions of *Vigna sublobata, Vigna umbellata, Vigna trilobata, Vigna adenantha, Vigna pilosa* and *Vigna aconitifolia* and the other with 13 accessions of *Vigna radiata, Vigna mungo* and *Vigna unguiculata*. Both the two major clusters had a similarity value of 0.31. Within *Vigna radiata-Vigna mungo-Vigna unguiculata* group, the two subspecies of *Vigna unguiculata* got isolated. Further, 7 genotypes of *Vigna mungo* and 4 genotypes of *Vigna radiata* formed 2 distinct groups sharing a similarity of about 53%.

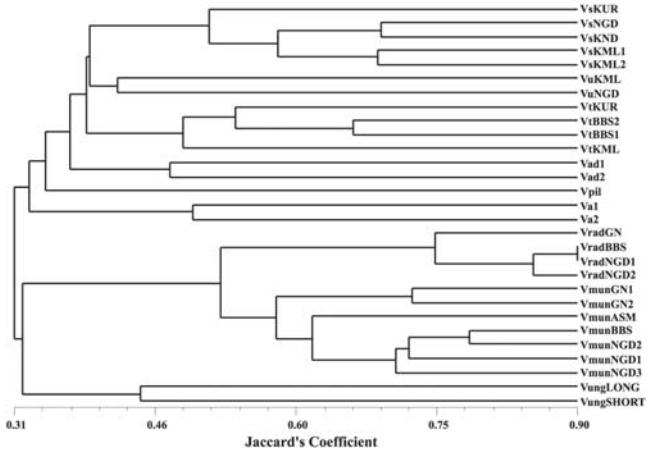

Fig 5. Dendrogram tree showing genetic relationship among *Vigna* species based on combination of RAPD and ISSR analysis.

Among the other group containing 16 accessions of 6 *Vigna* species, *V. aconitifolia* was the first to go as an out-group followed by *Vigna pilosa*. While *Vigna aconitifolia* had a genetic similarity of 33% with other members in the cluster, *Vigna pilosa* was similar by 35%. The clade was further sub-divided and 2 accessions of *Vigna adenantha* occupied a distinct place. Subsequently, accessions of *Vigna trilobata* and *Vigna umbellata* came out of the cluster leaving the 5 accessions of *Vigna sublobata* as a sub-group. *Vigna trilobata* accessions shared a similarity of 39% with *Vigna sublobata-Vigna umbellata* complex. Within each cluster accessions of a particular collection locality had closer genetic affinity among them.

4. Discussion

A total of 2426 fragments were amplified with both the primer sets, of which 2368 bands were of polymorphic nature. Eighteen unique bands and 58 monomorphic bands were also detected. One accession of *Vigna adenantha* was found to be highly polymorphic (109) and least polymorphism (63) was noted in case of a *Vigna aconitifolia*.

The phylogram generated using RAPD data clearly segregated *Vigna pilosa* from all other species and shared a similarity of mere 19%. The species *V. pilosa* is morphologically dissimilar from other species of the genus in its climbing habit and velvety nature of pods. Pandiyan *et al*. (2012) undertook diversity analysis of *Vigna* species through morphological markers and established *V. pilosa* as a divergent species. The above species was treated under a separate subgenus *Dolichovigna* and found it to be taxonomically quite distinct from other taxa (Bisht *et al.,* 2005) and later *Dolichovigna* was removed from *Vigna* and placed as a subgenus in *Dysolobium*. Marechal *et al*. (1978) transferred *V. pilosa* to the genus *Dysolobium* (Benth.) Prain and the species is better known today under the name *Dysolobium pilosum* (Willd.) Marechal (cf. Sanjappa, 1992). The result of RAPD analysis of the present work supported this view. However, the data obtained from ISSR and combined RAPD and ISSR markers could not be corroborated with the findings based on morphological characteristics (Marechal *et al*., 1978; Sanjappa, 1992; Bisht *et al*., 2005).

The intra-generic classification of Verdcourt (1970) as modified by Maréchal *et al.* (1978) recognised 7 subgenera namely, Vigna, Haydonia, Plectotropis, Ceratotropis, Lasiospron, Sigmoidotropis and Macrorhyncha. Of the *Vigna* species selected for the present work, the subgenus *Vigna* was represented by *Vigna unguiculata* and subgenus Ceratotropis by six species namely, *V. mungo, V. radiata, V. sublobata, V. aconitifolia, V. trilobata* and *V. umbellata*. *Vigna adenatha* was the only species under the subgenus Sigmoidotropis. During the present study, clustering of species in the dendrograms constructed using RAPD, ISSR and combined data was not in complete agreement with the intra-generic classification stated above but certain species followed the pattern of species placement stated above. On analysis of ISSR marker data, two subspecies of *Vigna unguiculata* (*V. unguiculata* ssp. *unguiculata* and *V. unguiculata* ssp. *cylindrica*) belonging to the subgenus Vigna got separated from members of two other subgenera viz. Ceratotropis and Sigmoidotropis in the phylogenetic tree.

The tree generated from combined RAPD and ISSR markers data revealed grouping of taxa into two distinct clusters, one with *Vigna radiata-Vigna mungo* belonging to the sect. Ceratotropis of the subgenus Ceratotropis along with *V. unguiculata* of the sect. Vigna of subgenus Vigna; and the other with members of sect. Leptospron of subgenus Sigmoidotropis and sect. Angulares and sect. Aconitifoliae of the subgenus Ceratotropis. Though both *V. aconitifolia* and *V. trilobata* were members of the sect. Aconitifoliae of the subgenus Ceratotropis, they were placed remotely. Using ISSR polymorphism, Ajibade *et al.* (2000) have also observed clear separation of *C. aconitifolia* from other species of *Vigna.*

Vigna sublobata, which is considered as a variety of *V. radiata* (Verdcourt, 1970; Saini and Jawali, 2009; Bisht *et al*., 2005; Dikshit *et al*., 2005), was found to be quite distinct from *V. radiata* and did not come even in the same clade. This authenticates the view of Bairiganjan *et al*. (1985) in treating this widely accepted variety as a true species. Morphological distinction, analysis of seed protein content, amino acid composition and crossability experiments by Babu *et al*. (1985) established *V. sublobata* as a putative progenitor of *V. mungo* and as remotely related to *V. radiata*. The F1 hybrid between *V. mungo* and *V. sublobata* and *V. radiata* and *V. sublobata* were observed to be sterile pointing at sterility barriers among these three species (Babu *et al*., 1985). UPGMA tree based on RAPD profiles placed *V. mungo*, *V. radiata* and *V. radiata* var. *sublobata* separately and were considered distinct taxonomic entities. Undal *et al*. (2011) found *V. radiata* and *V. radiata* var. *sublobata* (=*V. sublobata*) in two separate clusters in the dendrogram constructed on the basis of RAPD analysis further strengthened the above view.

Recently, the subgenus Ceratotropis has been divided into three sections Aconitifoliae, Angulares and Ceratotropis (Tomooka *et al*., 2002). However, in the present study using RAPD and ISSR in combination, *V. radiata* and *V. mungo* belonging to the section Ceratotropis came in one cluster but *V. sublobata* (*V. radiata* var. *sublobata*) of the same section formed a different clade with members of other sections in the phylogram. *V. aconitifolia* and *V. trlobata* of the section Aconitifoliae were grouped in a larger cluster but away from each other and intermingled with other taxa of the section Ceratotropis (*V. sublobata*) and Angulares (*V. umbellata*) and even unrelated species of subgenus Sigmoidotropis (*V. adenantha*). Therefore, the sectional classification of the subgenus Ceratotropis could not be resolved using either RAPD or ISSR markers. As remarked by Vir *et al.* (2010), the inability of ISSR markers to reveal sectional relationships could either be due to their inability to reflect the divergence within taxa owing to different evolutionary rates or alternatively, the earlier classification proposed (Verdcourt, 1970; Marechal *et al*., 1978; Tateishi, 1996) needs to be reinvestigated. Saini and Jawali (2009) studied the molecular evolution of 5S rDNA gene unit among 10 *Vigna* species belonging to the subgenus Ceratotropis and observed that the species relationship are not in agreement with sectional classification (Tomooka *et al*., 2002). In the present study, similar observations have been made and this necessitates reinvestigation of the sectional classification of the sub-genus Ceratotropis of genus *Vigna*.

Acknowledgement

The authors express their gratitude to the Chief Executive, Regional Plant Resource Centre, Bhubaneswar for providing laboratory facilities.

References

Ajibade, S. R., Weeden, N. F. and Chite, S. M. (2000). Inter simple sequence repeat analysis of genetic relationships in the genus *Vigna*. Euphytica 111: 47-55.

Arora, R. K. (1985). Diversity and collection of wild *Vigna* species in India. FAO/IBPGR Plant Genet. Resour. Newslett. 63: 26–35.

Babu, C. R., Johri, B. M. and Sharma, S. K. (1985). Leguminosae–Papilionoideae: Tribe–Phaseoleae. Bull. Bot. Surv. India 27: 1–28.

Bairiganjan, G. C., Panda, P. C., Choudhury, B. P. and Patnaik, S. N. (1985). Fabaceae in Orissa. J. Econ. Tax. Bot. J. Econ. Taxon Bot. 7: 249-276.

Bisht, I. S., Bhat, K. V., Lakanpaul, S., Latha, M., Jayan, P. K., Biswas, B. K. & Singh, A. K. (2005). Diversity and genetic resources of wild *Vigna* species in India. Genet. Resour. Crop Evolut. 52: 53-68.

Dikshit, H. K., Jhang, T., Singh, N. K., Koundal, K. R., Bansal, K. C., Chandra, N., Tickoo, J. L. and Sharma, T. R. (2005). Genetic differentiation of *Vigna* species by RAPD, URP and SSR markers. Biol. Plant. 51: 451–457.

Doyle, J. J. and Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Gherardi, M., Mangin, B., Goffinet, B., Bonnet, D. and Huguet, T. (1998). A method to measure genetic distance between allogamous populations of alfalfa (*Medicago sativa*) using RAPD molecular marker. Theor. Appl. Genet. 98: 406-412.

Goel, S., Raina, S. N. and Ogihara, Y. (2002) . Molecular evolution and phylogenetic implications of internal transcribed spacer sequences of nuclear ribosomal DNA in the *Phaseolus-Vigna* complex. Molec. Phylogenet. Evolut. 22(1):1-19.

Jaaska, V and V. Jaaska. (1990). Isoenzymme variation in Asian Beans. Bot. Acta 103: 281-290.

Jaccard, P. (1908). Nouvelles rescherches sur la distribution florale. Bull. Soc. Vaud. Sci. Nat. 44: 223-270.

Javadi, F., Tun Tun, Y., Kawase, M., Guan, K. and Yamaguchi, H. (2011). Molecular phylogeny of the subgenus Ceratotropis (genus Vigna, Leguminosae) reveals three eco-geographical groups and Late Pliocene–Pleistocene diversification: evidence from four plastid DNA region sequences. Ann. Bot. 108: 367–380.

Kaga, A., Tamooka, N., Egawa, Y., Hosaka, K. and Kamijima, O. (1996). Species relationships in subgenus *Ceratotropis* (genus *Vigna*) as revealed by RAPD analysis. Euphytica 88: 17-24.

Maréchal, R., Mascharpa, J. M. and Stainier, F. (1978). Etude taxonomique d'un groupe complexe d'espéces des genres *Phaselolus* et *Vigna* (*Papilionaceae*) sur la base de données morphologiques, traitées par l'analysis informatique. Boissiera 28: 1-273.

Pandiyan, M., Senthil, N., Anitha, M., Raveendran, M., Sudha, M., Latha, M., Nagarajan, P., Toomoka, N. and Balasubramanian, P. (2012). Diversity analysis of *Vigna sp* through morphological markers. Wudpecker J. Agric. Res. 1(8): 335 – 340.

Polhill, R. M., Raven, P. H. and Sirton, C. H. (1981). Evolution and systematics of the Leguminosae. In: Polhill, R. M. and Raven, P. H. (ed). Advances in Legume Systematics Vol.3. Royal Botanic Gardens, Kew, UK, pp. 22-88.

Prevost, A. and Wilkinson, M. J. (1999). A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theor. Appl. Genet. 98: 107-112.

Rohlf, F. J. (2000). NTSYS-pc: numerical taxonomy and multivariate analysis system, version 2.1. Exeter Software: Setauket, New York.

Saini, A. and Jawali, N. (2009). Molecular evolution of 5S rDNA region in Vigna subgenus Ceratotropis and its phylogenetic implications. Plant Syst. Evolut. 280:187–206.

Sanjappa, M. (1992). Legumes on India, Bishen Singh Mahendra Pal Singh, Dehra Dun, India.

Seehalak, W., Tomooka, N., Waranyuwat, A., Thipyapong, P., Laosuwan, P., Kaga, A. & Vaughan, D. A. (2006). Genetic diversity of the *Vigna* germ plasm from Thailand and neighboring regions revealed by AFLP. Genet. Resour. Crop Evolut. 53: 1043-1059.

Simon, M. V., Benko-Iseppon, A. M., Resende, L. V., Winter, P. and Kahl, G. (2007). Genetic diversity and phylogenetic relationships in Vigna Savi germplasm revealed by DNA amplification fingerprinting. Genome 50: 538-547.

Sneath, P. H. A. and Sokal, R. R. (1973). Numerical taxonomy: the principles and practice of numerical classification. Freeman, San Francisco.

Souframanien, J. and Gopalkrishna, T. (2004). A comparative analysis of genetic diversity in blackgram genotypes using RAPD and ISSR markers. Theor. Appl. Genet. 109: 1687-1693.

Tantasawat, P., Trongchuen, Prajongjai, T., Thongpae, T., Petkhum, C., Seehalak, W. & Machikowa, T. (2010). Variety identification and genetic relationships of mungbean and blackgram in Thailand based on

morphological characters and ISSR analysis. African J. Biotechnol. 9 (27): 4452-4464.

Tateishi, Y. (1996). Systematics of the species of *Vigna* subgenus Ceratotropis. In: Srinives P, Kitabamroong C, Miyazaki S. (ed.). Mungbean germplasm: collection, evaluation and utilization for breeding program. Japan: JIRCAS, 9–24.

Tomooka, N., Egawa, Y. and Kaga, A. (2000). Biosystematics and genetic resources of the genus *Vigna* subgenus Ceratotropis. In: Vaughan D., Tomooka N. and Kaga A. (ed.), The Seventh MAFF International Workshop on Genetic Resources. Part 1. Wild Legumes, Ministry of Agriculture, Forestry and Fisheries and National Institute of Agrobiological Resources, Japan, pp. 37–62.

Tomooka, N., Vaughan, D. A., Moss, H. and Maxted, N. (2002). The Asian *Vigna*: genus *Vigna* subgenus *Ceratotropis* genetic resources.' Kluwer Academic Publishers, Dordrecht, The Netherlands.

Undal, V. S., Thakare, P. V., Chaudhari, U. S., Deshmukh, V. P. and Gawande, P. A. (2011). Estimation of Genetic Diversity among wild *Vigna s*pecies revealed by RAPD markers. Ann. Biol. Res. 2 (4): 348-354.

Verdcourt, B. (1970). Studies in the *Leguminosae-Papilionoideae* for Flora of Tropical East Africa- IV. Kew Bull. 24:507–569.

Vir, R., Jehan,T., Bhat, K. V. and Lakhanpaul, S. (2010). Genetic characterization and species relationships among selected Asiatic *Vigna* Savi. Genet. Resour. Crop Evolut. 57: 1091-1107.

Zietkiewicz, E., Rafalski, A. and Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20: 176-183.

Plant Science Research 34 (1&2) : 10-16, 2012

Plant Science Research

ISSN 0972-8546

Biodiversity and seasonal distribution of fungal species in some soils of Odisha

B. K. Sethi[1Ψ], P. K. Nanda[2] and S. L. Sahoo[1]

[1] Post Graduate Department of Botany, Utkal University, Bhubaneswar-751004, India.

[2] Department of Botany, Saila Bala Women's College, Cuttack-753001, India.

ARTICLE INFO

Article history:
Received : 8 October 2012
Received in revised form : 01 December 2012
Accepted : 01 December 2012

Keywords:
Aspergillus
edaphic
isolation
mycoflora
Penicillium
Trichoderma

ABSTRACT

In this study, various soil samples of Odisha were evaluated for presence of instinctive fungal community. Edaphic properties of the soil were evaluated from February to June, 2011. There was not much disparity evident in temperature and pH of the soil samples. The water content was found steadily diminished from February to May. Water holding capacity was 67 % and 66.9 %, respectively. Organic carbon, organic matter, total nitrogen and phosphorous contents were 0.26 %, 0.45 %, 0.2g/kg % and 0.38mg/kg respectively. Soil mycoflora enumeration revealed utmost numbers of fungi including *Aspergillus* species from soils of the botanical garden of P.G. Department of Botany (3×10^8 CFU/g) followed by Mancheswar Industrial Estate (1×10^8 CFU/g). The soils of Joda Industrial Estate and Barbil Industrial Estate of Keonjhar were also affluent in fungal community. Among all 35 fungal taxa, *Aspergillus niger* was the most predominant microflora followed by *Aspergillus terreus*. A detailed investigation of mycoflora was undertaken from the garden soil, Department of Botany, Utkal University. Other fungal species like *Alternaria alternata*, *Aspergillus candidus, A. flavus, A. fumigatus, A. stellatus* and *Aspergillus* sp. were more prevalent in summer. The utmost incidence of *Fusarium oxysporum, Aspergillus niger, Aspergillus terreus* and *Mucor* sp. in this environment was found in rainy season. Species like *Aspergillus terreus, A. oryzae, A. niger, A. fumigatus*, *A. awamori*, *Penicillium* sp., *Mucor* sp. and *Trichoderma* sp. were abundant in winter. However, throughout the year maximum numbers of *Aspergillus* spp., *Alternaria* spp. and *Penicillium* spp. were observed.

1. Introduction

Commencing from the poles to equators, arid region to tropical and subtropical zones, fungi are plentiful in environment and display various inimitable characters to the climatic changes. More than one million species of fungi are estimated and numerous of the species are yet to be identified (Hawksworth, 1991). India possesses 27,000 species, the most prevalent biotic community after insects (Sarbhoy *et al*., 1996). About 205 new genera have been illustrated from India, of which 32% were credited to C. V. Subramanian of the University of Madras. To that, 12 new genera, 60 new taxa and 500 new fungi were contributed by Manoharachary *et al*. (2005). India contributes one third of fungal diversity of the entire globe. From 1.5 million fungi, only 50 % are typified and only 5–10% of fungi can be cultured *in vitro*. Besides to their magnificence, fungi perform vital role in human welfare with their exploitation in industry, agriculture, pharmaceuticals, food industry, textiles, bioremediation, natural cycling, as biofertilizers, secondary metabolite production, industrial enzymes (e.g. amylases, cellulases, lipases, glucoamylases, pectinases, phosphatases and proteases) and in countless other ways. Now-a-days, mycobiotechnology has become an indispensable part of the human wellbeing (Manoharachary *et al*., 2005). The interactions of fungi with other organisms have also played a vital role in the evolution of microorganisms, plants and animals.

Ψ *Corresponding author; Email:* reach4bijay@gmail.com

In spite of their noteworthy significance, the taxonomy of fungi is still not absolutely resolved and the species distribution in soil is not completely reported, though many fungi particularly Aspergilli are acknowledged to biosynthesize diverse groups of bio-active metabolites and enzymes. Keeping in view of the significance of fungal community, present study was undertaken to isolate, enumerate and to report about the abundance of aboriginal fungal strains as candidates to explore their prospective potentials for industrial exploitations.

2. Materials and methods

2.1 Chemicals and media

All the chemicals and microbiological grade media components implemented in the present study were of analytical reagent grade and purchased from Sigma Chemicals Co. (USA), Hi-Media Limited, SRL Pvt. Limited and Merck India Limited (Mumbai, India).

2.2 Selection of the experimental field and Collection of the soil sample

Experimental fields (5×5 m^2) of Bhubaneswar (Patia, Mancheswar Industrial Estate, Ganga Nagar, BDA Colony and garden soil of P.G. Department of Botany, Utkal University) and Keonjhar (Joda and Barbil) were selected. Soil samples (1 Kg each) were collected during February to June, 2011 in two different parts. One part was used for soil analysis and other part for microbial analysis and enumeration as per Mueller and Durrell (1957) with slight modifications.

2.3 Edaphic properties of selected soil samples

Physical parameters studied constituted soil texture, temperature, water content, water holding capacity and pH while chemical parameters of soil such as organic carbon, organic matter, total nitrogen and available phosphorous were recorded.

2.4 Isolation and identification of potent microorganisms by morphological analysis

A total of 35 samples from approximately 7 kg of different polluted and garden soil were collected from the selected experimental fields. Samples were serially diluted; pour-plate technique (Sohail *et al*., 2009) was performed for isolation and the plates incubated for 5 days separately on Potato dextrose agar (PDA), Sabouraud's dextrose agar (SDA) and Czapek's Dox agar (CZA) amended with sefixime (50 mg/100ml) under sterile conditions at 25 °C and 37 °C for 7 days. Isolated fungi were identified as per Alexopoulos and Mims (1979) and Watanabe (2002) based on macro and microscopic characteristics. The isolates were characterized and reconfirmed by National Center for Fungal Taxonomy, New Delhi, India. All isolates were maintained as pure cultures on PDA slants.

2.5 Statistical analysis

All experiments were carried out in triplicates and repeated three times. The samples collected from each replicate were tested for isolation of microorganisms. Each value is an average of three parallel replicates. Data were analyzed using analysis of variance (ANOVA) for a complete randomized design. Duncan's New Multiple range test (DMRT) (Gomez and Gomez, 1984) was used to indicate means with significant differences at $p \leq 0.05$.

3. Results and discussion

3.1 Edaphic properties of soil and fungal isolates

Surface soil up to one foot depth is very prolific precinct that holds highest number of soil dwelling microorganisms and all the microbial processes are performed at this stratum. Hence, all the soil samples were collected in between one foot depth.

Prior to the analysis, the temperature of the soil was recorded twice in a month and average of each month is presented in Table 1 depicting February with minimum temperature (24 °C) and June with maximum temperature (39 °C). There was not much disparity recorded in temperature of the soil as no rain fall occurred during the investigation period. The value of water content was least in the month of June and maximum in February. Water holding capacity was only determined twice during the total study period and it was found to be 67 and 66.9 % respectively (Table 1). The pH of the soil was recorded in beginning of every month with no variations between the values from February to June. Organic carbon and organic matter content of the soil were found to be 0.26 % and 0.45 %, respectively. Total nitrogen and phosphorous content were 0.2 g/Kg and 0.38 mg/Kg respectively.

Highest numbers of fungi including *Aspergillus* species were isolated from the botanical garden soil of P.G. Department of Botany (3×10^8 CFU/g) followed by Mancheswar Industrial Estate (Table 2; Fig. 1) by employing the dilution plate techniques. Lower counts in different garden soils were reported by Fleet and Mian (1987). The soils of Joda Industrial Estate and Barbil Industrial Estate of Keonjhar were rich in fungal community. All the fungal isolates were identified by the assistance of laboratory experiences, consultation of certain monographic books and NCFT, New Delhi (Thom and Raper, 1945; Alexopoulos and Mims, 1979; Watanabe, 2002).

Table 1
Physical and chemical properties (edaphic properties) of soil[a] taken in the study

Soil parameters	February	March	April	May	June
Texture	Sandy loam				
Temperature (°C)[b]	24	29	31	36	39
Water content (%)	6.0	5.7	5.6	5.5	5.0
Water holding capacity (%)		67.0		66.9	
pH[b]	5.2	5.1	5.2	5.2	5.3
Organic carbon (%)			0.26		
Organic matter (%)			0.45		
Total nitrogen (g/Kg)			0.2		
Phosphorus content (mg/Kg)			0.38		

[a] Represents the botanical garden soil of Post Graduate Department of Botany, Utkal University, Bhubaneswar, India.

[b] Temperature and pH of the soil samples were recorded at 11: 00 am in the beginning of every month.

Table 2
Colony forming units (CFU/g) in polluted soils of eastern and north-east regions of the state-Odisha

Soil collection sites	Total number of soil samples collected	Positive samples with maximum isolation	Percentage of positive samples	Colony forming units in original sample (CFU/g)
BHUBANESWAR				
Patia Industrial Estate	35	23	65.7[d]	5×10^7
Mancheswar Industrial Estate	35	27	77.1[c]	1×10^8
Ganga Nagar	35	29	82.9[b]	2×10^7
BDA Colony, CS Pur	35	26	74.3[c]	2×10^7
Botanical garden[a]	35	34	97.1[a]	3×10^8
KEONJHAR				
Joda Industrial Estate	35	24	68.6[d]	2×10^7
Barbil Industrial Estate	35	30	85.7[b]	7×10^7

[a] Botanical garden represents the botanical garden soil of Post Graduate Department of Botany, Utkal University, Bhubaneswar, Odisha, India. Data pooled from a total of 3 separate experiments each comprising of 3 replicates. Means within a column with different superscripts are significantly different at p d≤ 0.05 tested through Duncan's New Multiple Range Test.

Fig. 1. Mixed fungal cultures isolated from different soil samples.

In all soil samples, *Aspergillus niger* was the most dominated species followed by *Aspergillus terreus* (data not shown). A total of 35 fungal taxa were isolated belonging to 9 genera (Table 3; Fig. 2). *Curvularia* and *Alternaria* have represented by two taxa, *Fusarium* by two taxa, *Penicillium* by four taxa, *Rhizopus* by 3 taxa and *Trichoderma* by only one species were recorded (Fig. 3). There were respectively two and three isolates of *Mucor* sp. and *Rhizopus* sp. recorded from the sub division Zygomycotina. Even after a short treatment with U.V. radiation, only one fungal species was found never sporulated in the agar medium and was in white sterile mycelium. The observations were not sufficient for the identification up to the species level, but substantiated their affiliation up to the genus level. A specific identification is also required, which is underway. Fungi represent an array of microorganisms that are extensively spread in environment including soil (Boer *et al.*, 2005). Previously, Duarte and Costa-Ferreira (1994) reported that *Aspergillus* was the most copious genus of soil fungi.

In this finding, *Alternaria alternata*, *Aspergillus candidus, A. flavus, A. fumigatus, A. stellatus* and *Aspergillus* sp. were more frequent in summer. The highest occurrence of *Fusarium oxysporum, A. niger, A. terreus* and *Mucor* sp. in soil of garden environment was recorded in rainy season. *Penicillium* sp., *Mucor* sp., *Trichoderma aureoviride, Aspergillus terreus, A. oryzae, A. niger, A. fumigatus* and *A. awamori* were more abundant in winter. Maximum numbers of isolations were of genus *Aspergillus, Alternaria,* and *Penicillium* during all three seasons (Table 3; Fig. 3). de Ana *et al*. (2006) reported similar findings while investigating on seasonal distribution of *Alternaria, Aspergillus, Cladosporium* and *Penicillium* species in homes of fungal allergic patients.

Table 3
Prevalence frequency and seasonal variation of fungal isolates in soil collected from botanical garden of the department

Name of the fungus	Seasonal Variation		
	Summer	Rainy	Winter
Alternaria alternata	+++ [ac]	++	+
Alternaria tenuis	+	+	++
Aspergillus awamori	-	+	+++ [a]
Aspergillus brevipes	-	+	+
Aspergillus candidus	+++ [b]	++	++
Aspergillus clavatus	+	+	++
Aspergillus flavus	+++ [b]	++	+++
Aspergillus fumigatus	+++ [bc]	++	+++ [a]
Aspergillus japonicus	+	-	++
Aspergillus kanagawaensis	-	++	+
Aspergillus niger	++	+++ [d]	+++ [d]
Aspergillus nidulans	-	+	++
Aspergillus parasiticus	-	-	++
Aspergillus oryzae	-	++	+++ [ac]
Aspergillus stellatus	+++ [ac]	+	++
Aspergillus sydowii	+	++	++
Aspergillus tamarii	-	++	++
Aspergillus terreus	++	+++ [ac]	+++ [d]
Aspergillus versicolor	-	+	++
Aspergillus species	+++ [d]	++	++
Curvularia lunata	-	+	++
Curvularia pallescens	+	+	+

Fusarium oxysporum	-	+++ [ac]	-
Fusarium sp.	-	+	+
Mucor hiemalis	-	++	+++ [a]
Mucor sp.	+	+++ [c]	+++ [b]
Penicillium sp.1	+	++	+++ [d]
Penicillium sp.2	+	++	+++ [d]
Penicillium sp.3	+	++	+++ [d]
Penicillium sp.4	+	++	+++ [d]
Rhizopus nigricans	-	+	++
Rhizopus sp.1	-	+	+
Rhizopus sp.2	-	+	++
Trichoderma aureoviride	-	++	+++ [ab]
White sterile mycelium *	-	+	++

-: absent; +: less; ++: moderate; +++: abundant. [a] Beginning of the season; [b] mid of the season; [c] end of the season; [d] through out of the season. * The species was not sporulated in the agar medium even after a short treatment with UV radiation and was noted as white sterile mycelium.

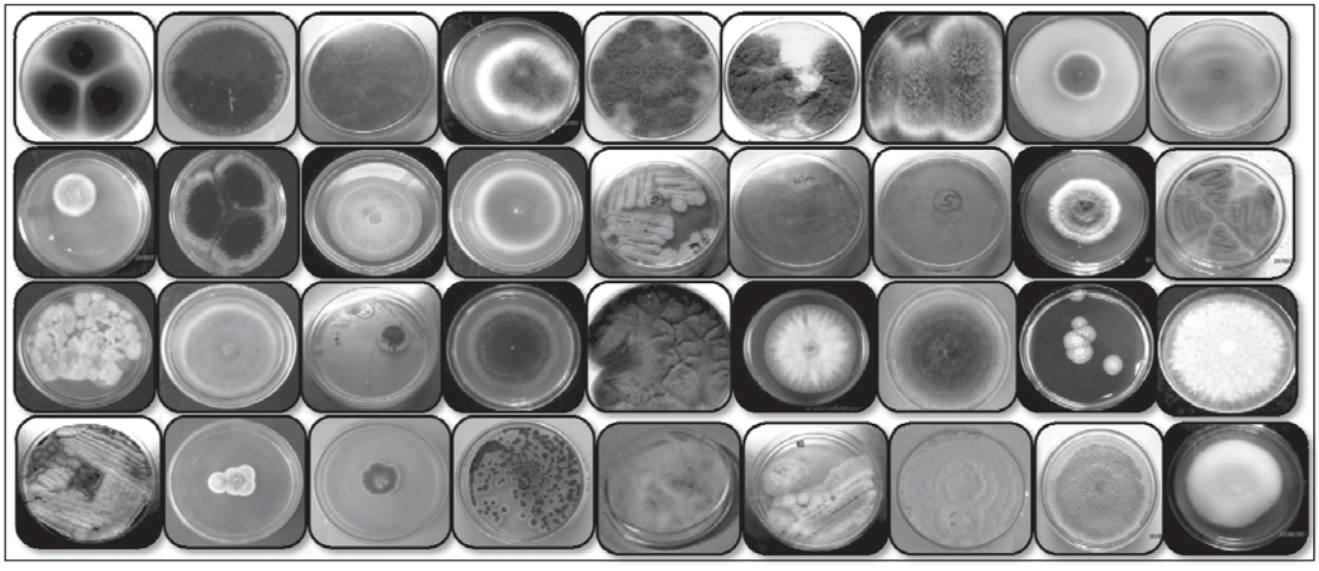

Fig. 2. Pure culture plates of isolated fungi; from left to right *Alternaria alternata, A. tenuis, Aspergillus awamori, A. candidus*, *A. clavatus*, *A. flavus, A. fumigatus, A. glaucus, A. japonicus, A. kanagawaensis, A. niger, A. nidulans, A. parasiticus*, *A. oryzae, A. stellatus, A. sydowii, A. tamari, A. terreus, A. versicolor, Aspergillus* sp., *Cladosporium* sp., *Curvularia lunata, C. pallescens, Fusarium oxysporum, Fusarium* sp., *Mucor hiemalis, Mucor* sp., *Penicillium* sp.1, *Penicillium* sp.2, *Penicillium* sp.3, *Penicillium* sp.4, *Rhizopus nigricans, Rhizopus* sp.1, *Rhizopus* sp.2, *Trichoderma aureoviride* and white sterile mycelium.

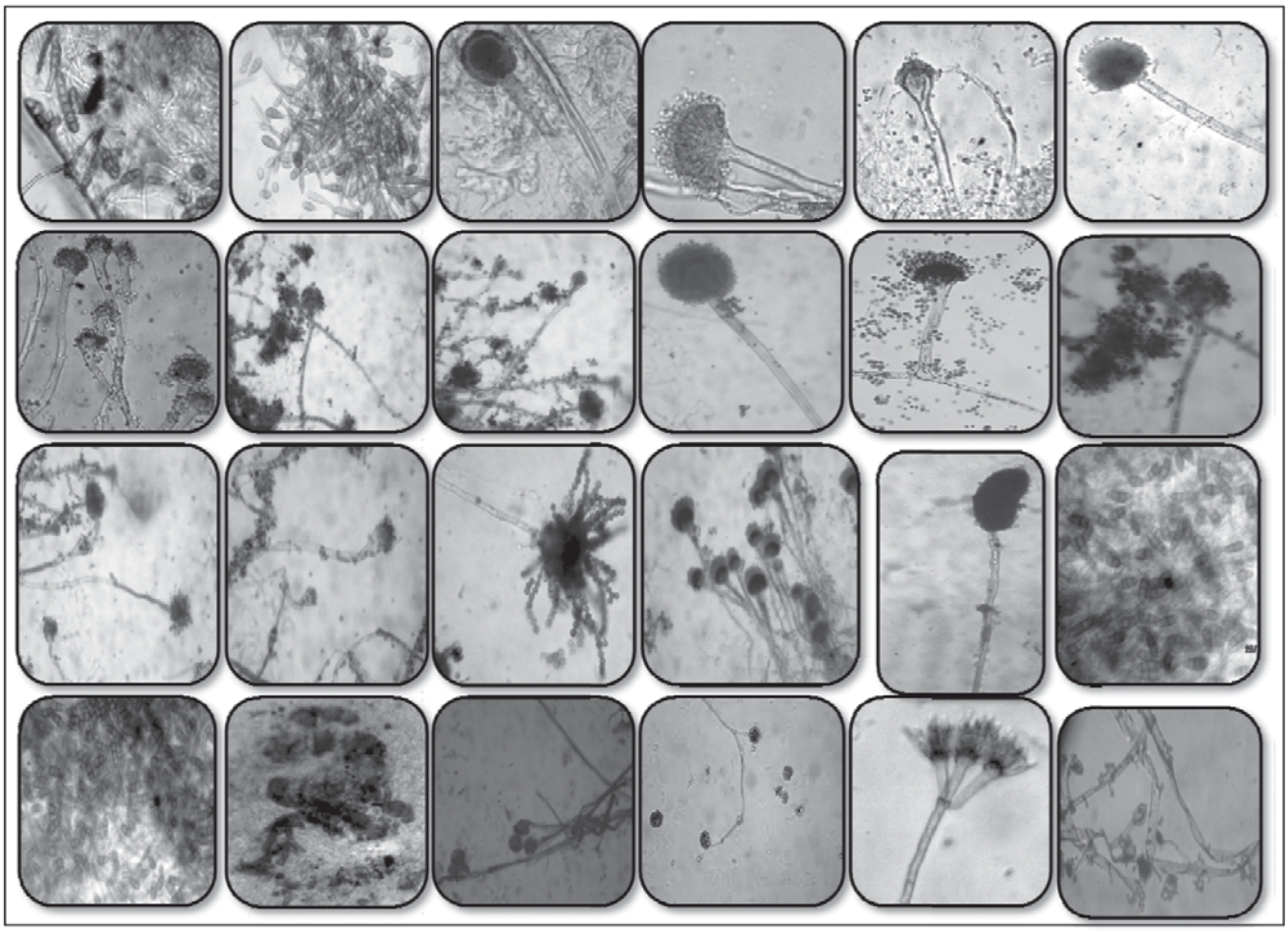

Fig. 3. Phase contrast microscopic view of isolated fungi (450 times magnified). From left to right : *Alternaria alternata, A. tenuis, Aspergillus awamori, A. candidus*, *A. flavus, A. fumigatus, A. glaucus, A. japonicus, A. kanagawaensis, A. niger, A. nidulans, A. parasiticus*, *A. stellatus, A. sydowii, A. tamari, A. terreus, Aspergillus* species, *Curvularia lunata, C. pallescens, Fusarium* sp., *Mucor hiemalis, Mucor* sp., *Penicillium* sp.1 and *Rhizopus* sp.2.

4. Conclusion

The genus *Aspergillus* is a very high flying and diverse community among all innate soil dwelling fungi. Affiliates of this genus are renowned for their impending and prospective attributes in biosynthesizing cluster of enzymes and metabolites at bountiful quantity by degrading various squanders and cheap substrates such as plant biomass, agro-industrial wastes and play pivotal place in managing ecosystem. Implementing these natural isolates, high valued-low cost products can be achieved from cheap substrates (wastes and effluents) addressing the wellbeing of mankind.

Acknowledgments

This work was financially assisted by UGC-RGNF Scheme, New Delhi, India. Authors gratefully acknowledge the generous support of Dr. P. N. Chowdhry, Principal Mycologist, National Center of Fungal Taxonomy, New Delhi and Prof. Narayan Behera, Former Visiting Professor, P.G. Department of Botany, Utkal University in identifying the isolated microorganisms. One of the author (BKS) is thanking the Head, P.G. Department of Botany, Utkal University for providing necessary laboratory facilities to carry out this research work.

References

Alexopoulos, C. J. and Mims, C. W. (1979). Introductory Mycology. 3rd Edn. John Wiley and Sons, New York.

Boer, W., Folman, L.B., Summerbell, R.C. and Boddy, L. (2005). Living in a fungal world: impact of fungi on soil bacterial niche development. FEMS Microbiol. Rev. 29: 795-811.

de Ana, S.G., Torres-Rodríguez, J.M., Ramírez, E.A., García, J. and Belmonte- Soler, J. (2006). Seasonal distribution of *Alternaria, Aspergillus, Cladosporium* and *Penicillium* species isolated in homes of fungal allergic patients. J. Investig. Allergol. Clin. Immunol. 16(6): 357-363.

Duarte, J.C. and Costa-Ferreira, M. (1994). Aspergilli and lignocellulosics: Enzymology and biotechnological applications. FEMS Microbiol. Rev. 13: 377-386.

Fleet, G.H. and Mian, M.A. (1987). The occurrence and growth of yeasts in dairy products. Int. J. Food Microbiol. 4: 145-155.

Gomez, K.A. and Gomez, A. A. (1984). Statistical Procedure for Agricultural Research. John Wiley and Sons, New York.

Hawksworth, D. L. (1991). The fungal dimension of biodiversity: magnitude and significance and conservation. Mycol. Res. 95: 641-655.

Manoharachary, C., Sridhar, K., Singh, R., Adholeya, A., Suryanarayanan, T. S., Rawat, S. and Johri, B. N. (2005). Fungal biodiversity: Distribution, conservation and prospecting of fungi from India. Curr. Sci. 89(1): 58-71.

Muller, K.E. and Durrel L.W. (1957). Sampling tubes for soil fungi. Phytopathol. 47: 243-245.

Sarbhoy, A. K., Agarwal, D. K. and Varshney, J. L. (1996). Fungi of India 1982–1992, CBS Publishers and Distributors, New Delhi.

Sohail, M., Siddiqi, R., Ahmad, A. and Khan, S. A. (2009). Cellulase production from *Aspergillus niger* MS82: Effect of temperature and pH. New Biotechnol. doi:10.1016/j.nbt.2009.02.002.

Thom, C. and Raper, K. B. (1945). A manual of the Aspergilli. Waverly Press Inc. The Williams and Wilkins Company, Baltimore, U.S.A.

Watanabe, T. (2002). Pictorial Atlas of Soil and Seed Fungi. 2nd Edn. CRC Press, London.

Plant Science Research 34 (1&2) : 17-22, 2012

Plant Science Research

ISSN 0972-8546

Isolation and characterization of a chlorpyrifos degrading bacterium from rice soil

S. Das and T. K. Adhya[Ψ]

Laboratory of Soil Microbiology, Division of Crop Production, Central Rice Research Institute, Cuttack - 753006, India

ARTICLE INFO

Article history:
Received : 01 November 2012
Received in revised form : 02 December 2012
Accepted : 03 December 2012

Keywords:
chlorpyrifos
biodegradation
rice soils
bacteria
Bacillus sp.

ABSTRACT

The organophosphorus insecticide, chlorpyrifos has been widely used in agriculture, in veterinary against house hold pests, and in field agriculture for subterranean termite control. Due to its slow rate of degradation in soil it can persist for extended periods in soil with significant concern to the environment. A soil bacterium capable of utilizing chlorpyrifos as the sole source of carbon and energy was isolated from rice soil enriched with repeated application of chlorpyrifos (10 mg/kg). The strain named CRRI NF3, was preliminarily identified as *Bacillus* sp. based on its morphological, physiological and biochemical tests as well as 16s rRNA gene sequence analysis. Bioremediation of chlorpyrifos was examined using CRRI NF3 inoculated to the soil treated with 10 mg/kg chlorpyrifos and resulted in faster degradation than control soils without any inoculation. The optimum pH, temperature could have promoted degradation.

1. Introduction

Organophosphorus (OP) compounds are used worldwide for pest control since 1937 (Dragun *et al.,* 1984) as substitute for highly persistent and toxic organochlorine pesticides. Organophosphorus insecticide chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridinol) phosphate; trade name Dursban or Lorsban], is used worldwide as an agricultural insecticide (Chao *et al.,* 2002) in veterinary against house hold pests, and in field agriculture for subterranean termite control. Extensive use of chlorpyrifos has led to widespread environmental contamination resulting in damage to non-target species. In the environment, chlorpyrifos undergoes hydrolysis to tri-chloro pyridinol (TCP) and diethyl thiophosphoric acid (Racke and Coates, 1987). TCP is considered to inhibit further hydrolysis of chlorpyrifos resulting into its accumulation in the environment (Somasundaram and Coates, 1990). Studies indicated that wide range of water and terrestrial ecosystem might be contaminated with chlorpyrifos (EPA, 1997; Sapozhnikova *et al.,* 2004; Yang *et al.,* 2005) and demanded the public choice to establish an efficient, safe, and cost effective method to remove or detoxify chlorpyrifos residues from contaminated environment. In a singular report, Chlorpyrifos was degraded co-metabolically in liquid medium by *Flavobacterium* sp. ATCC 27551 (Mallick *et al.,* 1999) that was isolated form diazinon enriched soil planted to rice paddy (Sethunathan and Yoshida, 1973).

Recently, chlorpyrifos has been reported to be more amenable to biodegradation. Singh *et al.* (2004) isolated one *Enterobacter* sp. Strain B-14 which was isolated from contaminated soil of Australia and having the capability to hydrolyze chlorpyrifos. The bacteria hydrolysed chlorpyrifos and metabolized di-ethylthio-phosphate (DETP) as carbon, phosphorus and energy source for growth and proliferation. Yang *et al.* (2005) isolated a *Alcaligenes faecalis* strain DSP3 from chlorpyrifos contaminated soil around a chemical factory that was able to degrade both chlorpyrifos and its hydrolysis product TCP. Li *et al.* (2008) reported that seven

[Ψ] *Corresponding author; E-mail:* adhyas@yahoo.com

bacterial isolates including *Sphingomonas* sp., *Stenotrophomonas* sp., *Bacillus* sp. and *Brevandimonas* sp. were capable of degrading chlorpyrifos using it as carbon and energy source. An aerobic bacterial consortium consisting of *Pseudomonas fluroscence*, *Bacillus melitensis*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*, developed from pesticide contaminated soil was able to degrade chlorpyrifos in liquid medium (Vidya Lakshmi *et al.,* 2008).

In the present study attempts were made to isolate, characterize and identify novel chlorpyrifos degrading bacteria capable of utilizing chlorpyrifos as the sole carbon and energy source, form rice soil enriched with chlorpyrifos and to assess the optimization of growth and degradation potential in liquid medium under the influence of different environmental factors like pH, temperature, etc.

2. Materials and methods

2.1 Soil

Soil from rice-growing areas of CRRI experimental field, was used in the study. The soil was air-dried in shade and ground to pass through a < 2-mm sieve before use. Soil sample was classified as alluvial and soil properties were as follows: sand, 52.5%; silt, 21.6%; clay, 25.9%; organic carbon content, 0.86%; cationic exchange capacity, 15.0 meq/100g soil; total nitrogen, 0.09%, and a pH of 6.21

2.2 Insecticide and chemicals

For studies under laboratory conditions and for greenhouse studies, a commercial formulation of 94% chlorpyrifos 20 EC (Force, Nagarjuna Chemicals, Hyderabad, India) was used. For degradation studies in culture medium, certified standard of chlorpyrifos (Dursban, AccuStandard Inc, New Haven, CT, USA) of 99.99% purity was used.

2.3 Enrichment of chlorpyrifos degrading bacteria

A mineral salt medium (MSM) (Mallick *et al.,* 1999) was used in preparing the enrichment of chlorpyrifos degrading bacteria from soils previously retreated with chlorpyrifos. Portions of 10 ml MSM containing chlorpyrifos (10 µg/ml) were inoculated with 100 µl suspension of soil from chlorpyrifos retreated flooded soil and incubated at room temperature ($30^0 \pm 2^0$C) on an orbital shaker at 110 rpm. After complete disappearance of chlorpyrifos from the inoculated medium in about 10 days, 1 ml of this medium was added to a fresh batch of 10 ml of MSM supplemented with chlorpyrifos as the sole source of carbon and energy and incubated for a further period of 5 days. This was repeated 4 times at 5 day intervals for selective enrichment of chlorpyrifos degrading bacteria. After 4th transfer, 1 ml sample was withdrawn aseptically from flask after 5 days incubation and analyzed for chlorpyrifos residue by GLC after the extraction with hexane. Un-inoculated medium served as control. Inoculated flask was incubated at room temperature at $30^0 \pm 2^0$C. After 5 days when chlorpyrifos disappeared completely, 100 µl sample from the culture was spread plated on MSM agar containing 10 µg/ml chlorpyrifos as the sole source of carbon and energy.

2.4 Characterization and identification of chlorpyrifos-degrading bacteria

The most efficient bacterial isolate growing on chlorpyrifos containing agar was subjected to morphological, cultural and biochemical characterization. The bacterial strain was further identified by using standard methods (Kreg and Holt, 1984), combined with 16S rDNA sequence analysis. Genomic DNA was isolated from bacterial cells grown overnight in MSM medium supplemented with glucose and peptone using standard CTAB method. The 16S rRNA gene was amplified by PCR using universal primers 5'-AGAGTRTGATCMTYGCTWAC-3' and 5'-CGYTAMCTTWTTACGRCT-3'. The purified PCR product was outsourced for sequencing of both the strands using ABI 3130 Genetic Analyzer and a Big Dye Terminator version 3.1"Cycle sequencing kit (ABI, USA). The determined sequence was compared with those available in the GenBank/EMBL database using the BLAST program (Altschul *et al.,* 1990). The nucleotide sequence coding for 16S rDNA sequence of the bacterium was deposited in the GenBank database with accession number.

2.5 Growth of chlorpyrifos-degrading bacteria vis-a-vis degradation of chlorpyrifos

One hundred µl of the bacterial suspension (10^6 cells/ml) was inoculated into MSM supplemented with 10 µg/ml chlorpyrifos and incubated for 3 days at room temperature at $30^0 \pm 2^0$C on a shaker at 110 rpm. At periodic intervals individual flask was sacrificed and the contents were used to determine the chlorpyrifos residues. All experiments were conducted in triplicate. Microbial growth was monitored by plate counting. Un-inoculated medium containing chlorpyrifos served as control.

2.6 Influence of various factors on chlorpyrifos degradation in liquid culture

To determine the effect of pH and temperature on biodegradation of chlorpyrifos by CRRI NF3, 10 ml of MSM supplemented with chlorpyrifos (10 µg/ml) was maintained at pH 6.0, 6.5, 7.0 and 7.5 at 30^0C. To determine the influence of temperature on chlorpyrifos biodegradation experiments were conducted in 100 ml flasks containing 10 ml of the MSM supplemented with chlorpyrifos (10 mg/ml) at pH 6.2

and maintained at 25, 30 and 35 ^{0}C respectively. All flasks were inoculated with 10^6 cell/ml and incubated at room temperature of $30^0 \pm 2^0$C in a BOD incubator. At periodic interval triplicate of the flasks were sacrificed and analyzed for microbial growth and chlorpyrifos degradation.

2.7 Degradation of chlorpyrifos in soil

Soil from rice-growing areas of CRRI experimental field, was used in the study. Soil sample (40 g) was taken in a 250 ml conical flask and sterilized thrice on consecutive days and treated with chlorpyrifos (10 mg/kg). One set of soil was inoculated with CRRI NF3 (10^8 cells/ml) while another set without inoculation was kept as control. The inoculum was thoroughly mixed into the soil under sterile condition. The soil moisture was adjusted by the addition of sterile distilled water to 60% of its water holding capacity. The soil were incubated at room temperature in a laboratory condition. Chlorpyrifos was extracted from whole conical flask and determined by gas chromatography.

2.8 Extraction and analysis of chlorpyrifos residues

In the experiment on the degradation of chlorpyrifos in mineral salt medium, the whole flasks were sacrificed and extracted with equal volume (10 ml) of hexane dried over sodium sulfate (pinch of amount) and analyzed by GLC. Extraction of residues and sample preparation were done in the laboratory in diffuse light.

Chlorpyrifos residues extracted in hexane were analyzed by Perkin-elmer AutoSystem XL (USA) with electron-capture detector (ECD) for separation and quantitative analysis. The GLC fitted with metal packed column (2 m length, 3 mm OD) packed with 3% OV-17 on Chrome WHP 80/100 mesh. The operating conditions were as follows: carrier gas was nitrogen XL grade (99.99% purity), carrier flow was 20 ml/min, injector temperature was 240^{0}C, column temperature fixed at 220^{0}C, and detector temperature was 350^{0}C.Under these condition, the retention time of chlorpyrifos was 4.1 min. All the results presented are the mean of duplicate observations.

2.9 Statistical analysis

Mean and standard deviation of the data were calculated using Excel (Microsoft,USA). The kinetics data of all treatment were obtained using a first order model. Individual data sets on residues of chlorpyrifos was statistically analyzed using statistical package CropStat Ver. 7.2 (International Rice Research Institute, Philippines).

3. Results and discussion

3.1 Isolation, selection and characterization of strains

The degradation of chlorpyrifos was rapid in the CRRI soil after repeated application of chlorpyrifos (data not shown). A chlorpyrifos degrading bacterium was isolated by enrichment culture from such soil retreated with chlorpyrifos. The bacterial strain (coded CRRI NF3) was gram positive, aerobic and positive to oxidase and catalase activity. It was motile rod and its colony was white in colour. The biochemical characterization of the bacterium is given in table 1. Sequence analysis its 16S rRNA gene showed that strain CRRI NF3 was most closely related to *Bacillus* sp (GenBank Accession No JN592473). Bacterila speices belonging to genus *Bacillus* have previously been reported to degrade chlorpyrifos (Li *et al.,* 2008; Vidya Lakshmi *et al.,* 2008, Rani *et al.,* 2007, Anwar *et al.,* 2009), parathion (Yasuno *et al.,* 1965), methyl parathion and fenitrothion (Miyamoto *et al.,* 1966; Sharmila *et al.,* 1989, Ou and Sharma, 1989).

3.2 Growth and degradation ability Bacillus *sp. CRRI NF3*

Degradation pattern of bacterial strain was studied in the liquid culture medium with chlorpyrifos as the sole source of carbon. The time course of chlorpyrifos degradation by *Bacillus* sp. CRRI NF3 is shown in Fig.1, complete disappearance of 10 µg/ml was observed within 12 days with regular increment of bacterial population which could grow taking this insecticide as their energy sources.

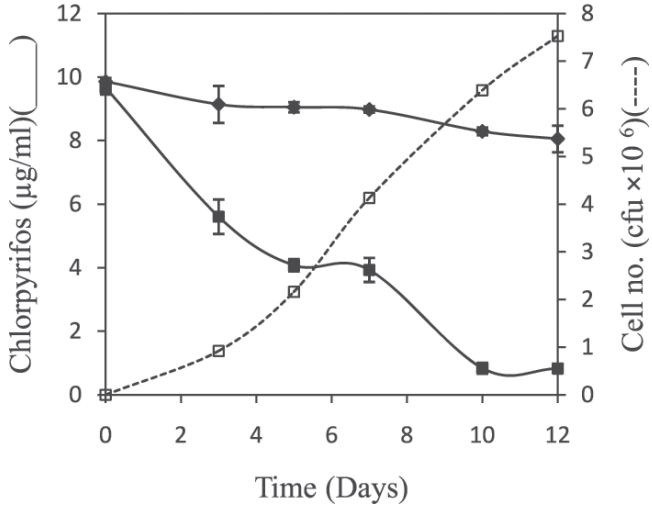

Fig. 1 Degradation of chlorpyrifos by strain CRRI NF3 in mineral salts medium. Each value is the mean of three replicates with error bars representing the standard deviation of the mean. Legends: control (◆), CRRI NF3 (■), cell no. (□)

Table 1
Biochemical characterization of chlorpyrifos degrading strain CRRI NF3

Characters	Isolate
Morphological	
Gram reaction	+
Cell shape	rod
Cell length	2.0±0.1
Colony color	White
Motility	+
Biochemical	
MR	+
MRVP	+
Nitrate reduction	+
Oxidase	+
Catalase	+
Tributyrin hydrolysis	+
Trehalose	+
Melibiose	+
ONPG	+
Esculin hydrolysis	+
D-arabinose	+
Malonate utilization	+
Dextrin	+
Gelatine	+
D-gluconic acid	+
Chemical sensitivity assay	
PH 6	+
PH 5	+
1% NaCl	+
4% NaCl	+
8% NaCl	+
1% Sodium lactate	+
Lithium chloride	+
Potassium tellurite	+
Aztreonum	+
Sodium butyrate	+
Sodium bromate	+

3.3 *Effect of pH on biodegradation of chlorpyrifos*

The degradation patterns of chlorpyrifos in mineral salt medium at different pH are presented in Fig. 2. After 12 days of incubation at 30^{0}C the degradation rates of chlorpyrifos were found to varied with pH and there was corresponding variation of half-lives (Table 2). The hydrolysis percentages of chlorpyrifos were less than 5% in all controls. It is well known that the rates of base-catalysed hydrolysis for many organophosphorus insecticides are often greatly accelerated in water at pH values above 7.5 (Greenhalgh *et al.,* 1980). Wang *et al.* (2006) had also reported that the degradation rate of chlorpyrifos by *B. latersprorus* DSP in pure cultures was affected by higher as well as low pH.

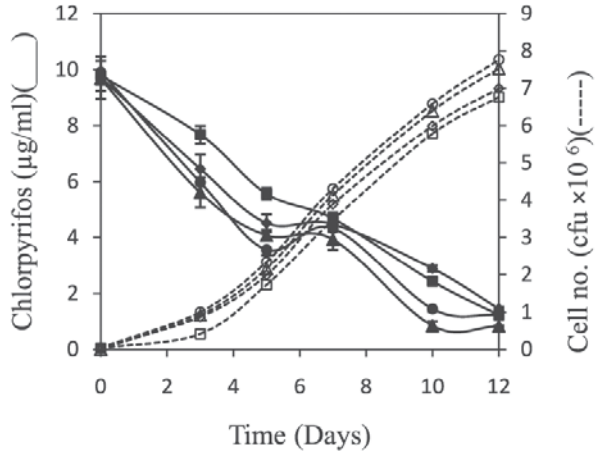

Fig.2 Effect of pH on degradation of chlorpyrifos in the mineral salt medium. Each value is the mean of three replicates with error bars representing the standard deviation of the mean. Legends: CRRI NF3 at (■) 6.0, (▲) 6.5, (●) 7.0 and (♦) 7.5; Cell no. at (□) 6.0, (△) 6.5, (○) 7.0 and (◇) 7.5

Table 2
Kinetic data of chlorpyrifos degradation by the bacterial strain CRRI NF3

Impact Factor			$t_{1/2}$ (days)	r^2
Chlorpyrifos concentration (µg.ml-1)	pH	Temperature (^{0}C)		
10	6.0	30	4.12	0.9397
10	6.5	30	3.10	0.8707
10	7.0	30	3.93	0.9372
10	7.5	30	4.83	0.9412
10	6.5	25	5.80	0.9552
10	6.5	30	5.15	0.9291
10	6.5	35	3.91	0.9400

3.4 *Effect of temperature on biodegradation of chlorpyrifos*

The effect of different temperatures (25, 30 and 35 ^{0}C) on chlorpyrifos biodegradation in mineral salts medium of

pH 6.5 is presented in Fig. 3. After 12 days of incubation, the degradation rate of chlorpyrifos at 25, 30 and 35^{0}C were examined to be 0.120, 0.135, and 0.177 and the corresponding half-lives were 5.80, 5.15 and 3.91, respectively (Table 2). Degradation rate at 35^{0}C was faster than lower temperature. The ANOVA analysis confirmed that half-lives of chlorpyrifos at 35^{0}C were significantly shorter than other two temperatures ($P < 0.05$). The results showed that the increase in temperature enhances the degradation rate of chlorpyrifos. Liu *et al.* (2003) reported that degradation of chlorpyrifos by *Aspergillus* sp. and *B. latersprorus* DSP (Wang *et al.,* 2006) was enhanced with the increase in temperature.

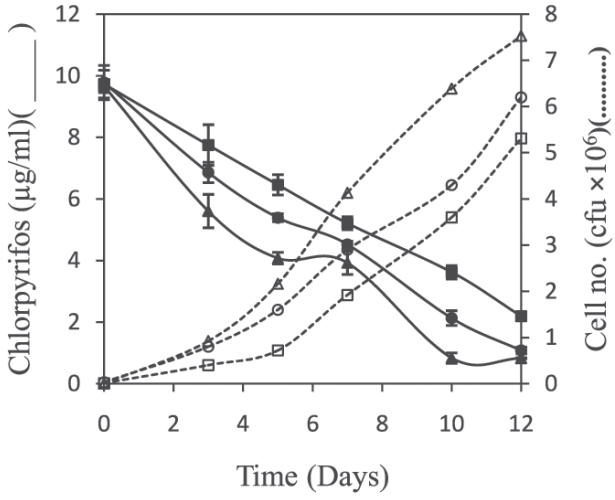

Fig. 3 Effect of temperature on degradation of chlorpyrifos in the mineral salt medium. Each value is the mean of three replicates with error bars representing the standard deviation of the mean. Legends: CRRI NF3 at (■) 25^{0}C, (▲) 30^{0}C and (●) 35^{0}C; Cell no. at (□) 25^{0}C, (△) 30^{0}C and (○) 35^{0}C.

3.5 Degradation of chlorpyrifos in laboratory soil

Inoculation of CRRI NF3 to sterile field soil and incubated under laboratory conditions exhibited a more rapid degradation of chlorpyrifos than that by un-inoculated sterile soil (Fig. 4). The concentration of chlorpyrifos in inoculated flasks reduced to 2 mg/kg upon incubation for 25 days while in uninoculated soil, concentration of chlorpyrifos came down to only 8 mg/kg indicated enhanced degradation of chlorpyrifos upon inoculation with the chlorpyrifos-degrading *Bacillus* sp.

4. Conclusion

A *Bacillus* sp. degrading chlorpyrifos was obtained from a rice soil following enrichment by repeated addition of chlorpyrifos. Rice soil represents a unique ecosystem and acts as reservoir of unique microbial population. Rice plants with its actively growing roots released organic

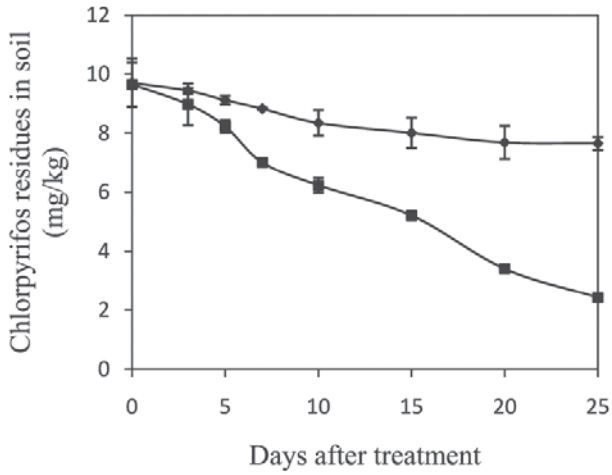

Fig.4 Degradation of chlorpyrifos by the bacterial strain CRRI NF3 on CRRI soil in the laboratory condition. Each value is the mean of three replicates with error bars representing the standard deviation of the mean. Legends: (◆) sterile soil suspension, (■) inoculated with CRRI NF3.

compounds into the rhizosphere that supported growth of the microbial community in the rhizosphere. The role of microorganisms specially *Bacillus* sp., was studied in the degradation of chlorpyrifos in liquid culture medium and in the soil. It is known that microorganisms are a major component of the ecosystem and play a considerable role in the degradation of several xenobiotics including chemical insecticide molecules. However, more research is needed to better understand the interactions among the rice plants, the rhizosphere bacterial communities and the population dynamics of the chlorpyrifos degrading bacterium.

Acknowledgements

This work was supported in part by the ICAR Networking Project, "Application of Microorganisms in Agriculture and Allied Sciences (AMAAS) - theme Agrowaste Management, Bioremediation and Microbes in Post-Harvest Processing" by the Indian Council of Agricultural Research, New Delhi.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). A basic local alignment search tool. J. Mol. Biol. 215: 403-410.

Anwar, S., Liaquat, F., Khan, Q. M., Khalid, Z. M. and Iqbal, S. (2009). Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol by *Bacillus pumilus* strain C2A1. J. Hazard. Mat. 168: 400-405.

Chao, C. M., Mulchandani, A. and Chen, W. (2002). Bacterial cell surface display of organophosphorus hydrolase

for selective screening of improved hydrolysis of organophosphate nerve agent. Appl. Environ. Microbiol. 68: 2026-2030.

Dragun, J., Kuffner, A. C. and Schneiter, R. W. (1984). Groundwater contamination . 1. 1 Transport and transformation of organic chemicals. Chem. Eng. 91: 65-70.

EPA, (1997) Review of Chlorpyrifos Poisoning Data. EPA, Washington, DC.

Greenhalgh, R., Dhavan, K. L. and Weinberger, P. (1980). Hydrolysis of fenitrothion in model and natural aquatic system. J. Agric. Food Chem. 28: 102-105.

Krieg, N. R. and Holt, J. G., (ed.) (1984). Bergey's Manual of Systematic Bacteriology, Vol. 1. The Williams and Wilkins Co., Baltimore, MD.

Li, X., Jiang, J., Gu, L., Ali, S.W., He, A. J. and Li, S. (2008). Diversity of chlorpyrifos degrading bacteria isolated from chlorpyrifos contaminated samples. Int. Biodeterior. Biodegr. 62: 331-335.

Liu, X., You, M. S., Wei, Y. Z., Liao, J. Y., Ye, L. L. and Chen, J. (2003). Isolation of chlorpyrifos degrading *Aspergillus* sp. Y and measurement of degradation efficiency. Chinese J. Appl. Env. Biol. 9: 78-80.

Mallick, B. K., Banerji, A., Shakil, N. A. and Sethunathan, N. (1999). Bacterial degradation of chlorpyrifos in pure culture and in soil. Bull. Environ. Cont. Toxicol. 62: 48-55.

Miyamoto, J., Kitagawa, K. and Sato, Y. (1966). Metabolism of organophosphorus insecticides by *Bacillus subtilis*, with special emphasis on Sumithion. Jpn. J. Exp. Med. 36: 211-225.

Ou, L. T. and Sharma, A. (1989). Degradation of methyl parathion by a mixed bacterial culture and a *Bacillus* sp. isolated from different soils. J. Agric. Food Chem. 37: 1514-1518.

Racke, K. D. and Coats, J. R. (1987). Comperative degradation of organophosphorus insecticides in soil: Specificity of enhanced microbial degradation. J. Agric. Food Chem. 36: 193-199.

Rani, M. S., Lakshmi, K. V., Devi, P. S., Madhuri, R. J., Aruna, S., Jyothi, K., Narasimha, G. and Venkateswarlu, K. (2008). Isolation and characterization of a chlorpyrifos degrading bacterium from agricultural soil and its growth response. African J. Microbiol. Res. 2: 26-32.

Sapozhnikova, Y., Bawardi, O. and Schlenk, D. (2004). Pesticides and PCBs in sediments and fish from the Salton Sea, California, USA. Chemosphere 55: 797-809.

Sethunathan, N. and Yoshida, T. (1973). A *Flavobacterium* sp. that degrades diazinon and parathion. Can. J. Microbiol. 19: 873-875.

Sharmila, M., Ramanand, K. and Sethunathan, N. (1989). Effect of yeast extract on the degradation of organophosphorus insecticides by soil enrichment and bacterial cultures. Can. J. Microbiol. 35: 1105-1110.

Singh, B. K., Walker, A., Morgan, J. A. W. and Wright, D. J. (2004). Biodegradation of chlorpyrifos by *Enterobacter* strain B-14 and its use in bioremediation of contaminated soils. Appl. Environ. Microbiol. 70: 4855-4863.

Somasundaram, L. and Coats, J. R. (1990). Influence of pesticide metabolites on the development of enhanced biodegradation. In: Racke, K. D. and Coats, J. R. (ed.) Enhanced Biodegradation of Pesticides in the Environment. American Chemical Society, Washington, D.C.

Vidya Lakshmi, C., Kumar, M. and Khanna, S. (2008). Biodegradation of chlorpyrifos in soil by Enriched Cultures. Curr. Microbiol. 58: 35-38.

Wang, X., Chu, X. Q., Yu, Y. L., Fang, H., Chen, J. and Song, F. M. (2006). Characteristics and function of *Bacillus latersprorus* DSP in degrading chlorpyrifos. Acta Pedologica Sinica 43: 648-654.

Yang, L., Zhao, Y.-H., Zhang, B.-X., Yang, C.-H. and Zhang, X. (2005). Isolation and characterization of a chlorpyrifos and 3,5,6-trichloro-2-pyridinol degrading bacterium. FEMS Microbiol. Lett. 251: 67-73.

Yasuno, M., Hirakoso, S., Sasa, M. and Uchida, M. (1965). Inactivation of some organophosphorus insecticides by bacteria in polluted water. Jpn. J. Exp. Med. 35: 546-563.

Plant Science Research 34 (1&2) : 23-26, 2012

Plant Science Research

ISSN 0972-8546

Genetic divergence in linseed (*Linum usitatissimum* L.) germplasm under late sown condition

J. Dash[1], B. S. Naik[2] and U. B. Mohapatra[3Ψ]

[1] Department of Botany, Jashipur College, Mayurbhanj, India

[2] AICRP, RRTTSS, Chipilima, Sambalpur, India

[3] Dept. of Biotechnology, Govt. of Odisha, Bhubaneswar, India

ARTICLE INFO

Article history:
Received : 12 November 2012
Received in revised form : 07 December 2012
Accepted : 08 December 2012

Keywords:
cluster means
linseed
Mahalanobis D^2 statistics
variance

ABSTRACT

One hundred forty genotypes were studied for their genetic divergence in late sown condition through cluster analysis. The results revealed that there were significant differences among linseed germplasm for eight traits. Under late sown conditions, days to 50% flowering and number of capsules per plant are two important characters to be selected to increase seed yield. Intercrossing of selected genotypes from both the distant cluster IV and I would provide enough scope for recombination breeding programme.

1. Introduction

Linseed or flax (*Linum usitatissimum* L.), is popularly known as *Atasi*, *Pesi*, *Phesi* or *Tisi* in Odiya. In Odisha, linseed is cultivated in 0.264 lakh hectares with an annual production of 0.119 lakh tonnes and the productivity level is 451kg / ha. (Anonymous*,* 2011). The low productivity of linseed is mainly due to low yield potential of the existing cultivars with poor crop husbandry. The important linseed growing districts are Mayurbhanj, Kalahandi, Nawapara, Nowrangpur, Keonjhar and Puri.

Though linseed is an important *rabi* oilseed crop of Mayurbhanj district, a significant number of farmers are forced to sow linseed about one month late due to excess moisture in the field (Dash *et al.,* 2011*)*. Now a days, farmers of Mayurbhanj district are sowing linseed one month later than the recommended schedule of the crop i.e. mid October. So an experiment was laid out to study genetic diversity of linseed genotypes in late sown condition, which will help to identify genetically diverse parents for recombination breeding programme.

2. Materials and methods

The crop was sown one month late during November i.e. on 22.11.2006 and 22.11.2007. The field trial was laid out in a Randomized Complete Block Design (RCBD) with two replications. Each genotype was sown in a single row of 3m length with a spacing of 30cm X 5cm between and within the row, respectively. The fertilizers were applied as basal and weedings were done at the right stage following recommended package of practices. Ten randomly selected competitive plants were used to record biometric observations of plant height (cm), number of primary branches / plant, number of capsules / plant, number of seeds / capsule and seed yield / plant (g). But days to 50% flowering, days to maturity, 1000 seed weight (g), reaction to *Alternaria* blight and reaction to wilt were recorded on whole row basis.

3. Results and discussion

Analysis of variance revealed highly significant differences among the genotypes for all the eight characters. 140 linseed genotypes were tested for genetic divergence to ascertain the nature of genetic variability existing among the genotypes. Classification using multivariate analysis of

Ψ *Corresponding author; Email:* ubmbotnou@gmail.com

genetic divergence aims at grouping the genotypes precisely and objectively into various clusters by adopting different methods. In the present investigation simultaneous variations in all the eight characters of 140 genotypes of linseed were tested for assessing the nature of genetic divergence among them following Mahalanobis (1928) D^2 statistics as described by Rao (1952) and group constellation was done following Tocher's method (Singh and Chaudhary, 1977).

Following the cluster analysis, by Tocher's method 140 linseed genotypes were grouped into 11 groups or clusters consisting of 2 to 68 genotypes. Cluster XI retained the highest number (68 genotypes) followed by cluster VI (27 genotypes). Cluster VIII contained 14 genotypes followed by cluster I, VII, and X contained 10, 6 and 5 genotypes respectively. Cluster II, III, IV, V and IX which had only 2 genotypes in each are presented in Table 1.

In the past, eco-geographical diversity has been largely relied as an index of genetic diversity. This criterion being only inferential, obviously, cannot be used for discrimination among the populations of same or similar geographical region. Published results are highly conflicting with regard to geographical distribution and genetic diversity. A number of workers indicated somewhat close relationship between the two (Sinha *et al*., 2001). On the other hand, a large number of crop plants including linseed with different breeding systems showed no parallelism between genetic diversity and eco-geographical distribution (Murthy and Arunachalam, 1966; Verma, 1996; Singh *et al*., 1999; Datta and Mani, 2003).

In the present study, all the eleven clusters contained genotypes from different locations. The clustering pattern revealed that the tendency of genotypes from diverse geographic regions to group together in one cluster might be due to similarity in requirements and selection approaches followed under domestic cultivation (Arunachalam and Ram, 1967). It was also observed that the genotypes belonging to the same state were distributed in different clusters indicating wide genetic diversity among genotypes

Table 1
Clustering pattern of 140 Linseed genotypes pooled over two years.

Cluster	No.of Genotypes	Genotypes
I	10	OL 3-1, OL 98-12-1,OL98-2-5, OL98-1-4, ACC.NO.442, 1052/RLC-27, RL-771, OLC-61, LCK-9814., LMH-78.
II	02	LIN-99289, PCA-12.
III	02	RLC-28, OL98-2-4.
IV	02	BAULK-2, JRF-4.
V	02	LCK-241, LMC-926.
VI	27	OL93418-2-2, OL98-11-4, LCK-14, RL-87, LMH-42, OL98-8-1, LC-1038, OL98-18-4, LC-54, LHCK-10, OL98-8-8, OL-4-1, RL-17, OL98-7-5, 1216/JRF-5, OL2-4, OL2-7, MLH-12, LMH16-5, RLC-3, OL98-2-2, LCK-206, OL98-3-I, BAUL-4-4, RLC-1, P650, OLC-22.
VII	6	JLT-32, Mayurbhanj Local, RLC-41, OL98-2-1, LC-1009, LCK-8901.
VIII	14	NML-4, LCK10-10, ACNO-1396, OL98-2-3, OL98-9-4, NL-129, LC-1049, OL98-1-2, GS-234,OL98-1-4, JRF-3, NL-9, OL98-17-6, RL-1011.
IX	02	OL98-18-3, OL98-7-2.
X	05	OL98-8-3, RLC-29, RLC-6, PCA-8, LCM-1020.
XI	68	OLC-58, LIN-2, LCK-3707, PLP-1, OL93418-1, PCA-18, LW36-3, POLF-19, RLC-27, OL92-16-3, LCK-119, OL98-16-7, LHCK-82, LCK-9733, RLA-71, SLS-27, JRF-5, RRL-1, LCK-216, SPS72-23-10, LIN-12, OL98-5-3, CI-1466, PCA-89, OL2-5, LCK-233-1, RLC-42, OL98-18-5, EC-41563, BAULK-1, LCK-9436, NL-142, NDL8804, OL98-11-2, PCA-7, LCK-8523, BAU-4708, LHCK-176, OL98-3-2, OL1-3RLC-44, OL7-7, OL3-2, LCK8132, CHIPILIMA-6, LMH-43, PKDL-8, NL-97, OL98-17-6, CHIPILIMA-3, SLS-26, 1396, LCK9816, LMH-16-5, OLC-37, OL98-5-1, LCK875, LMH-90-7, OL98-5-6, EC-1392, KL49-47, A95-13, LS-2323, PCA-9, BAULK-8, Padmimi, OLC-11, RLC-2.

Table 2
Estimates of average intra and inter cluster distances for 11 clusters involving 140 genotypes of linseed.

Cluster	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
I	4.999	4.814	4.834	4.332	4.436	5.149	5.189	5.32	5.643	4.932	5.169
II		1.016	2.643	2.701	1.628	4.315	3.668	4.39	2.289	4.22	3.907
III			1.033	2.861	2.4	4.429	4.527	4.559	3.677	4.422	4.058
IV				1.106	2.784	4.319	4.177	4.393	3.617	3.954	3.831
V					1.127	3.944	3.529	4.204	2.896	3.886	3.723
VI						5.221	5.058	5.327	5.18	5.036	5.081
VII							4.652	5.419	4.073	5.087	4.910
VIII								5.45	5.311	5.003	5.206
IX									1.438	4.907	4.613
X										5.051	4.897
XI											4.874

originating from the same geographic regions. The clustering pattern, thus, revealed lack of strict correspondence between genetic divergence and geographic distribution. This could be due to genetic drift and selection in different environments which caused greater diversity than geographic distances. Previous research work also observed non parallelism between genetic diversity and geographic distribution of the genotypes in linseed (Murthy and Arunachalam, 1966; Jeswani *et al.,* 1970; Asthana and Pandey, 1980; Sarkar, 2005).

The estimates of average intra and inter cluster distances among the 140 genotypes have been presented in Table 2. Using Mahalanobis D^2 statistics the highest intra-cluster distance was noticed in cluster VIII (5.45) followed by cluster VI (5.221). The least intra-cluster distance was reported in cluster II (1.016). The spectrum of intra cluster distance revealed that Cluster VIII contained 14 genotypes, but showed maximum intra cluster distance (5.45), which would be due to heterogeneous nature of the genotypes included in the cluster i.e some genetic divergence still existed among the genotypes.

The spectrum of intercluster distance ranges from 1.628 (Cluster II and V) to 5.643 (Cluster I and IX). The highest inter cluster distance of 5.643 was obserced between cluster IX and cluster I followed by 5.419 (Cluster VII and VIII) and 5.327 (Cluster VI and VIII). This indicated that the genotypes of cluster IX were distantly related to those of clusters I, and those of VIII to VII and VI. On the other hand, minimum intercluster distance was observed between cluster V and cluster II (1.628) indicating less divergence between the genotypes.

As regards the inter-cluster distance cluster IX showed maximum genetic distance from cluster I suggesting wide diversity between these groups. Crosses involving genotypes from these clusters are likely to produce wider and desirable recombinants and this could help in producing wider variable progeny. It has been pointed that selection of parent for hybridization should be done from two clusters having wider inter cluster distance to get maximum variability in segregating generations (Mahto, 1995; Singh *et al*., 1995; Mahto and Singh, 1997; Payasi, 2000; Sarkar, 2005; Naik, 2008).

The intra cluster means of eight yield parameters for different clusters revealed that Cluster I had maximum values in number of primary branches per plant (0.702), number of capsules per plant (18.382), and seed yield per plant (0.697g). Cluster II had minimum days to 50% flowering (68.38). Cluster III was characterized with minimum values in plant height (50.775 cm), number of seeds per capsule (6.813) and seed yield per plant (0.411g). Cluster IV had maximum days to 50% flowering (72.25) and minimum values in 1000 seed weight (4.887g). Cluster V showed maximum days to maturity (119.38) and plant height (54.975 cm) Cluster VII had maximum number of seeds per capsule (8.058). Cluster IX was characterized with minimum days to maturity (116.88), number of primary branches per plant (0.100) and number of capsules per plant (11.400) and maximum 1000 seed weight (6.913).

Under late sown conditions, days to 50% flowering and number of capsules per plant are two important criteria for selection to increase seed yield. Cluster IV and I had highest cluster means for days to 50% flowering and number of capsules per plant, respectively. Two genotypes,*viz.* BAULK- 2 and JRF- 4, whose mean number of capsules per

plant are 16.30 and 16.50 respectively were selected for days to 50% flowering from cluster IV. Similarly, four genotypes,*viz* OL3-1, OL98-2-5, OL98-1-4 and RL-771, whose mean for days to 50% flowering are 80.25, 74.75, 73.00 and 71.00 were selected for number of capsules per plant from cluster I , keeping the score of days to 50% flowering above the grand mean of 70.25. Inter crossing of selected genotypes from both the distant clusters IV and I would provide enough scope for recombination breeding programme to have varieties with higher days to 50% flowering and number of capsules per plant for improving seed yield in late sown conditions.

References

Anonymous (2011). Annual Report, All India Coordinated Research Project on Linseed (ICAR), Hyderabad.

Arunachalam, V. and Ram, J. (1967). Geographical diversity in relation to genetic divergence in cultivated sorghum. Indian J. Genet. 27: 369-378.

Asthana, A.N. and Pandey, V.K. (1980). Genetic divergence in linseed. Indian J. Genet. 40: 247-250.

Dash, J., Naik, B. S. and Mohapatra, U. B. (2011). Path coefficient analysis for seed yield in linseed (*Linum usitatissimum* L.) under late sown condition. J. Oilseeds Res. 28(1): 45-47.

Datta, S. and Mani, S. C. (2003). Genetic divergence in elite genotypes of basmati rice (*Oryza sativa* L.). Indian J. Genet. 63 (1) 73-74.

Jeswani, L. M., Murthy, B. R. and Mehra, R. B. (1970). Divergence in relation to geographical origin in a world collection of linseed. Indian J. Genet. 30:11-25

Mahalanobsis, P. C. (1928). A. Statistical study Chinese head measurement. J. Asiatic Soc. Bengal 25: 301-377.

Mahto, J. L. (1995). Genotype Environmental interaction, stability and genetic diversity study in linseed for yield and yield attributes under dry land situation. Madras Agric. J. 82 (11): 601-605.

Mahto, J. L. and Singh, S. N. (1997). Character association and genetic diversity in linseed under rainfed environments. J. Moharastra Agric. Univ. 21 (3): 471-477.

Murthy, B. R. and Arunachalam, V. (1966). The nature of genetic divergence in relation to breeding system in some crop plants. Indian J. Genet. 26 A: 188-198.

Naik, B. S. (2008). Euclidean cluster analysis in late sown linseed, (*Linum usitatissimum* L.). J. Oilseeds Res. 25 (2): 188-189.

Rao, C. R. (1952). Advanced Statistical Methods in Biometric Research. John Wiley and Sons, lnc. New York.

Singh, N. P., Singh, P. K. and Kumar, M. (1995). Genetic Variability and analysis of yield components in linseed germplasm. Annals Agric. Res. 16(2): 164-167.

Singh, R. K. and Chaudhary, B. D. (1977). Biometrical Methods in Quantitative Genetic Analysis. Kalyni Publishers, Ludhiana.

Singh, U. K. Mishra, S. B. and Thakur, R. (1999). Genetic divergence in boro rice. Oryza 36 (1): 76-77.

Sinha, P. K., Prasad, C., Prasad, K. and Mandal, N. P. (2001). Genetic divergence in upland rice varieties, Oryza 38(1&2): 28-34.

Payasi, S. K. (2000). Genetic divergence in linseed. Crop Research, 19 (1): 158-161.

Sarkar, T. K. (2005). Genetic Variability and character association in linseed (*Linum usitatissimum* L.) M. Sc (Ag) Thesis Odisha University of Agriculture and Technology, Bhubaneswar, Orissa, India.

Verma, O. P. (1996). Genetic divergence in linseed (*Linum usitatissimum* L.). J. Oilseeds Res. 13 (2): 225-228.

Plant Science Research 34 (1&2) : 27-33, 2012

Plant Science Research

ISSN 0972-8546

Diversity of plant growth promoting rhizobacteria (PGPR) in rice soils of Odisha

H. B. Bal[1Ψ] and T. K. Adhya[2]

[1] Laboratory of Soil Microbiology, Division of Crop Production, Central Rice Research Institute, Cuttack-753006, India

[2] School of Biotechnology, KIIT University, Bhubaneswar-751024, Odisha, India

ARTICLE INFO

Article history:
Received : 11 November 2012
Received in revised form : 26 November 2012
Accepted : 06 December 2012

Keywords:
plant growth promoting rhizobacteria
ACC deaminase
indole acetic acid
P-solubilization

ABSTRACT

Numerous species of soil bacteria which flourish in the plant rhizosphere, but may grow in, on, or around plant tissues, stimulate plant growth by various direct and indirect mechanisms. These bacteria are collectively known as PGPR (plant growth promoting rhizobacteria). The search for PGPR and investigation of their modes of action are increasing at a rapid pace as efforts are being made to exploit them commercially as biofertilizers. In this study a total of 27 bacteria having unique colony and cell morphology were isolated from the rice rhizosphere soil. These isolates were screened for plant-growth promoting (PGP) traits, including indole acetic acid (IAA) production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, P-solubilization, and siderophore, cyanide (HCN) and ammonia (NH_3) production. Percentage of isolates having IAA, ACC deaminase, P-solubilization, siderophore, HCN and NH_3 activities was 85.2%, 18.5%, 44.4%, 37%, 25.9% and 48.2% respectively. 11.1% of the total isolates did not have any PGP traits whereas the percentage of isolates having one and six PGP traits was 22.2% and 3.7% respectively. There was a positive correlation (0.630, $p<0.05$) between phosphate solubilizers and NH_3 producers and there was no significant relationship among other PGP traits in rhizobacteria. Further evaluation of the isolates exhibiting multiple PGP traits on soil–plant system is needed to uncover their efficacy as effective PGPR.

1. Introduction

Plant growth-promoting rhizobacteria (PGPR) offer an environment-friendly means for increasing productivity and sustainability in agriculture. A diverse array of free living bacteria including *Acetobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Klebsiella, Pseudomonas, and Serratia* are reported to enhance plant growth by increasing seed emergence, plant biomass, and crop yield (Glandorf *et al.,* 1994; Rodriguez *et al.,* 2008). These bacteria inhabit plant roots and affect plant growth promotion by mechanisms ranging from a direct influence such as increased solubilization of mineral phosphates and other nutrients, their uptake and/or production of plant growth regulators like indole acetic acid, gibberellic acid, cytokinins etc to indirect effects such as suppression of plant pathogens by producing siderophores, antibiotics (Glick, 1995; Lucy *et al.,* 2004; Raaijmakers *et al.,* 2009), chitinase, b-1,3-glucanase, protease, or lipase (Chet and Inbar, 1994). Many PGPR contains the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which cleaves ACC the physiological precursor of ethylene to ammonia and α-ketobutyrate and reduce the level of stress ethylene, conferring resistance and resulting better growth of plants under various abiotic stress (Glick *et al.,* 2007).

In the last 10 years, the number of PGPR that have been identified has seen a great increase, mainly because the role of the rhizosphere as an ecosystem has gained importance in the functioning of the biosphere and also because mechanisms of action of PGPR having been deeply studied. The beneficial effects of PGPR have been

Ψ *Corresponding author; E-mail:* himadribhubneswar@gmail.com

demonstrated for many crops. However, inconsistency in their field performance, attributed mainly to poor rhizosphere competence and lack of multiple plant growth-promoting activities, is the major limiting factor in realizing the full potential of these microorganisms (Lottmann *et al.,* 2000; Ahmad *et al.,* 2008). To get maximum benefits from the inoculation, the selection of the most-effective PGPR is a pre-requisite as the use of rhizobacterial isolates directly in the field without screening is a highly laborious procedure. However, there has been no standard approach for the selection of effective PGPR.

Isolation and study of native strains that are adapted to their environment may contribute to the formulation of an inoculant to be used in regional crops. Therefore, this study was designed to isolate rhizospheric bacteria from the soil samples of agricultural fields planted to rice and to screen for their multiple plant growth promoting (PGP) traits like phosphate mobilization, production of siderophore, indole acetic acid (IAA), ammonia, hydrogen cyanide (HCN) and ACC deaminase activity in order to identify potent strain(s) to be used as candidate PGPR with rice crop.

2. Materials and methods

2.1 Sampling and characterization of soils

The root adhering soil (RAS) samples were collected from the experimental rice fields at CRRI, Cuttack during September, 2009 at the tillering stage of the monsoon season (kharif) rice. The rice plants were carefully uprooted along with the soil and brought to the laboratory in polythene bags in portable cool chambers (~4°C). The non-rhizosphere soil was removed by vigorously shaking the uprooted rice hills leaving behind the rhizosphere soil strongly adhering to the roots (Ramakrishna and Sethunathan, 1982). The soil sample was analyzed for physic-chemical characteristics according to Spark *et al.* (1996). The soil was a typic haplaquept having pH 6.16, electrical conductivity 0.5 dS/m, cation exchange capacity 15.0 meq/g soil, organic carbon 0.86 %, total nitrogen 0.09 % and contained 25.9 % clay, 21.6%slit and 52.5%sand. The rhizosphere soil was used for the isolation of bacteria.

2.2 Isolation and characterization of rhizospheric bacteria

For the isolation of rhizospheric bacteria, 1 g of closely associated rhizospheric soil was added to 9 ml of sterile water and shaken for 30 min on a mechanical rotary shaker. Six fold dilutions were made and plated on to seven media, Jensen's N free medium, Luria Bertani (LB) agar medium, nutrient agar (NA), tryptose soy yeast extract agar (TSY), 1/1000 dilution of TSY media (TSY/1000), casein–peptone–starch–glycerol agar (CPSG) and soil extract agar (SEA) and incubated for 72 hours at 30^0C (Reichardt *et al.,* 1997). Rhizobial colonies were chosen based on their colony morphology and purified by streaking on nutrient agar plates (Holt *et al.,* 1994). Bacterial cultures were maintained on the respective slants at 4 ^{0}C and in 65% glycerol at -80 ^{0}C till further use.

Morpho-physiological and biochemical characters of the bacterial isolates were examined according to the Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994). Individual cultures grown on NA medium at 30^0C were examined for the colony morphological features. Motility and morphology were studied by phase contrast microscopy (Olympus BX-51, Olympus America Inc., USA). Gram staining was performed as per standard procedures with exponentially growing cultures. The bacterial isolates were tested for biochemical characteristics using standard methods (Cappuccino and Sherman, 1992).

2.3 Screening of bacterial isolates for their plant growth promoting traits

IAA production by the isolates in the presence of 0.2% L-Tryptophan (L-Trp) was detected by the method of Salkowski (Glickmann and Dessaux, 1995). Uninoculated control was kept for comparison. The quantity of IAA produced was determined by UV-VIS spectrophotometry (Specord 200, Analytic Jena, Germany) against a standard curve of IAA ranging from 0.01 to 0.1 mM (Leveau and Lindow, 2005). All measurements were made in five replicate samples and averaged.

Screening of bacterial isolates for ACC deaminase activity was based on their ability to use ACC as a sole nitrogen source in the minimal medium. The cultures were spot inoculated on DF salts minimal medium supplemented with 3 mM ACC as a nitrogen source. The plates were incubated for 3-4 days at 28 ± 2^0C. Isolates growing on DF minimal medium with ACC were purified and assayed for enzymatic activity by monitoring the amount of α-ketobutyric acid generated from the cleavage of ACC (Penrose and Glick, 2003). The ACC deaminase activity was expressed as the amount of α-ketobutyrate produced per mg of protein per hour.

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48-72 h at 28 ± 2^0C. Development of brown to yellow colour following nesslerization was a positive test for ammonia production (Cappuccino and Sherman, 1992).

All the isolates were screened for the production of

hydrogen cyanide by adopting the method of Lorck (1948). Briefly, nutrient broth was amended with 4.4 g glycine/l and bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 28 ± 2^0C for 4 days. Development of orange to red colour indicated HCN production.

Bacterial isolates were assayed for siderophores production on the Chrome azurol S agar medium (Sigma, Ltd.) described by (Louden *et al.,* 2011). Chrome azurol S agar plates were prepared and divided into equal sectors and spot inoculated with test organism (10 ml of 10^6 CFU/ml) and incubated at 28 ± 2°C for 48-72 h. Development of yellow-orange halo around the growth was considered as positive for siderophore production.

For studying phosphate solubilization, 5 il of overnight grown culture was spotted onto Pikovskaya's agar plates containing 2% tricalcium phosphate. The plates were incubated at 28°C for 24-48 h and observed for the appearance of the solubilization zone around the bacterial colonies (Mehta and Nautiyal, 2001).

3. Result and discussion

3.1 Isolation and characterization of bacteria

A total of 46 bacteria were isolated using seven different culture media. Highest numbers of the bacteria (9) were isolated using LB agar medium which was followed by Jensen's agar medium (8) and equal numbers of bacteria (6) were isolated by using NA, TSY, TSY/1000 and CPSG agar medium and lowest number of bacteria (5) were isolated using SEA media. After growing all the isolates on NA medium to study colony morphology and cell morphology under phase contrast microscope, 27 numbers of bacteria having unique colony morphology and cell morphology were selected for further characterization Table 1. Morphological and biochemical characteristics of the 24 isolates having PGP activities were studied (Table 2). Except CR6, 7 and 46 all the strains were gram positive in reaction and almost all the isolates were rod shaped except CR29 and 33.

3.2 Plant growth promoting traits of selected isolates

Extensive research has demonstrated the potential of PGPR in plant growth improvement (Govindarajan *et al.*, 2006; Cakmakci *et al.*, 2007). The abilities of PGPR bacterial isolates on production of plant growth promoting substance (Khalid *et al.*, 2004; Bal *et al.*, 2012) have been well documented and were focused in this study. Screening results of PGP traits have been depicted in Table 1 and 3. The results showed that 11.1% of the total isolates did not have any PGP traits and 3.7% isolates showed all the six PGP traits studied. Highest number of the rhizobacteria (29.6%) showed three PGP traits. Percentage of rhizobacteria displaying single PGP traits was 22.2 % which was followed by 14.8% bacteria having five PGP traits, 11.1% bacteria having two PGP traits and 7.4% bacteria having four PGP traits.

IAA production was reported in 85.2% of the isolates (Fig.1) highlighting the enormous potential these organisms have to contribute to a plant's endogenous pool of IAA. Generally, microorganisms isolated from the rhizosphere and rhizoplane of various crops have revealed higher potential of IAA production than those from the root free soil (Sarwar and Kremer, 1995a, b; Arshad and Frankenberger, 1998).

Table 1
Isolation of bacteria from rice rhizosphere and screening for PGP traits

Media	No. of bacteria isolated	IAA	ACCd	P-Solubilization	Siderophore	HCN	NH_3
TSY (%)	4/27(14.8%)	3/23(13.0%)	1/5(20%)	2/12(16.7%)	1/10(10%)	1/7(14.3%)	2/13(15.4%)
TSY/1000(%)	6/27(22.2%)	6/23(26.1%)	0	3/12(25.0%)	2/10(20%)	2/7(28.6%)	3/13(23.1%)
NA(%)	3/27(11.1%)	3/23(13.0%)	0	1/12(8.3%)	1/10(10%)	0	2/13(15.4%)
LB(%)	2/27(7.4%)	1/23(4.3%)	0	0	1/10(10%)	0	1/13(7.7%)
Jensen's(%)	5/27(18.5%)	4/23(17.4%)	1/5(20%)	3/12(25.0%)	2/10(20%)	2/7(28.6%)	2/13(15.4%)
CSGP(%)	4/27(14.8%)	3/23(13.0%)	2/5(40%)	2/12(16.7%)	1/10(10%)	1/7(14.3%)	2/13(15.4%)
SEM(%)	3/27(11.1%)	3/23(13.0%)	1/5(20%)	1/12(8.3%)	2/10(20%)	1/7(14.3%)	1/13(7.7 %)
SUM (%)	27	23/27(85.2%)	5/27(18.5%)	12/27(44.4%)	10/27(37.0%)	7/27(25.9%)	13/27(48.1%)

Table 2
Biochemical characterization of the isolates

Biochemical test	Isolates											
	CR1	CR5	CR6	CR7	CR8	CR9	CR10	CR11	CR12	CR14	CR15	CR16
Gram reaction	+	+	-	-	+	+	+	+	+	+	+	+
Cell shape	Rod	Rod	Rod	Rod	Rod	Thin, rod	Rod	Rod	Rod	Thick, Rod	Rod	Rod
Colony Color*	W	Y	LO	Y	P	T	T	P	W	Y	W	Y
Motility	+	+	+	-	-	+	+	+	+	+	-	+
MR	+	+	+	-	+	-	+	+	+	-	+	-
MRVP	+	-	-	+	+	-	-	-	+	+	-	+
Citrate	-	+	+	-	+	+	+	-	+	-	-	+
Nitrate	-	-	+	+	+	+	-	-	-	+	+	-
Oxidase	+	+	+	-	+	-	+	+	+	-	+	-
Catalase	-	-	-	-	+	+	-	-	+	+	-	+
Starch	-	-	+	+	-	-	+	-	-	+	-	+
Tributyrin	+	-	-	+	-	+	-	+	-	+	-	-
Tween 80	-	+	-	+	-	-	-	+	-	-	+	+
Gelatin	-	-	+	-	-	+	-	-	+	-	-	+
Casein	-	+	-	-	+	-	+	+	-	-	+	-
Urease	+	-	-	-	+	+	+	-	+	+	+	+

Biochemical test	Isolates											
	CR21	CR28	CR29	CR33	CR34	CR36	CR37	CR39	CR41	CR43	CR44	CR46
Gram reaction	+	+	+	+	+	+	+	+	+	+	+	-
Cell shape	Rod	Rod	Coccus	Coccus	Rod	Rod	Thick rod	Rod	Thick rod	Rod	Rod	Rod
Colony Color*	T	W	W	W	W	W	BT	W	BT	W	ST	O
Motility	-	+	+	-	-	+	-	+	+	+	-	-
MR	+	-	+	+	+	-	+	-	+	+	+	-
MRVP	+	+	-	-	-	+	+	-	+	-	-	+
Citrate	-	+	-	+	+	+	-	+	-	-	+	-
Nitrate	+	-	+	+	-	+	+	-	-	+	+	-
Oxidase	-	+	+	-	-	-	-	+	+	+	-	+
Catalase	+	+	-	-	+	-	+	-	-	+	+	-
Starch	-	-	+	-	-	+	-	+	-	-	-	+
Tributyrin	+	-	-	+	-	-	+	+	-	+	+	-
Tween 80	+	+	-	-	+	-	-	-	+	+	-	+
Gelatin	-	-	+	+	-	-	+	+	-	+	+	-
Casein	+	-	+	+	-	+	-	-	-	+	-	+
Urease	-	-	+	+	+	-	-	-	+	+	-	-

*Colony color; T-Translucent; W-White; BT-Blue and translucent; ST-Semi-translucent; O-Orange; Y-Yellow; LO-Light orange; P-Pink

Table 3
PGP activities of the selected isolates from rice rhizosphere

Isolates	IAA	ACCd	P-Solubilization	Siderophore	HCN	NH_3
CR1	+	+	+	+	+	+
CR3	-	-	-	-	-	-
CR5	+	-	+	-	-	+
CR6	+	-	-	-	-	-
CR7	+	-	+	+	+	+
CR8	+	-	-	+	-	+
CR9	+	-	+	-	-	-
CR10	+	-	+	-	+	+
CR11	+	-	-	-	-	-
CR12	+	-	-	-	-	-
CR14	+	-	-	+	-	+
CR15	+	-	+	-	-	+
CR16	+	-	-	-	-	-
CR20	-	-	-	-	-	-
CR21	+	-	-	+	-	+
CR28	+	-	+	+	+	+
CR29	+	+	+	+	+	-
CR30	-	-	-	-	-	-
CR33	+	-	+	-	-	+
CR34	+	-	-	-	-	-
CR36	+	+	+	-	+	+
CR37	-	-	+	+	-	+
CR39	+	+	-	-	-	-
CR41	+	-	-	-	-	-
CR43	+	-	+	+	-	+
CR44	+	-	-	+	+	-
CR46	+	+	-	-	-	-

Microorganisms having ACC deaminase activity enhances plant growth indirectly by reducing the stress ethylene level in plant in presence of different abiotic stress. In this study only 18.5% of the rhizobacterial strains developed colony on DF salt minimal medium containing ACC as the sole nitrogen source implying presence of ACC deaminase activity in these isolates. However, presence of ACC deaminase activity was much less common than any other PGP traits. This is in agreement with earlier reports that only a minority of soil microorganisms possess ACC deaminase (Honma and Shimomura, 1978; Glick *et al.,* 1999).

Phosphorus is one of the major nutrients, second only to nitrogen in requirement for plant growth. The capacity to solubilize precipitated phosphates and enhance phosphate availability to rice is a promising attribute for the selection of bacteria capable of increasing available P in rhizosphere under field conditions (Verma *et al.*, 2001). In comparison to non-rhizospheric soil, a considerably higher concentration of phosphate-solubilizing bacteria is commonly found in the rhizosphere. In this study 44.4% of the total isolates were having P-solubilizing activity (Fig.1). Another important trait of PGPR, that may indirectly influence the plant growth, is the production of siderophores. They bind to the available form of iron Fe^{3+} in the rhizosphere, thus making it unavailable to the phytopathogens and protecting the plant health. In the present investigation 37% of the total isolates were siderophore producing rhizobacteria (Fig.1).

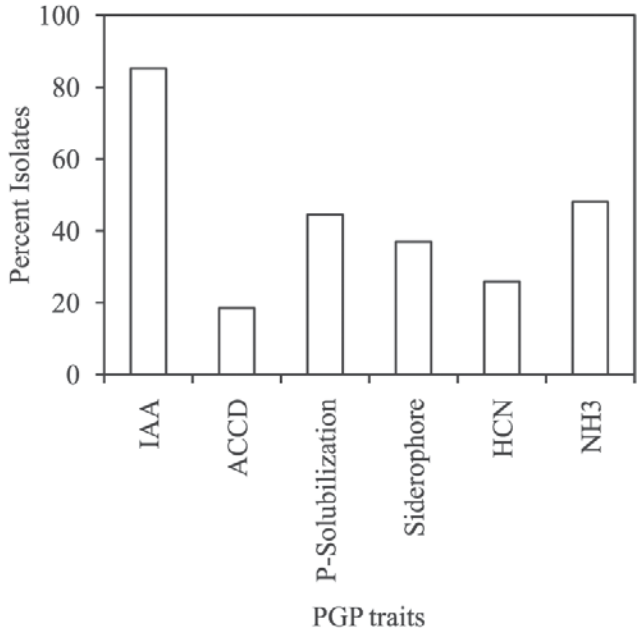

Fig.1. Percent of isolates having different PGP activities

solubilization and NH_3 production PGP traits of the isolates. Nevertheless, there was no significant relationship among other PGP traits possessing rhizobacteria.

Some of the above-tested isolates could exhibit more than two or three PGP traits, which may promote plant growth directly or indirectly or synergistically. Multiple PGP activities among PGPR have been reported by other workers while such findings on indigenous isolates of India are less commonly explored (Gupta *et al.*, 1998). Further studies on the performance of such native isolates and their mutants on the growth of plant will uncover the mechanism and potential of these PGPR exhibiting multiple PGP traits.

4. Conclusion

The search for a diverse group of rhizobacteria with useful PGP traits from various crop sources paves the way for the reduction of costs associated with the use of fertilizer nutrients as well as minimizes the risk of pollution from continuous application of chemical fertilizers. Use of strains with multiple PGP properties in particular would help to increase crop productivity on a sustainable basis. The effects of PGPRs on the growth of plants are well known for many crops. Indigenous wild isolates exhibiting multiple PGP traits *in vitro* are expected to influence plant growth and yield of the crops alone or in combination with other PGPR.

Table 4
Correlation coefficients of analysis of relationship among all the six PGP traits of the isolated rhizobacteria

	IAA	ACCd	P Solubilization	Siderophore	HCN	NH_3
IAA	1					
ACCd	0.199	1				
P- Solubilization	0.163	0.149	1			
Siderophore	0.104	0.029	0.240	1		
HCN	0.247	0.371	0.491	0.421	1	
NH_3	0.193	-0.078	0.630*	0.489	0.276	1

*Significant at $p \leq 0.05$

In the present investigation 25.9% and 48.1% of the total isolates were positive for HCN and NH_3 production (Fig.1). These two traits are very important when considering field applications, as plant resistance in a non-sterile environment will be potentially increased if the associated bacteria having HCN production potential. The capacity of some bacterial species to produce NH_3 also enhances plant growth. These traits can influence plant growth in various ways, although it is probably the combination of the diverse PGP traits of the used bacterial strain that is responsible for increase in growth of the cultivated crop.

Table 4 shows the results of the correlation analysis among the PGP traits possessing rhizobacteria. There was a negative correlation (-0.078, $p \leq 0.05$) between ACC deaminase and NH_3 production traits of the isolates. There was a positive correlation (0.630, $p \leq 0.05$) between phosphate

References

Ahmad, F., Ahmad, I. and Khan, M. S. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth-promoting activities. Microbiol. Res. 163: 173-181.

Arshad, M. and Frankenberger, W. T. (1998). Plant growth-promoting substances in the rhizosphere: microbial production and function. Adv. Agron. 62: 45-151.

Bal, H. B., Nayak, L., Das, S. and Adhya, T. K. (2012). Isolation of ACC deaminase producing PGPR from rice rhizosphere and evaluating their plant growth promoting activity under salt stress. Plant Soil, doi:10.1007/s11104-012-1402-5.

Cakmakci, R., Erat, M., Erdogan, U. and Donmez, M. F. (2007). The influence of plant growth-promoting rhizobacteria on growth and enzyme activities in wheat and spinach plant. J. Plant Nutr. Soil Sci. 170: 288-295.

Cappuccino, J. C. and Sherman, N. (1992). Negative staining. In: Cappuccino, J. C., Sherman, N. (ed.), Microbiology: A Laboratory Manual. 3rd edn. Benjamin/ Cummings Pub Co. Redwood City. pp. 125-179.

Chet, I. and Inbar, J. (1994). Biological control of fungal pathogens. Appl. Biochem. Biotechnol. 48: 37-43.

Glandorf, D. C. M., Sluis, I. V., Anderson, A. J., *et al.* (1994). Agglutinization, adherence, and root colonization by fluorescent pseudomonads. Appl. Environ. Microbiol. 60: 1726-1733.

Glick, B. R. (1995). The enhancement of plant growth by free-living bacteria. Can. J. Microbiol. 41: 109-117.

Glick, B. R., Patten, C. L., Holguin, G. and Penrose, D. M. (1999). Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria. Imperial College Press, London, United Kingdom.

Glick, B. R., Cheng, Z. Y., Czarny, J. and Duan, J. (2007). Promotion of plant growth by ACC deaminase producing soil bacteria. Eur. J. Plant Pathol. 119: 329-339.

Glickmann, E. and Dessaux, Y. (1995). A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. Appl. Environ. Microbiol. 61: 793-796.

Govindarajan, M., Revathi, G. and Lakshminarasimhan, C. (2006). Improved yield of micropropagated sugarcane following inoculation by endophytic *Burkhoderia vietnamiensis*. Plant Soil 280: 239-252.

Gupta, A., Saxena, A. K., Murali, G. and Tilak, K. V. B. R. (1998). Effect of plant growth promoting rhizobacteria on competitive ability of introduced *Bradyrhizobium* sp. (Vigna) for nodulation. J. Sci. Ind. Res. 57: 720-725.

Honma, M. and Shimomura, T. (1978). Metabolism of 1-aminocyclopropane-1-carboxylic acid. Agricul. Biol. Chem. 42: 1825-1831.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. and Williams, S. T. (1994). Bergy's Manual of Determinative Bacteriology, Ninth ed. Williams and Wilkins Pub., MD, USA.

Khalid, A., Arshad, M. and Zahir, Z. A. (2004). Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. J. Appl. Microbiol. 96: 473-480.

Leveau, J. H. J. and Lindow, S. E. (2005). Utilization of the plant hormone indole-3-acetic acid for growth by *Pseudomonas putida* strain 1290. Appl. Environ. Microbiol. 71: 2365-2371.

Lorck, H. (1948). Production of hydrocyanic acid by bacteria. Physiol. Plant. 1: 142-146.

Lottmann, J., Heuer, H., de Vries, J *et al.* (2000). Establishment of introduced antagonistic bacteria in the rhizosphere of transgenic potatoes and their effect on the bacterial community. FEMS Microbiol. Ecol. 33: 41-49.

Louden, B. C., Haarmann, D. and Lynne, A. M. (2011). Use of blue agar CAS assay for siderophore detection. J. Microbiol. Biol. Edu. 12: 51-53.

Lucy, M., Reed, E. and Glick, B. R. (2004). Applications of free living plant growth-promoting rhizobacteria. Antonie van Leeuwenhoek 86: 1-25.

Mehta, S. and Nautiyal, C. S. (2001). An efficient method for qualitative screening of phosphate solubilizing bacteria. Curr. Microbiol. 43: 51-56.

Penrose, D. M. and Glick, B. R. (2003). Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol. Plant. 118: 10-15.

Raaijmakers, J. M., Paulitz, T. C., Steinberg, C., Alabouvette, C. and Moënne-Loccoz, T. (2009). The rhizosphere: a playground and battlefield for soil-borne pathogens and beneficial microorganisms. Plant Soil 321: 341-361.

Ramakrishna, C. and Sethunathan, N. (1982). Stimulation of autotrophic ammonium oxidation in rice rhizosphere soil by the insecticide carbofuran. Appl. Environ. Microbiol. 44: 1-4.

Reichardt, W., Mascariña, G., Padre, B. and Doll, J. (1997). Microbial communities of continuously cropped, irrigated rice fields. Appl. Environ. Microbiol. 63: 233-238.

Rodriguez, H., Vessely, S., Shah, S. and Glick, B. R. (2008). Effect of a nickel-tolerant ACC deaminase-producing *Pseudomonas* strain on growth of non-transformed and transgenic canola plants. Curr. Microbiol. 57: 170-174.

Sarwar, M. and Kremer, R. J. (1995a). Determination of bacterially derived auxins using a microplate method. Lett. Appl. Microbiol. 20: 282-285.

Sarwar, M. and Kremer, R. J. (1995b). Enhanced suppression of plant growth through production of L-tryptophan-derived compounds by deleterious rhizobacteria. Plant Soil 172: 261-269.

Spark, D. L., Page, A. L., Summer, M. E., Tabatabai, M. A. and Helmke, P. A. (1996). Methods of soil analysis, part 3, chemical methods. American Society of Agronomy, USA.

Verma, S. C., Ladha, J. K. and Tripathi, A. K. (2001). Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. J. Biotechnol. 91: 127-141.

Plant Science Research 34 (1&2) : 34-38, 2012

Plant Science Research

ISSN 0972-8546

Diversity study of predominant fungi from the sediments of mangroves at Mahanadi delta and its adjoining areas

K. Sahoo[1Ψ], N. K. Dhal[1] and R. Das[2]

[1] Environment and Sustainability Department, CSIR-Institute of Mineral and Materials Technology, Bhubaneswar-751013, Odisha

[2] P.G. Dept. of Botany, Utkal University, Bhubaneswar-751004, Odisha

ARTICLE INFO

Article history:
Received : 02 November 2012
Received in revised form : 27 November 2012
Accepted : 29 November 2012

Keywords:
Mangrove
Aspergillus
Trichoderma
Penicillium
Acremonium
Fusarium

ABSTRACT

Microorganisms in mangrove areas perform complex interactions for nutrient and ecological balances. Marine fungi play an important role in nutrient regeneration cycles as decomposers of dead and decaying organic matter. Since a very little is known about fungal populations in Mahanadi delta mangroves, the present study has been conducted to analyze the fungal diversity in relation to soil physico-chemical properties in the Mahanadi delta and its adjoining areas, a tropical mangrove ecosystem in India. In the study, ten sediment samples have been collected from different mangrove areas and the physico-chemical as well as fungal diversity study has been carried out. The physico-chemical parameters varied significantly among all sites. The pH was maximum (pH=8.3) at MHS-2 and minimum (pH=4.3) at DVS-1, organic carbon content was maximum (48.48mg/gm soil) and minimum (14.1 mg/gm soil) at MHS-5, salinity was maximum (3.84 PSU) at DVS-1 sediments. The fungal diversity was maximum ($8.56\pm0.48 \times 10^5$ cfu/gm soil) at DVS-4 sediment sample. The most dominant genera among all the fungi was *Aspergillus*. The occurrence of other genera such as *Trichoderma, Penicillium, Acremonium, Fusarium* etc. were also found in the different sampling sites. This study revealed the presence of diverse fungi in the mangroves of Mahanadi delta which provides information regarding better utilization of the industrially potent marine fungal groups for valuable product formation such as antibiotics, surfactants, antioxidants, industrial enzymes, metal-tolerant enzymes, stress proteins, food preservatives etc.

1. Introduction

Mangrove forests are among one of the world's most productive tropical ecosystems (Kathiresan, 2000). The mangrove ecosystem is composed of various organisms such as bacteria, fungi, actinomycetes, microalgae, invertebrates, birds, mammals etc. Mangrove forests are biodiversity "hotspots" for marine fungi (Shearer *et al.,* 2007). Mangrove trees are fascinating study objects for the mycologist because the bases of their trunks and aerating roots are permanently or intermittently submerged. Thus, terrestrial fungi and lichens occupy the upper part of the trees and marine species occupy the lower part (Kohlmeyer, 1969). At the interface there is an overlap between marine and terrestrial fungi (Sarma and Hyde, 2001). Mangrove fungi constitute the second largest ecological group of the marine fungi (Sridhar, 2004). The latest estimate of marine fungi is 1,500 species, which excludes those form lichens, and many of them are new or inadequately described species (Hyde *et al.,* 1998, Zhong-shan *et al.,* 2009). Fungi play important role in the ecological processes occurring in mangroves and which are also involve in organic matter decomposition pathways, despite the fact that a large fraction of the carbon processing in mangroves happens in the anoxic bulk sediment. Mangrove communities are recognized as highly productive ecosystems that provide large quantities of organic matter to adjacent coastal water in the form of detritus. Microbial activity is responsible for major nutrients transformations within a mangrove ecosystem. The biochemical versatility and diversity of rare microorganisms represent an enormous variety of genes that are still unknown. So it is open up new areas of

Ψ *Corresponding author*; E-mail: kalpanactc@gmail.com

biotechnological explorations, which drive the necessity to isolate and culture these organisms. The objectives of the present study include the study of physico-chemical characterization and fungal diversity of mangrove sediments of Mahanadi delta and its adjoining areas of Odisha which is less explored.

2. Materials and method

2.1 Study area

Samples have been collected from Mahanadi delta and its adjoining areas located at Long. 20^0 17' N to Lat. 86^0 42' E occupying an area of 9,000 sq. km. The mangrove vegetation is luxuriant near the estuaries of the Devi river situated at Long. 19^0 58' N and Lat. 86^0 22' E which are the adjoining areas of Mahanadi and in the Protected Forest along the creeks of Boman nadi of Bitikolia estuary. Since the mangrove forest of the Mahanadi delta receives freshwater from three rivers such as Mahanadi, the Brahmani and Baitarani, they are rich in species diversity and dense. The sediment is generally sandy clay in the adjoining areas.

2.2 Collection of samples

The sediment samples were collected in sterile condition with the help of a borer. Samples were collected in big zipper polythene bags. Two sets of sample for physico-chemical and microbiological analysis were collected from each site. After collection the samples were labled properly, marked well and kept in ice box and carried to the laboratory immediately. In the laboratory the samples were stored at a temperature of 4^0C till the time of analyses/study. Samples were collected from Kansardi (MHS-1), near light house (MHS-2 & MHS-3), Ghangholia jora site (MHS-4) and MHS-5 of Mahanadi estuary whereas Machamachikuda (DVS-1), Bandar (DVS-2), Nadiakhia (DVS-3), Nentai (DVS-4) and Kiakhala (DVS-5) of Devi mangrove estuary of Odisha (Fig.1).

2.3 Analysis of physicochemical parameters

pH and conductivity of the sediment samples were measured by using a digital pH and conductivity meter. Salinity, alkalinity and total phosphorous were analyzed following the methodology of APHA (1998). The oven dried sediment samples were powdered with grinder (Tetsch, model, RM 100). Then required amount of grounded sample was taken for total OC analysis following the methodology by Wakely and Black (1934).

2.4 Isolation and identification of fungi

The sediments were air dried aseptically for 7-10 days. Isolation of fungi from mangrove sediments was carried out by spread plate method. The samples were plated on different solid medium i.e. Potato Dextrose Agar for the isolation fungal colonies and kept in incubator for growth at 28-30^0C for 48-72 hrs. After enumeration by counting the Colony forming units (CFU) of the microbes, the isolates were sub-cultured repeatedly for isolation of pure culture and maintained in agar slants at 4^0C. After isolation, the fungal colonies were identified by studying their morphological and microscopical characteristics. Morphological characterization was done by studying the upper and lower surface of the culture plate whereas microscopical study has been done by using Lactophenol cotton blue (LPCB) staining.

3. Results and discussion

Different physico-chemical parameters of the mangrove sediments were investigated such as pH, conductivity, salinity, OC, alkalinity and TP of the sediments (Fig. 2). The pH of DVS-1 was least 4.32±0.12 and maximum i.e. 7.36±0.21 in DVS-4; conductivity was minimum i.e. 1327±113 at DVS-2 and highest i.e. 1675±138 µS/cm in DVS-5; temperature

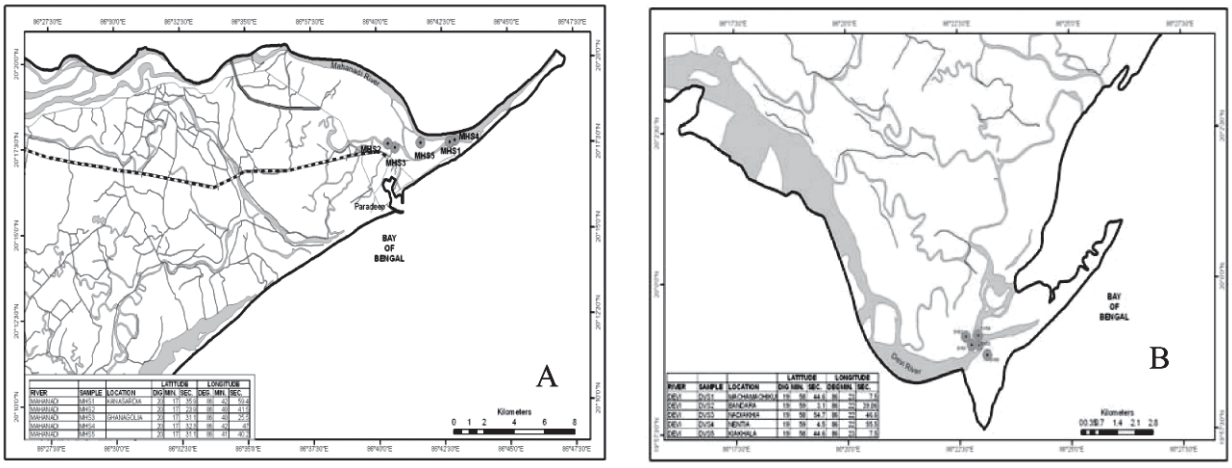

Fig.1. Sampling sites of Mahanadi delta (A) and its adjoining areas (B) of mangrove estuary

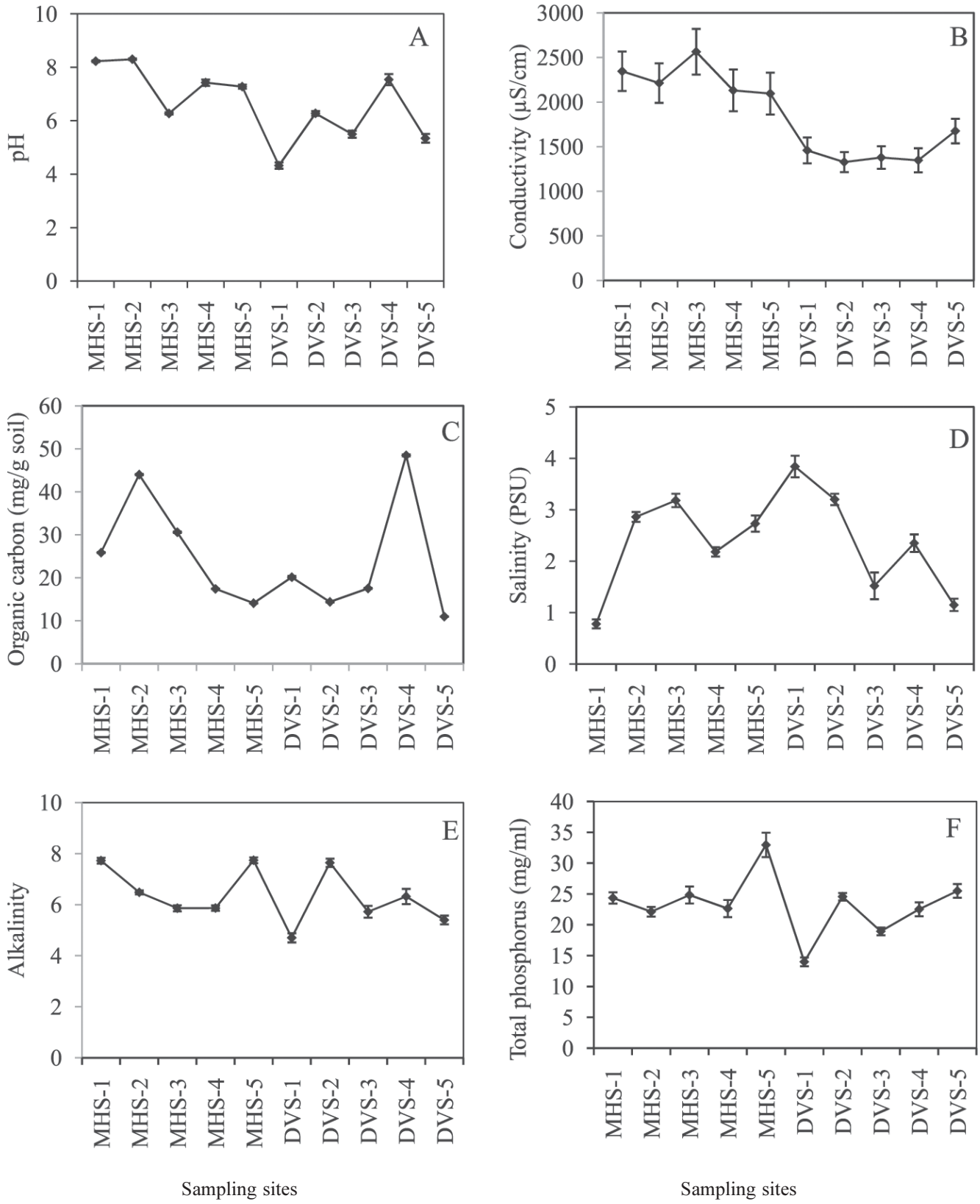

Fig.2. Physico-chemical characterization of sediment samples ; A-pH, B-Conductivity, C-Organic carbon content, D-Salinity, E-Alakalinity, F-Total Phosphorus content

ranged from 22±1.05 to 24±0.98 ^{0}C at DVS-1 and DVS-3 respectively; salinity varied between 1.15 ±0.12 PSU at DVS-5 to 3.84±0.21 PSU at DVS-1; organic carbon varied from 10.98±0.055 at DVS-5 to 48.48±0.05mg/gm at DVS-4; alkalinity was maximum at DVS-1 i.e. 4.7±0.18 to 7.64±0.16 at DVS-2; total phosphorus varied from 14.23±0.61 to 25.5±1.11 mg/ml at DVS-1 and DVS-5 (Fig.2). The total colony forming unit varied a lot in five different sampling sites of Devi. The total CFU was 3.56±0.39, 6.76±0.29, 5.14±0.11, 1.42±0.36, 0.92±0.02, 5.12±0.33, 1.84±0.26, 4.18±0.26, 8.56±0.48 and 0.94±0.03 (x 10^5) at sediments of MHS-1, MHS-2, MHS-3, MHS-4, MHS-5, DVS-1, DVS-2, DVS-3, DVS-4 andDVS-5 respectively (Fig.3). The fungal diversity study has been carried out for the collected sediments. The fungal diversity of sediments of MHS-2 and DVS-4 were maximum. Among all the fungi the most dominant were found to belong to the genus

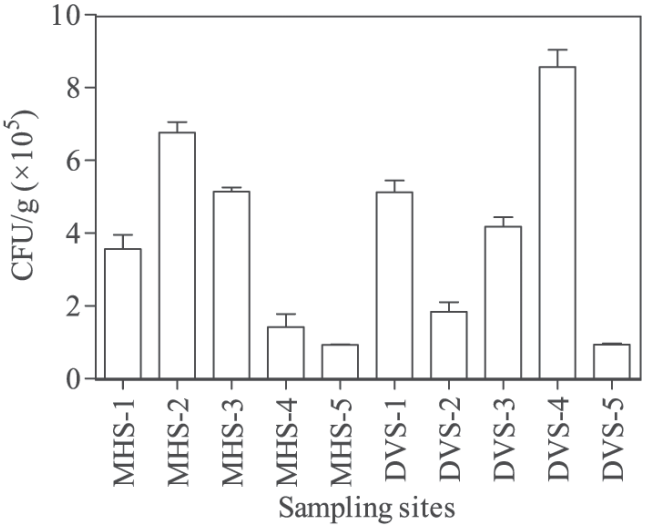

Fig.3. Total CFU (x 10^5/g) of sediment fungi from sediments

Table 1
Predominant fungal diversity in different sampling sites of mangrove area of Mahanadi delta

Sl. No.	Name of the strains	MHS-1	MHS-2	MHS-3	MHS-4	MHS-5	DVS-1	DVS-2	DVS-3	DVS-4	DVS-5
1.	*Aspergillus niger*	+	+	+	++	+	+	-	+	++	+
2.	*Aspergillus fumigatus*	+	+	+	++	-	+	+	+	++	-
3.	*Acremonium kiliense*	+	-	-	+	+	++	-	-	+	+
4.	*Penicillium citrinum*	+	-	+	-	+	+	-	+	-	+
5.	*Trichoderma viride*	++	+	-	+	-	++	+	-	+	-
6.	*Aspergillus oryzae*	+	+	-	+	-	+	-	-	+	-
7.	*Penicillium chrysogenum*	+	+	+	-	+	+	+	-	-	+
8.	*Aspergillus flavus*	-	+	-	-	+	-	-	-	+	-
9.	*Trichoderma* sp.	-	+	+	+	-	+	-	+	-	-
10.	*Aspergillus* sp.	+	-	+	-	+	-	+	-	-	-
11.	*Aspergillus* sp.	-	+	+	+	-	-	-	-	-	-
12.	*Aspergillus flavus*	-	-	-	+	-	+	-	-	+	+
13.	*Penicillium* sp.	+	+	+	-	+	-	-	+	-	+
14.	*Fusarium oxysporum*	-	+	-	-	-	+	-	-	-	-
15.	*Trichoderma virens*	-	+	-	-	-	+	-	+	-	-

Aspergillus. Other fungi belonging to the genera *Penicillium, Trichoderma, Acremonium, Fusarium* were also observed in the mangrove sediments which may be due to the presence of different nutrients in the sediments (Table 1).

Mangrove ecosystem environmental parameters affecting the community of soil fungi have been studied over many years (Holguin *et al*., 1999). Microbial activity is responsible for major nutrient transformations within a mangrove ecosystem (Alongi *et al.*, 1993; Holguin *et al.,* 1999). Organic carbon represents the organic matter in the sediments and this is of potential significance for aquatic productivity. The mangrove ecosystem of the study area had shown significant variation in pH and salinity. Despite inherent limitations, viable count of fungal population in our study as it would reflect relatively abundant and functionally dominant microbial communities (Nannipieri *et al.,* 2003, Das and Dangar, 2008). By consuming the dissolved organic carbon present in interstitial waters, microbial population in mangrove sediments prevent the export of this form of carbon to adjacent ecosystems, such as pelagic

food or adjacent coastal areas (Alongi *et al.,* 1993; Boto *et al.*, 1989). Microbial diversity comprises a wide range of microbes than any other living group of organisms of the world. Average fungal population in mangrove sediments was maximum at Nentai of Devi mangrove sediments i.e. $8.56\pm0.48x10^5$ cfu/g soils (Das and Dangar, 2008). However, Gonzalez-Acosta *et al*. (2006) have recorded more microbes (10^9- 10^{11}cfu/ml water) in a Mexican mangrove forest. Out of microbial populations fungal diversity in the estuarine and marine sediments vary in density with varying regions and also among various sites. Thus, they have worldwide distribution which indicates adaptability to extremely varied environmental conditions.

4. Conclusion

A range of fungi occur frequently in the mangrove ecosystem, although these differ as to their location. Some fungi certainly occur more frequently than others. Many factors will have an effect on species/frequency of occurrence either individually or synergistically. This could be attributable to the geographical proximity and similar climatic factors prevailing in both the delta systems. However, minor differences could be due to subtle local environmental factors. However this study is a preliminary study of fungal diversity in Mahanadi and its adjoining areas of Odisha. Correlation study showed that microbial diversity is strongly related to organic carbon. The most dominant fungi belong to the genus *Aspergillus*. It requires further study to establish relation between microbes and plants which is very much essential for conservation point of view as well as to protect the coastal areas against cyclones, storm and also tsunami.

Acknowledgement

The authors are thankful to Prof. B. K Mishra, Director, Institute of Minerals and Materials Technology, Bhubaneswar for providing necessary facilities for carrying out the research work.

References

Alongi, D. M., Christoffersen, P. and Tirendi, F. (1993). The influence of forest type on microbial-nutrient relationships in tropical mangrove sediments. J. Exp. Mar. Biol. Ecol. 171: 201-223.

APHA (American Public Health Association). (1998). US Standard methods for the examination of water and waste water, 19th edition, American Public Health Association, Washington DC.

Boto, K. G., Alongi, D. M. and Nott, A. L. J. (1989). Dissolved organic carbon-bacteria interactions at sediment-water interface in a tropical mangrove system. Mar. Ecol. Prog. Ser. 51: 243-251.

Das, J. and Dangar, T. K. (2008). Microbial population dynamics, especially stress tolerant Bacillus thuringiensis, in partially anaerobic rice field soils during post-harvest period of the Himalayan, island brackish water and coastal habitats of India. World J. Microbiol. Biotechnol. 24: 1403-1410.

Gonzalez-Acosta, B., Bashan, Y., Hernandez- Saavedra, N. Y., Ascencio, F. and Agüero, G. C. (2006). Seasonal seawater temperature as the major determinant for populations of culturable bacteria in the sediments of an intact mangrove in an arid region. FEMS. Microbiol. Ecol. 55: 311-321.

Holguin, G., Bashan, Y., Mendoza Salgado, R. A., Amador, E., Toledo, G., Vazquez, P. and Amador, A. (1999). La Microbiologia de los manglares. Bosques en la frontera entre el mary la tierrra. Ciencia Desarrollo. 144: 26-35.

Hyde, K. D., Jones, E. B. G., Leano, E., Pointing, S. B., Poonyth, A. D. and Vrijmoed, L. L. P. (1998). Role of fungi in marine ecosystems. Biodiv. Conserv. 7: 1147-1161

Kathiresan, K. (2000). A review of studies on Pichavaram mangrove, southeast India. Hydrobiologia. 430: 185-205.

Kohlmeyer, J. (1969). Ecological notes on fungi in mangrove forests. Transactions of the British MycoIogical Society. 53: 237-250.

Nannipieri, P., Ascher, J., Ceccherini, M. T., Landi, L., Pietramellara, G. and Renella, G. (2003). Microbial diversity and soil functions. Euro. J. Soil. Sci. 54: 655-670.

Sarma, V. V. and Hyde, K. D. (2001). A review on frequently occurring fungi in mangrove. Fung. Divers. 8: 1-34.

Shearer, C. A., Descals, E., Kohlmeyer, B., Kohlmeyer, J., Marvanová, L., Padgett, D., Porter, D., Raja, H. A., Schmit, J. P., Thorton, H. A. and Voglymayr, H. (2007). Fungal diversity in aquatic habitats. Biodiv. Conserv. 16: 49-67.

Sridhar, K. R. (2004). Mangrove fungi in India. Curr. Sci. 86: 1586-1587.

Wakley, A. and Black, I. A. (1934). An Examination of The Degtjareff Method for determining Organic Carbon in Soil: Effect of variations in Digestion conditions and of inorganic soil constituent. Soil Sci. 63: 251-262.

Zhong-shan, C., Jia-Hui, P., Wen-cheng, T., Qi-jin, C. and Yong-cheng, L. (2009). Biodiversity and biotechnological potential of mangrove-associated Fungi. J. Forestry Res. 20: 63-72.

Plant Science Research 34 (1&2) : 39-42, 2012

Plant Science Research

ISSN 0972-8546

Bioleaching of Alumina from Partially Lateritised Khondalite using Iron oxidizing bacteria

M. Mishra[1Ψ], R. C. Mohanty[2] and L. B. Sukla[1]

[1] Institute of Minerals and Materials Technology, Bhubaneswar-751013, India

[2] Department of Botany, Utkal University, Bhubaneswar-751004, India

ARTICLE INFO

Article history:
Received : 13 November 2012
Received in revised form : 11 December 2012
Accepted : 14 December 2012

Keywords:
A. ferrooxidans
bioleaching
alumina

ABSTRACT

The chemolithotrophic bacteria *Acidithiobacillus ferrooxidans* has been exploited world wide for various metallurgical phenomenon. Since these microorganisms thrive well in extreme environmental conditions hence these bacteria show certain tolerance limit to various heavy metal ions. In the current study bioleaching of alumina was carried out from partially laterized khondalite (PLK) using these iron oxidizing bacteria. Various leaching parameters such as pulp density, pH and temperature were studied to evaluate their effects on the leaching efficiency.

1. Introduction

Employment of microorganisms to recover metal values is free from environmental concerns unlike conventional hydrometallurgical methods. The main advantage in the bio-hydrological techniques is the ease of operation as well as limited use of process control, thus making the operation more users friendly (Pradhan *et al*., 2010).

Bio-hydrometallurgy is commercially exploited in the recovery of copper and uranium and is also used in the recovery of finely disseminated gold from refractory ore like pyrite and arsenopyrite (Pinches *et al.*, 1997; Schnell, 1997; Brierley and Brierley, 2001). Bacterial oxidation of sulphide ores using chemoauto-trophic bacteria such as *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* is a well-known process in bio-hydrometallurgy and thought to occur by a combination of direct and indirect mechanisms (Jain and Sharma, 2004).

Acidithiobacillus ferrooxidans is an acidophilic bacterium which can either grow on reduced sulphur compounds or on ferrous iron. It utilises energy obtained from oxidation of inorganic sulphur compounds (e.g. Fe_2S, CuFeS) as well as ferrous iron dissolved in a liquid medium. Ferrous iron is oxidised to ferric iron in acidic medium by means of *A. ferrooxidans*, while its chemical oxidation by means of oxygen is extremely low (Das *et al.,* 1998).

Partially Laterized khondalite is a waste material generated during the mining of bauxite. About 5 million tones of Bauxite is being processed by NALCO each year, for this amount of bauxite a lot of mine waste is generated and dumped at the mining site (Swain *et al.,* 2010). Most of these mining waste materials are PLK rock containing kaolinite, bauxite in varying proportions with minor amounts of hematite, goethite and rutile. The PLK contains about 45–56% Al_2O_3, 0.3–30% Fe_2O_3, 0.97–3%, TiO_2, 20–30% SiO_2 and 20–30% LOI. (Swain *et al.,* 2010).

The aim of this work is to evaluate the possibility of leaching alumina by iron oxidizing bacterial strains. There are many factors which can affect bioleaching kinetics. Some of those factors are pH, nutrient concentration, pulp density, reaction time, metal toxicity, temperature, etc. The present study discusses the effect of various leaching parameters on the efficiency of a mixed culture of iron oxidizing bacteria predominantly *Acidithiobacillus ferrooxidans*.

Ψ *Corresponding author; Email:* mousumi_official@yahoo.co.in

2. Materials and methods

2.1 Mineralogical characterization

PLK samples were procured from bauxite mines of National Aluminium Company (NALCO), Damanjodi, Orissa, India. The mineralogical studies were carried out by X-ray diffraction methods. The mineralogical phase analysis was carried out using PANalytical X-pert X-ray Diffractometer with Mo-Ká radiation (ë=0.709 ú) from 6^0 to 40^0 scanning angle at a scanning rate of 0.02^0/sec. Complete chemical analysis of the sample was carried out by X-ray florescence (Mishra *et al*., 2011).

2.2 Microorganisms

A mixed laboratory stock culture consisting predominantly *Acidithiobacillus ferrooxidans* was used for the experiment. The generation time was reduced and the ferrous oxidation rate was fastened by repeated subculture of the consortia in $9K^+$ medium (Table 1). The growth pattern of the bacteria was analyzed by estimating the ferrous in the growth medium. The ferrous concentration was estimated by a titration method using, 0.1 N potassium dichromate as titrant and barium diphenylamine -4-sulfonate (BDAS) as a redox indicator.

Table 1
Composition of $9K^+$ medium used to culture *Acidithiobacillus ferrooxidans*

Components	Amount (g/l)
$(NH_4)SO_4$	3
KH_2PO_4	0.5
KCl	0.1
$MgSO_4.7H_2O$	0.5
$FeSO_4.7H_2O$	44

2.3 Bioleaching studies

All bioleaching experiments were performed 250 ml Erlenmeyer flasks. Each flask contained 90ml of $9K^+$ medium and 10ml of inoculum. A centrifugal incubator- shaker was used for the bioleaching experiments with a fixed speed of 150 rpm. Samples were collected at regular intervals for the analysis of pH and concentration of metal ions. The analysis of the metals was carried out by ICP-OES. Effects of various parameters on the leaching process were studied. Parameters like contact time, pulp density, pH, temperature and particle size were studied to obtain optimum conditions for the maximum recovery of the targeted metals from the waste. Other than the specified parameters rest of the parameters were maintained at: Fe (II), 10 g/l; pH, 2; pulp density, 10%, temperature 35^0C, particle size, 200 µm and 150 rpm.

3. Result and discussion

3.1 Mineralogical analysis

The sample was light yellow in colour. Mineralogy by *X*-ray diffraction pattern studies indicated that the PLK rock samples consist of mostly aluminium-bearing minerals like gibbsite, Kaolinite. Some other associated opaque minerals like goethite, hematite, ilmenite/altered ilmenite, rutile and graphite were also identified. Quartz, orthoclase and minor amounts of biotite form the silicate gangue minerals (Fig. 1). Microscopic studies showed that a higher percentage of quartz is present as the cavity fillings whereas biotite flakes of are present along with the orthoclase. It is observed in many places that orthoclase has been completely altered to a mass of kaolin. Gibbsite usually occurs as coarse to fine grains or clusters.

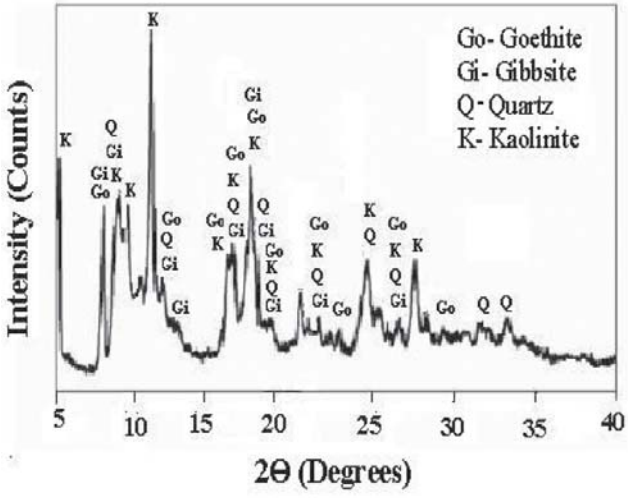

Fig. 1. Mineralogical analysis of PLK by XRD

3.2 Bioleaching studies

Pulp density is an important parameter involved in determining the process feasibility. The leaching studies were carried out by varying the pulp density from 5 to 25% (5-25 g of ore in 100 ml of medium).The leaching efficiency decreased with the increase of pulp density (Fig. 2). 63% alumina leaching was observed at 5% pulp density which decreased to 35% at 25% pulp density. The Eh values of the solution at 5 and 25% pulp density were around 600 and 500 mV respectively, showing less oxidizing condition at higher pulp density. The iron precipitation rate also increased with the increase of pulp density. This may have occurred due to improper mixing of the particles with the lixiviant, and

inadequate diffusion of oxygen which may have decelerated bacterial growth or improper growth of bacteria in higher pulp density. The solubility of oxygen in water is 8 g/m^3 and it decreased with the increased ionic concentration in the

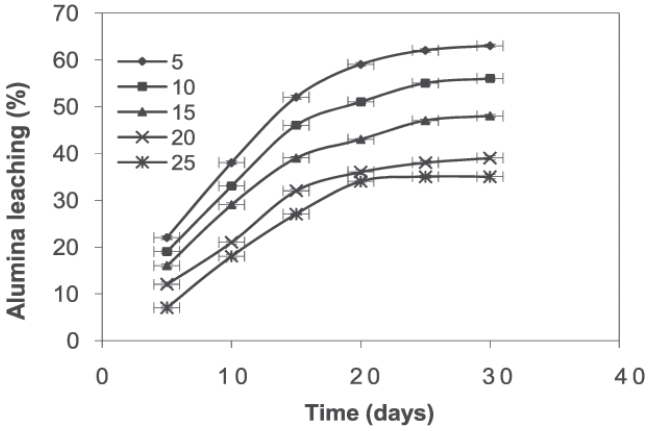

Fig. 2. Effect of pulp density (g/100ml) on alumina leaching from PLK

solution (Karavaiko *et al.,* 1988) which hinders the further growth of the bacteria. Poor heat and mass transfer might be the other reasons for the lower leaching at higher pulp density.

To evaluate the effects of acidity, the initial pH was varied from 1.5 to 3. The leaching efficiency was higher at lower pH values. The alumina leaching was 58% at 1.5 pH which gradually decreased with increase in pH (Fig. 3) The iron precipitation rate increased with the increase of pH. Numerous studies have demonstrated that bioleaching of the minerals occur favorably pH< 3. At pH<3 the regeneration of Fe^{3+} via biological oxidation of Fe^{2+} is of

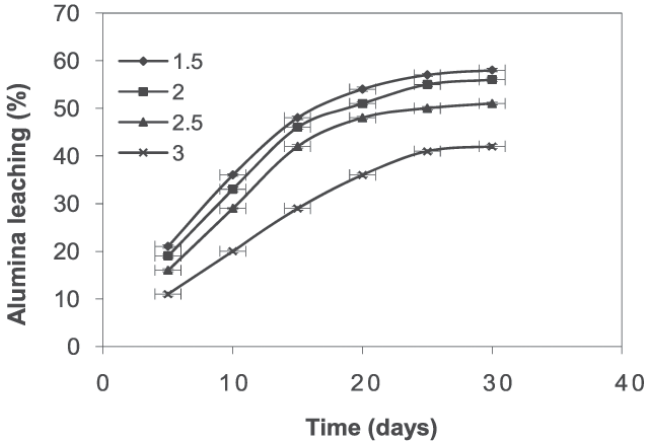

Fig. 3. Effect of pH on alumina leaching from PLK

great importance due to negligible abiotic oxidation of Fe^{2+} (Stumm and Morgan, 1981) The Eh of the solution in all cases varied in the range 550–650 mV indicating good oxidizing conditions.

Temperature is an important environmental factor that influences bacterial activities in biological leaching operations (Kelly and Tuovinen, 1998). In the present study the temperature was varied from 15 to 45^{0}C. The leaching efficiency increased with the increase of reaction temperature, thus indicating the dissolution process to be endothermic in nature. At the highest temperature the leaching decreased due to lesser adaptability of the organisms. At the lower temperatures the organisms used in the experiment became metabolically dormant but at higher temperatures they were rapidly destroyed as they are mesophilic in nature and therefore gave the best leaching results at 30-35^{0}C. The maximum leaching of alumina obtained at the moderate

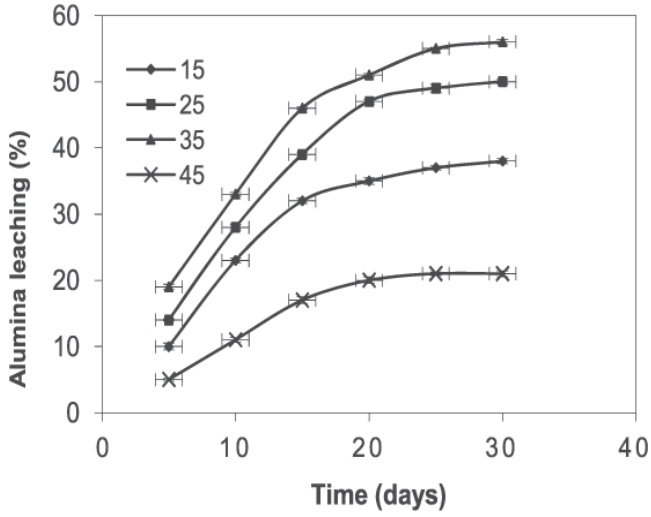

Fig.4. Effect of temperature (^{0}C) on alumina leaching from PLK

temperate range was around 55-56%. The leaching results are shown in Fig. 4. The iron precipitation rate increased with the increase of reaction temperature.

Leaching of alumina from different ores and waste materials using heterotrophic microorganisms have been reported through many studies (Mishra *et al*., 2009) but there are few studies showing extraction of alumina using mesophilic chemolithoautotrophs. Solisio *et al.* (2002) reported bioleaching of Zn and Al from industrial waste sludges using *A. ferrooxidans.* They achieved around 78% of alumina extraction. Bojinova and Velkova (2001) worked with industrial waste product for extraction of different valuable metals. A maximum of 71% of extraction was observed on 28th day of the experiment with *A. ferrooxidans*.

4. Conclusion

In this study, experiments were conducted to evaluate the leaching efficiency of Alumina from Partially Laterized Khondalite using iron oxidizing bacteria. The dissolution

process followed dual kinetics, i.e., an initial faster rate followed by a slower one, attaining equilibrium after 10 days of reaction time. The initial concentration of Fe(II) played an important role in determining the leaching efficiency. The leaching efficiency decreased with increase of pulp density, probably due to improper mixing or an increase of iron precipitation rate or lack of oxygen or combination of all these factors. Acidity and temperature also played an important role in determining the leaching efficiency.

Acknowledgement

The authors are thankful to Professor B.K. Mishra, Director, for his kind permission to carry out the work and publish this paper.

Reference

Bojinova, D.Y. and Velkova, R.G. (2001). Bioleaching of Metals from mineral waste product. Acta Biotechnol. 21(3): 275-282.

Brierley, J. A. and Brierley, C.L. (2001). Present and future commercial applications of biohydrometallurgy. Hydrometallurgy 59(2): 233–239.

Das, T., Roychaudhury, G. and Ayyappan, S. (1998). Use of *Thiobacillus ferroxidans* for iron oxidation and precipitation. Biometals 11(2): 125-129.

Jain, N. and Sharma, D.K. (2004). Biohydrometallurgy for non-sulphidic mineral- A review. Geomycrobiol. J. 21 (3): 135-144.

Karavaiko, G. L., Golovacheva, R. S., Pivovarova, I. A., Izaplina, I. A. and Vartanjan, N. S. (1988). Themophillic bacteria of genus *Sulphobacillus.* In: Norris, P. R. and Kelly, D.P. (ed.) Biohydrometallurgy, Science and Technology Letters, pp. 29-41

Kelly, B. C. and Tuovinen, O. H. (1998). Microbiological oxidation of minerals in mine tailings. In: Salomons, W. and Frostner, U. (ed.) Chemistry and Biology of Solid Waste: Degraded Material and Mine Tailings, Verlag AG, Berlin, pp. 33-53.

Mishra, M., Sukla, L. B. and Mishra, B. K. (2009). Microbial removal of alumina and silica from low grade iron ores. SGAT Bull. 10 (1): 1-11.

Mishra, M., Pradhan, M., Sukla, L. B. and Mishra B. K. (2011). Microbial beneficiation of Salem iron ore by *Penicilliun purpurogenum.* Metallurg. Mat. Transact. B 42(B): 13-19.

Pinches, A. Neale, J. W., Kruger, P. P., Van Staden and P. F., Miller, P. C. (1997). The Mintek-Bac-Tech bioleach process for copper sulfide concentrates, Proc. Copper Hydromet Roundtable Conf., Vancouver, British Columbia, pp.5-50.

Pradhan, D., Kim, D.J., Roychaudhury G. and Lee, S. W. (2010). Bio-dissolution of Ni, V and Mo from spent petroleum catalyst using iron oxidizing bacteria. J. Environ. Sci. Hlth. Part A 45(4): 476 — 482

Schnell, H.A. (1997). Bioleaching of copper. In: Rawlings, D. E. (ed.) Bioleaching: Theory, Microbes and Industrial Processes. New York, USA.

Solisio, C., Lodi, A. and Veglio, F. (2002). Bioleaching of zinc and aluminlum from industrial waste sludges by means of *Thiobacillus ferrooxidans.* Waste Manage. 22: 667-675.

Stumm, W. Morgan, J. J. (1981). Aquatic Chemistry: An Introduction Emphasizing Chemical Equilibria in Natural Waters. New York, USA.

Swain, R., Rao, D. S, Vasumathy, N. Mahapatra, R.and Rao R. B. (2010). Characterization of partially laterized khondalite for value added material. Int. J. Mining Mineral Engg. 1: 1-14.

Plant Science Research 34 (1&2) : 43-46, 2012

Plant Science Research

ISSN 0972-8546

Effect of environmental factors on spore shedding in *Gracilaria corticata* J.Agardh

G. Subba Rangaiah[Ψ], G. M. Narasimha Rao and K. Deepthi Lavanya

Department of Botany, Andhra University, Visakhapatnam-530 003, India

ARTICLE INFO

Article history:
Received : 18 July 2012
Received in revised form : 04 November 2012
Accepted : 19 November 2012

Keywords:
Gracilaria corticata
spore shedding
environmental factors

ABSTRACT

Gracilaria corticata is an agarophyte which occurs abundantly throughout the year in the intertidal rocky surfaces of Visakhapatnam coast, India. Spore shedding experiments such as effect of environmental factors on spore shedding and on diurnal periodicity of spore shedding were conducted during 2007 and 2008. Maximum numbers of tetraspores were released when fronds were in submerged condition, exposed to dark condition and at 30 ‰ salinity. Neither acceleration nor delay in the peak shedding of tetraspores in *G. corticata* was found even after exposure to various periods of desiccation, salinities and different light intensities.

1. Introduction

Seaweeds are commercially valuable for the sources of food, fodder, fertilizer and extraction of important phycocolloids and biofuels. Spores in seaweeds are single celled reproductive bodies and are capable of growing into new plants. Studies on sporulation play a vital role in the field of mariculture to generate the algal populations in the natural habitats. Several authors have studied the spore shedding from marine algae of tropical waters (Katada, 1955; Srinivasa Rao, 1971; Umamaheswara Rao and Kaliaperumal, 1983; Subba Rangaiah, 1983, 1984, 1985; Umamaheswara Rao and Subba Rangaiah, 1986; Narasimha Rao and Subba Rangaiah, 1991. In the present investigation studies were made on the spore shedding from *G. corticata* in different environmental conditions.

2. Material and methods

Visakhapatnam is situated on the east coast of India between the latitude 17^0 40' 30" and 17^0 45' N longitudes 83^0 16' 25" and 83^0 21' 30"E. The coastline is sandy with outcrops of rocky boulders in different regions. Materials for this study were collected during the spring tide periods from VUDA park regions where large accessible boulders occur with dense growth of algae. *Gracilaria corticata* J.Agardh was collected for carrying the laboratory experiments during the years 2007 and 2008. Experiments were conducted on the effect of environmental factors such as desiccation, salinity and light intensity on spore shedding and diurnal periodicity of spore shedding from the above candidate. In the experiments conducted to study the exposure to air, the tetrasporophytic fronds were blotted to remove the water on the surface of the fronds and exposed to air in the laboratory and also in the open air during the day time. At the time of conducting these experiments, the temperature in the laboratory was 32± 2 ^{0}C and the relative humidity varied from 62 to 67%. In the open air where these experiments were conducted, the temperature was 34±2 ^{0}C and relative humidity ranged from 58 to 63%. At 15 minute intervals the materials thus exposed to air were transferred to Petri-dishes filled with seawater and the spore output was estimated after 24 hours as mentioned in the earlier works (Subba Rangaiah, 1983). Seawater collected from the inshore area was adjusted to 80‰ salinity by exposing to sun light to make up the stock solution. Lower grades were prepared from this stock solution by the addition of requisite quantity of distilled water. Spore output was estimated at

[Ψ] *Corresponding author; Email:* gaddamsranga2006@yahoo.co.in

0‰ to 70‰ salinities, maintaining the Petri-dishes at room temperature (32±2) ^{0}C under 8 hours day length with 9 µ E/ m^2 s day light fluorescent illumination. Effect of light intensity on spore output were investigated at room temperature using light intensities of 0 (dark) to 36 µ E/ m^2 s. Based on the changes observed in the spore output per day, experiments on diurnal periodicity were conducted selecting certain periods of exposure to air (0 to 60 minutes), salinities (10 to 60 ‰) and light intensities (0 to 36 µ E/ m^2 s).

3. Results

3.1 Factors influencing spore output

Changes were observed in the tetraspore output of *Gracilaria corticata* , in control (i.e., in fronds submerged for 2 hours), maximum spore output was observed and with increase in the time of exposure the quantity of spores liberated gradually decreased. The spore output declined rapidly from 15 to 45 minutes of exposure to air at room temperature in air (Figs. 1). In general the spore production was less in the fronds exposed to open air (Fig.1 A) when compared to shade condition. There was no spore discharge in the fronds exposed after 75 minutes (Fig.1B).

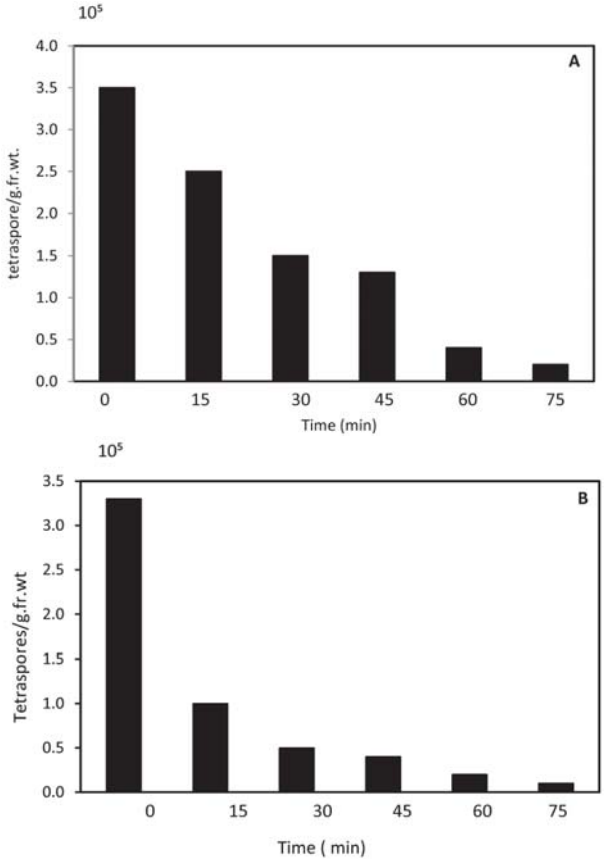

Fig.1. Rate of the spore output of gracillaria corticata at ambient (A) outdoor and (B) indoor temperature on exposure to air

Effect of salinity on spore shedding was shown in Fig. 2. There was no spore shedding at 0 ‰ and 70 ‰ salinities. Spore liberation was seen in salinities from 10- 60 ‰ and maximum spore shedding was observed at 30 ‰ followed by 20 ‰ and 40 ‰ salinities. Minimum spore shedding was seen in salinities at 10 ‰ and 60 ‰.

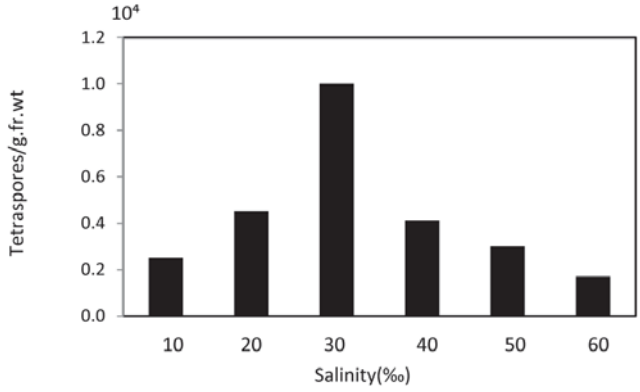

Fig.2. Rate of spore output *Gracilaria corticata* at different salinities

Fig.3 shows the quantity of spores liberated in darkness and at three different light intensities ranging from 9 µE/ m^{2}s to 36 µ E/ m^2 s. In *Gracilaria corticata* spore output was observed in complete darkness. The values obtained at 9 µ E/ m^{2}s were slightly less than those obtained in darkness

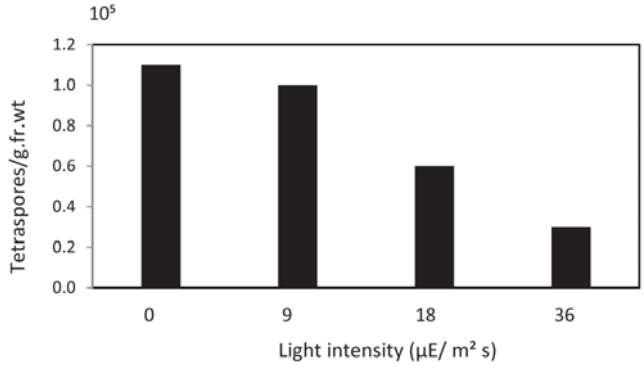

Fig.3. The effect of irradiance on spore output of *Gracilaria corticata*

and the spore output decreased gradually with increasing light intensity staring from 18 ì E/ m^{2}s.

3.2 Factors influencing diurnal periodicity

Diurnal periodicity curves obtained on *Gracilaria corticata* exposed to air for 0 (control), 15, 30, 45 and 60 minutes are shown in Fig.4. The diurnal periodicity of tetraspores did not vary in *Gracilaria corticata* in control and in the fronds exposed to air for a period of 15 to 60 minutes. Since exposure of the fronds to air affect the spore shedding, the quantity of spores liberated at different times of the day also decreased from control to 60 minutes exposure but the diurnal periodicity curve did not change (Fig.4).

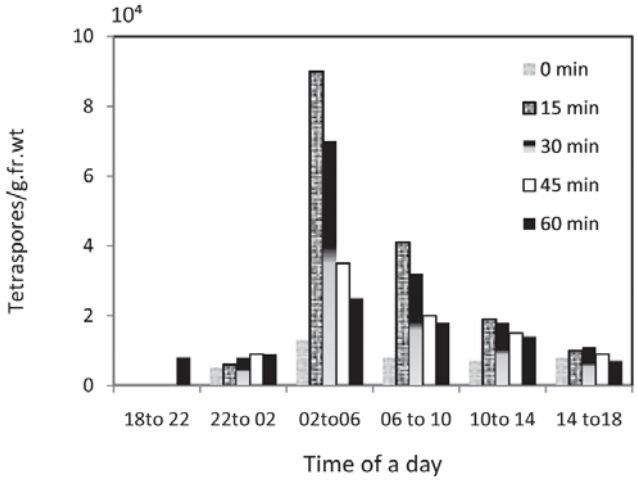

Fig.4. Diurnal periodicity in the spore output of *Gracilaria corticata* on exposure for different duration (min) to air.

Maximum liberation of spores was observed in all five different exposures with peak output between 02. 00 hour and 06.00 hour in the morning.

In order to show the effect of salinity on the diurnal periodicity, data collected at five different salinities (10 to 50‰) showed similar rhythm in the daily liberation of spores in all the selected salinities with peak output between 02.00 h and 06.00 h (Fig.5). As observed in the total spore output per day, the quantity of spores liberated varied in different salinities in the diurnal periodicity experiments also. Peak output was obtained in *Gracilaria corticata* at 30 ‰ (Fig. 5).

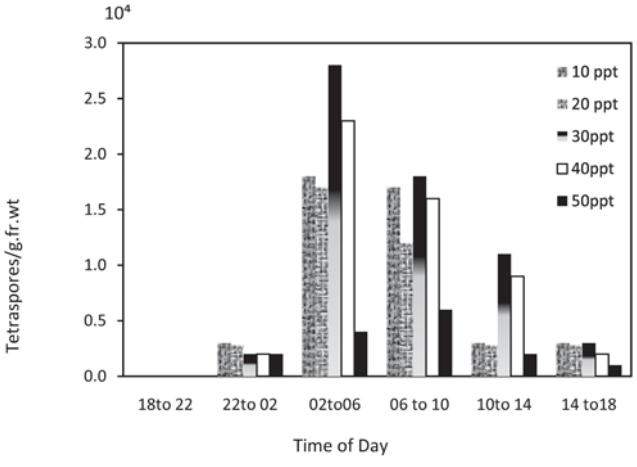

Fig.5. Diurnal periodicity in the spore output of *Gracilaria corticata* on incubation in water with different salinities (ppt)

Diurnal periodicity curves obtained at 0, 9 µE/m^{2}s and 18 µ E/ m^{2}s light intensities are shown in Fig. 6. In these experiments also the diurnal periodicity of spores was not affected either in the dark or in the two light intensities used. The maximum shedding was observed between 02.00 h and 06.00.h (Fig.6).

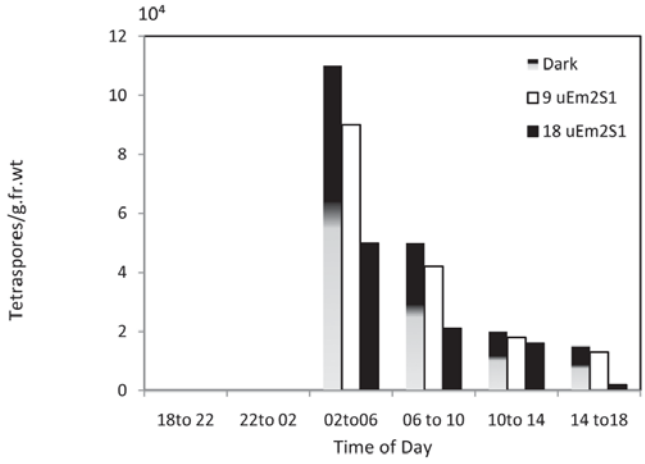

Fig. 6. Diurnal periodicity in the spore output of *Gracilaria corticata* at different irradiance (µE/m^{2}s).

4. Discussion

In the intertidal habitats, the environmental factors such as desiccation, salinity, temperature and light are considered as some of the important factors in controlling growth, reproduction and spore shedding of algae (Narasimha Rao and Subba Rangaiah, 2009, 2010). In the present study the effect of temperature on spore shedding was not conducted because of lack of proper facility. The eco-physiological aspect of spore discharge was studied on some Indian algae, viz. *G. corticata, G. textorii* and *Gracilariopsis sjoestedtii* (Subba Rangiah, 1983, 1984, 1985). In the present study, effect of environmental factors such as exposure to air on spore shedding of *Gracilaria corticata* showed a decreasing trend of tetraspores output when the algae were exposed to air and subjected to desiccation (Figs. 1). It is therefore; likely that exposure of fronds during low tides affects the quantity of spores produced. A similar response was reported in Gelidiales (Katada, 1955; Srinivasa Rao, 1971; Umamaheswara Rao and Kaliaperumal, 1983), Gigarinales (Umamaheswara Rao and Subba Rangaiah, 1986), Bangiales and Ectocarpales (Narasimha Rao and Subba Rangaiah, 1991). Some cultural studies showed that the release of spores /gametes in seaweeds is induced by desiccation and when wetting the fertile thalli (Luning, 1980; Sheath and Cole, 1980). In the present study the tetraspore shedding in *G. corticata* was observed for 75 min exposure in the outside and inside the laboratory which is almost coinciding the previous studies.

It was also observed that the degree of salinity of the seawater influenced spore shedding in *G. corticata*. The optimum salinity range observed for the maximum shedding in *G. corticata* was 30 ‰ (Fig. 2). Several studies recorded the influence of salinity on spore production and release in various algae of the Visakhapatnam coast and reported different optimum ranges (Subba Rangiah *et. al.*1975; Subba

Rangiah,1986; Umamaheswara Rao and Subba Rangaiah, 1986; Narasimha Rao and Subba Rangaiah, 1991. The tetraspore liberation occurred in *G. corticata* under photon flux densities raging from 0 to 36 μE/m^2s. The observations of the present study was in agreement with the previous studies made by Umamaheswara Rao and Subba Rangaiah (1986), where in the peak discharge of tetraspores in *G. corticata* was observed in complete darkness.

Environmental factors play an important role in the formation and liberation of spores from marine algae occurring in different vertical heights of the rocky substratum (Narasimha Rao and Subba Rangaiah, 2009; 2010). Like this, environmental factors influence the diurnal periodicity of spore shedding in the marine algal populations. In the present study neither acceleration nor delay in the peak shedding of tetraspores in *G. corticata* was found even after exposure to 60 minutes (Fig.4). The present study on *G. corticata* is in agreement with the observations made by Umamaheswara Rao and Kaliaperumal (1983) on Gigartinales. Variations in salinity did not affect diurnal periodicity pattern in the members of Gigartinales, Gelidiales and Cryptonemiales (Umamaheswara Rao and Subba Rangaiah, 1981; Umamaheswara Rao and Kaliaperumal, 1987). In this respect the present study on *G. corticata* agrees with the above findings. When the thalli of *the alga was* exposed up to 18 to 36 μE/m^2s light intensities, there was no change in the peak period of shedding of tetraspores. Umamaheswara Rao and Kaliaperumal (1987) observed pre-ponement of the time of peak shedding of spores by 4 hours in *Gelidiopsis variabilis* at 3000 lux but not at 500 and 1400 lux and also in the dark. But in the present study, it seems that photon flux intensity did not have any effect on the diurnal periodicity of tetraspores shedding in *G. corticata*.

5. Conclusion

It is interesting to note that the quantity of spores liberated in *G. corticata* of the present study is almost less than half when compared to the studies made by Subba Rangaiah (1983) in the same alga almost 30 years back. This change may be due to increase in the temperature (2-3 ^{0}C) in the nature, and indiscriminate discharge of industrial effluents in to the sea. If this process continues, we do hope that in future there will be a drastic change in the seaweeds of Visakhapatnam towards decrease in the vegetation as well as in spore shedding capacities. So, we request the Government and NGOs to take necessary steps to conserve the seaweeds of Visakhapatnam by taking proper steps.

References

Katada, M. (1955). Fundamental studies on the propagation of gelidiaceous algae with special reference to shedding and adhesion of the spore, germination, growth and vegetative reproduction. Shimonoseki Coll. Fish J. 5: 1-87.

Luning, K. (1980). Critical levels of light and temperature regulating the gametogenesis of three *Laminaria* species (Phaeophyceae). J. Phycol. 16:1-15.

Narasimha Rao, G. M. and Subba Rangaiah, G. (1991). Control of spore shedding from some marine algae of the Visakhapatnam coast, India. Br. Phycol. J. 26: 356-360.

Narasimha Rao, G. M. and Subba Rangaiah, G. (2009). Spore shedding in Marine red algae of Indian Coast: A review. Adv. Pollen Spore Res. 27 : 43-53.

Narasimha Rao, G. M. and Subba Rangaiah, G. (2010). Sporulation in marine brown algae of the Indian Coast: A review. Adv. Pollen Spore Res. 28: 63-70.

Sheath, R.G. and Cole, K.M.(1980). Distribution and salinity adaptations of *Bangia atropurpurea* (Rhodophyta) a putative migrant into the Laurentian great lakes. J. Phycol. 16: 412-420.

Srinivasa Rao, P. (1971). Studies on *Gelidiella acerosa (*Forsskal) Feldman *et.* Hamel. IV. Spore studies. Bull. Japan. Soc. Phy. Col. 19: 9-14.

Subba Rangaiah, G. (1983). Seasonal growth, reproduction and spore shedding in *Gracilaria corticata* J. Agardh of the Visakhapatnam coast. Proc. Indian Nat. Sci. Acad. B49: 711-718.

Subba Rangaiah, G. (1984). Growth, reproduction and spore shedding in *Gracilaria textorii* (Sur.) J. Ag. of the Visakhapatnam coast. Phykos. 23: 246-253.

Subba Rangaiah, G. (1985). Spore shedding in *Gracilariopsis sjoestedtii* (Kylin)Dawson (Rhodophyta, Gigartinales). In: Krishnamurthy, V. and Untawale, A. G. (ed.) Proc. All India Symp. Mar. Plants, their Biology, Chemistry, Utilization. Seaweed Research and Utiln. Assoc. India: pp. 59-64.

Subba Rangaiah, G. (1986). Effects of environmental factors on the shedding of monospores from *Porphyra vietnamensis* Tanaka et. Ho of the Visakhapatnam coast. Phykos., 25: 29-35.

Subba Rangaiah, G., Umamaheswara Rao, M. and Rao, B. G. S. (1975). Effect of salinity on spore shedding in *Gracilaria corticata*. Curr. Sci. 44: 717-718.

Umamaheswara Rao, M. and Kaliaperumal, N. (1983). Effect of environmental factors on the liberation of spores from some red algae of Visakhapatnam coast. J. Exp. Mar. Biol. Ecol. 70: 45-53.

Umamaheswara Rao, M. and Kaliaperumal, N. (1987). Effect of thermal stress on spore shedding in some red algae of Visakhapatnam coast. Indian J. Mar.Sci., 16: 201-202.

Umamaheswara Rao, M. and Subba Rangaiah, G. (1986). Effect of environmental factors on the shedding of tetraspores of some Gigarinales (Rhodophyta). Proc. Symp. Coastal Aquacult. 4: 1199-1205.

Plant Science Research 34 (1&2) : 47-53, 2012

Plant Science Research

ISSN 0972-8546

Tolerance of *Anabaena* sp. PCC 7119 to cypermethrin measured through photosynthetic pigment fluorescence

Y. Chandrakala and P. K. Mohapatra[Ψ]

Department of Botany, Ravenshaw University, Cuttack – 753 003

ARTICLE INFO

Article history:
Received : 19 September 2012
Received in revised form : 29 November 2012
Accepted : 30 November 2012

Keywords:
pyrethroid
cypermethrin
Anabaena sp PCC 7119
OJIP
fluorescence

ABSTRACT

Insecticides are used worldwide in agriculture in vast amount every year. Use of pyrethroid insecticides has increased during past two decades due to their high knock down action and considerably low mammalian toxicity. Residual toxicity of the insecticides has also gone up due to their extensive use making it necessary to develop strategies for their rapid detoxification in the environment. The present study was undertaken to study the response of normal and cypermethrin pre-exposed *Anabaena* sp. PCC 7119 strains against the pyrethroid insecticide. Assay for 5 days showed enhanced growth performance of the tolerant strain up to 40 µM cypermethrin compared to that of normal strain. The stress indicating fluorescence parameters, viz., relative variable fluorescence at J level (V_j), net rate of PSII closure (M_0) and effective dissipation of active RC (DI_0/RC) were low in the strain having tolerance than of a normal one. Similarly, there was enhancement of the photosynthetic efficiency measured through the estimation of variable fluorescence (F_v) and fluorescence yield. The plant efficiency measured through derived fluorescence parameters, viz., maximum trapping rate of active PSII (TR_0/RC), trapping probability (TR_0/Abs), electron transport probability (ET_0/TR_0), and electron transport in active RC (ET_0/RC) showed higher values in the tolerant strain than in the normal one.

1. Introduction

Pyrethroids are widely used as pest control agents in a wide array of indoors and out door applications, including medicinal, veterinary and agriculture sector and are the synthetic derivative of natural pyrethrins with greater potenticy and environment stability (Elloiott and Janes, 1978). Cypermethrin was introduced on the market in late 1970s and has since been used on a wide range of crops due to its high pesticidal activity and low mammalian toxicity relative to other insecticides. Several recent studies show that pesticide residues frequently occur in surface water and soil in agricultural areas (Larson *et al.,* 1999). Many aquatic and soil algal and cyanobacterial species are known to be affected by the non-target effects of pesticides (Mohapatra and Mohanty, 1992; Mohapatra *et al.,* 1996, 2003; Barata *et al.,* 2006; Jena *et al.,* 2012). Aquatic ecosystems in agricultural fields are thus at risk of being negatively affected by these chemicals (Kallqvist and Ramstad, 1994; Ahmad, 2008; Cycon *et al.,* 2012; Tandon *et al.,* 2012; Jena *et al.,* 2012).

Insecticide resistance is an important feature under natural condition and many organisms ranging from the target insect(s) to bacteria are known to develop resistance on prolonged exposure to non-cidal concentrations of the insecticides (Kostaropoulos *et al.,* 2001; El-Latif and Subrahmaniyam, 2010). Pyrethroid resistance in insects has been recorded worldwide (Agosin, 1985; Wendt-Rasch *et al.,* 2003). Resistance has been found in bacteria though the exact mechanism of resistance is yet to be properly deciphered. Cytochrome P450 mediated detoxification of OP insecticides has been recorded in bacteria and cyanobacteria (Rodriguez *et al.,* 1993; Cheikh *et al.,* 1998). There are also informations on esterase mediated detoxification of OP chemicals and both general and specific esterases have

[Ψ] *Corresponding author; Email:* pradiptamoha@yahoo.com

been found effective to metabolically hydrolyse the insecticides (Sogorb *et al.,* 1998; Sogorb and Vilanova, 2002; Trovaslet-Leroy *et al.,* 2011). However, there is hardly any information of such activity against pyrethroid insecticides in cyanobacteria though the same has been recorded in many other organisms (Qu *et al.,* 2000; El-Latif and Subrahmaniyam, 2010; Tandon *et al.,* 2012). Moreover resistance of cyanobacteria against pyrethroid insecticides has not yet been documented. The purpose of the present study was to examine the effects of cypermethrin, a pyrethroid insecticide, on the normal and 30 µM cypermethrin tolerant *Anabaena* sp PCC 7119. The paper focuses on the effects of exposure to cypermethrin on the OIJP fluorescence transient and photosynthetic activity.

2. Materials and methods

The filamentous and heterocystous cyanobacterium *Anabaena* sp. PCC 7119 of normal and 30 µM cypermethrin tolerant strain was chosen as test organism for this work. The organism was maintained in non absorbent cotton stopper 250 ml flasks containing 250 ml B.G-11 medium (Rippka *et al.,* 1979). Actively growing cells were taken at an initial inoculums density with absorbance of 0.05 at 678 nm (3.664 ± 0.113 µg Chl *a* / 10^7 cells). The inocula were prepared in a sterile growth medium 2 h before the experiment. All stocks and experimental cultures were grown in a culture room at 29 ± 2^0C with a continuous irradiance of 35 µE/m^2 s. The stock solution (10 mM) of cypermethrin 25 EC [(R, S)-α – Cyano – 3 – phenoxybenzyl – 2, 2 – dimethyl (1R, 1S) cis, trans – (2, 2- dichlorovinyl) cyclopropane carboxylate] was freshly prepared by dissolving the commercial formulation of the insecticide in equal volume of acetone and diluted with freshly prepared aqueous medium. Required volumes of the stock solution were added aseptically to the experimental culture flasks/ tubes to achieve desired treatment concentrations (5-40 µM). The cultures were grown for 5 days under the growth conditions same as that of the stock cultures and then the effect was measured.

The absorbance of the homogenized culture suspension was measured at 678 nm and 630 nm, using UV -1700 pharmaspec UV - Vis spectrophotometer (Shimadzu, Japan), as growth parameters. The extraction of photosynthetic pigment was made in methanol following the standard extraction protocol for cyanobacteria and absorbance values of extracts were converted to biomass (mg pigment/ l of culture) following the equation of Hirschberg and Chamoviz (1994). Extraction and quantification of the cellular protein content was done by following the protocol of Lowry *et al.* (1951). The absorbance values were converted to biomass of protein using a standard curve prepared through same procedure taking bovine serum albumin as substrate. Measurement of carbohydrate content of cultures was made following the anthrone reagent method of Roe (1955). The absorbance values were converted to biomass of carbohydrate through the regression equation of a standard slope prepared by using glucose.

The chlorophyll *a* fluorescence was measured through the liquid culture attachment of a plant efficiency analyzer (Handy PEA, Hansatech Instruments, Norfolk, UK) following the protocol of Jena *et al.* (2012). The homogenized culture of the cyanobacterium was taken in 2 ml capacity glass vials fitted with aluminum screw cap and the vials were placed in dark for 15 minutes for complete relaxation of PS II RCs. Each vial was then thoroughly shaken and placed in the measuring chamber of the attachment. The measuring gain of the attachment and the PEA were fixed at 1.0 and 0.7 (rel units), respectively. The fluorescence was induced by a saturating white light pulse at an irradiance of 1500 µE/ m^2s provided from the internal light source of the liquid culture attachment. The fluorescence rise of O, J, I and P levels were recorded after 50 µs (F_0 µs), 2 ms (F_J), 30 ms (F_I) and T_{fm} (F_m), respectively. Several fluorescence parameters, viz., variable fluorescence (F_v), relative variable fluorescence at J level (V_j), net rate of PSII closure (M_0), maximum quantum yield of primary photochemistry (φP_0), probability of exciton movement beyond Q_A (Ψ_0), quantum yield of electron transport (φE_0), quantum yield of energy dissipation (φD_0), maximum trapping rate of active PSII (TR_0/RC), electron transport in active RC (ET_0/RC), effective dissipation of active RC (DI_0/RC), and the performance index of primary photochemistry (PI_φ) were calculated from the OJIP fluorescence transient following the equations of Force *et al*. (2003) and Strasser *et al.* (2005). The photosynthetic flux parameters, absorption flux, trapped energy flux, electron transport flux and dissipation flux per RC and the performance of primary photochemistry were also analyzed for estimation of the photosynthetic efficiency of cells.

The samples were taken in triplicate and treatments were made with graded concentrations of the insecticide. In all cases the stock culture used was the one, which was continuously maintained at 30 µM insecticide and other untreated normal strain. The data presented in tables and figures are the means of replicates of two successive experiments. Comparison among means have been done wherever necessary using standard statical methods (Gomez and Gomeez, 1984).

3. Results and discussion

Significant reduction in the culture absorbance at 678 nm and 630 nm on treatment of normal cultures with graded

concentrations of the insecticide. Concurrently there was similar pattern of the cellular carbohydrate and protein contents in the normal cultures (Table 1). On the other hand, the growth of the tolerant cultures were found significantly enhanced by the insecticide at concentrations ≤ 10 mM. The other higher concentrations ≤ 30 mM caused only insignificant reduction of growth and biochemical content of cells during the treatment period.

In normal strain of cyanobacterium a significant reduction of chl *a* and carotenoid content was observed with increase in the concentration of cypermethrin indicating down regulation of photosynthetic pigments for survival. The Chl *a*/ Car ratio did not differ significantly even though a slight increase in the ratio in the treated cultures was observed. This proved that both the photosynthetic pigments were more or less equally affected by the insectides. This is in agreement with the results observed on treatment with other insecticides (Mohapatra *et al.,* 1992; 1996; 2003; 2010; Jena *et al.,* 2012). A comparatively high amount of pigment content in the tolerant strains on treatment the same concentrations of the insecticide showed that the cyanobacterium was not adversely affected by the insecticide concentrations taken in the study though significant inhibition of growth and biochemical contents of cells were reported at these concentrations in the normal strain.

The cellular protein and carbohydrate content of normal strain decreased with increase in cypermethrin concentrations whereas the tolerant strain of cyanobacterium showed no significant change in the metabolite content (Table 1). This strengths the fact that the cyanobacterium was metabolically active in the presence of the insecticide concentrations, that are found growth inhibitory to the normal strain. Growth enhancement indicated that the cyanobacterium not only tolerated the insecticide in the applied concentration but also utilized the same as a nutrient source. Such phenomena have been reported in bacteria and cyanobacteria at low insecticide concentrations. It has also been reported that OP insecticides accelerate the growth of soil algae and cyanobacteria at field concentration (Mohapatra and Schiewer, 2000; Li *et al.,* 2010).

The variable fluorescence is an indicator of photosynthetic activity of green plants and the fluorescence responses are highly correlated with the chlorophyll content of the photosynthetic structure (Strasser *et al*., 1995; Elias *et al.,* 2004; Strasser *et al.,* 2005; Marques da Silva, 2007). Healthy plants show high variable fluorescence than of stressed ones (Strasser *et al.,* 1995; 2005; Marques da Silva, 2007). Cyanobacteria often show low variable fluorescence as the fluorescence rise in such organisms is significantly low compared to green plants (Mohapatra and Schiewer, 2000; Li *et al.,* 2010). It is because of the fact that a significant part of the antenna pigments in cyanobacteria are phycobiliproteins (organized as membrane complexes called phycobilisomes) which do not absorb the red light applied to generate fluorescence.

The shape of OJIP fluorescence rise has been found to be very sensitive to stress caused by changes in different environmental conditions, e.g., light intensity, temperature, drought, atmospheric CO_2 concentration and chemical influences (Tsimilli-Michael *et al.,* 2000; Strasser *et al.,* 2005; Antal and Rubin, 2008; Li *et al.,* 2010; Mohapatra *et al.,* 2010; Jena *et al.,* 2012). The observations showed that the shape of the OJIP rise and also that of the relative variable fluorescence [V(t)] was significantly changed only in normal strain (Fig.1) but such change was not reported in the tolerant strains in response to the treatment with the selected

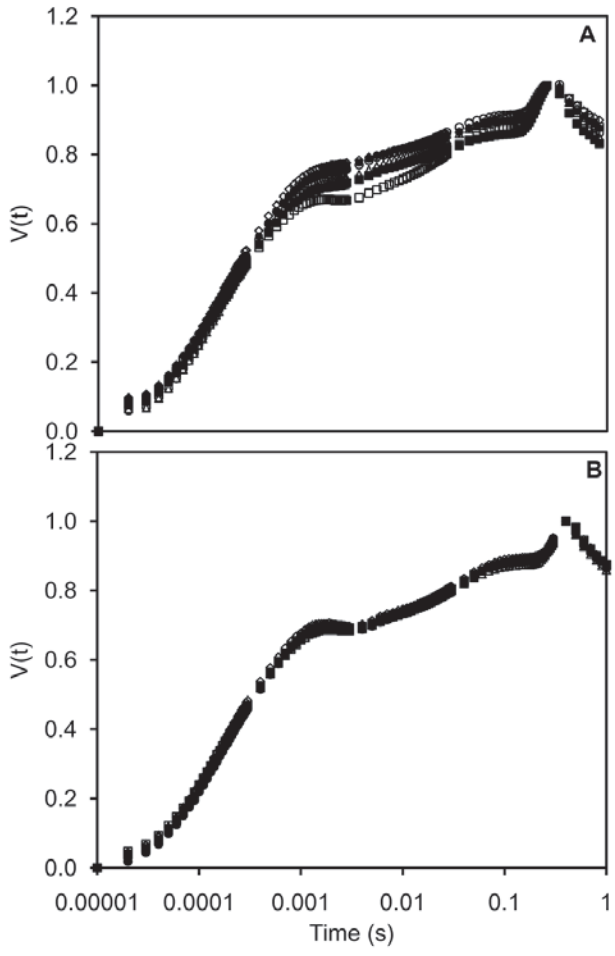

Fig.1. The OJIP fluorescence transient of normal and cypermethrin tolerant *Anabaena* sp. PCC 7119 strains exposed to different concentrations of the insecticide for 5 days. The transients represent the relative variable fluorescence [V(t)]. Cypermethrin concentrations (μM): (□)0, (■) 5, (△) 10, (o) 20, (●) 30, and (◇) 40.

Table 1
Effect of cypermethrin on growth and pigment content of normal and cypermethrin tolerant *Anabaena* sp. PCC 7119 strains during 5 days of incubation. C1 and C2 are the normal and tolerant control cultures, respectively.

Cypermethrin (μM)	678 nm	630 nm	678/630 nm	Chl *a* (mg/l)	Carotenoids (mg/l)	Chl *a*/Car	Carbohydrate (mg/l)	Protein (mg/l)
C1	0.742±0.007^{a}	0.736±0.004^{a}	1.008±0.044^{a}	6.019±0.085^{a}	1.602±0.044^{a}	3.76±0.116^{b}	104.2±3.4^{a}	332.1±12.8^{a}
5	0.631±0.004^{b}	0.654±0.004^{b}	0.965±0.038^{ab}	5.244±0.073^{b}	1.297±0.035^{b}	4.04±0.148^{a}	87.4±3.9^{b}	283.2±13.9^{b}
10	0.617±0.005^{c}	0.626±0.006^{c}	0.986±0.041^{a}	5.108±0.105^{c}	1.263±0.028^{c}	4.04±0.204^{a}	84.2±3.1^{b}	273.5±11.6^{c}
20	0.527±0.006^{d}	0.579±0.007^{d}	0.911±0.028^{ab}	4.352±0.093^{d}	1.092±0.031^{d}	3.98±0.182^{a}	71.1±4.0^{c}	231.5±14.2^{d}
30	0.461±0.002^{e}	0.508±0.007^{e}	0.906±0.046^{b}	3.337±0.082^{e}	0.873±0.037^{e}	3.83±0.179^{ab}	51.5±3.2^{d}	172.4±5.8^{e}
40	0.372±0.019^{f}	0.473±0.005^{f}	0.786±0.029^{c}	2.825±0.066^{f}	0.704±0.034^{f}	4.01±0.227^{a}	39.7±1.4^{e}	132.6±5.7^{f}
C2	0.743±0.005^{b}	0.747±0.006^{b}	0.995±0.043^{a}	5.987±0.142^{b}	1.611±0.069^{b}	3.72±0.213^{b}	101.3±4.1^{b}	328.1±12.9^{c}
5	0.880±0.006^{a}	0.870±0.006^{a}	1.011±0.051^{a}	7.127±0.133^{a}	1.859±0.073^{a}	3.83±0.128^{ab}	123.1±3.9^{a}	397.4±17.3^{a}
10	0.874±0.003^{a}	0.871±0.006^{a}	1.003±0.032^{a}	6.921±0.097^{a}	1.769±0.022^{ab}	3.91±0.187^{ab}	117.4±4.2^{a}	376.1±18.3^{b}
20	0.739±0.005^{b}	0.725±0.007^{c}	1.019±0.037^{a}	5.922±0.109^{b}	1.588±0.038^{c}	3.72±0.117^{b}	104.4±5.2^{b}	388.7±11.2^{ab}
30	0.735±0.005^{b}	0.718±0.006^{cd}	1.023±0.042^{a}	5.864±0.117^{b}	1.579±0.059^{c}	3.71±0.132^{b}	101.2±5.6^{b}	332.4±14.7^{c}
40	0.704±0.001^{c}	0.702±0.002^{d}	1.002±0.029^{a}	5.589±0.138^{c}	1.388±0.042^{c}	4.02±0.185^{a}	94.2±3.2^{c}	286.4±11.2^{d}

Note: Means superscripted by same letter in a column are not significantly different from each other at p = 0.05; comparison was done separately for normal and tolerant strain by applying LSD.

Table 2
The OJIP fluorescence derived parameters of normal and cypermethrin tolerant *Anabaena* sp. PCC 7119 strains exposed to different concentration of the insecticide for 5 days. C1 and C2 are as mentioned in table 1.

Cypermethrin (μm)	Fv	V_j	M_0	φP_0	ψ_0	φE_0	φD_0	TR_0/RC	ET_0/RC	DI_0/RC	PI_φ
C 1	478[a]	0.582[d]	1.425[c]	0.281[a]	0.418[a]	0.117[a]	0.719[e]	2.448[b]	1.023[a]	6.265[f]	0.391[a]
5	428[b]	0.615[c]	1.576[c]	0.232[b]	0.385[b]	0.089[b]	0.768[d]	2.563[a]	0.987[b]	8.483[e]	0.302[b]
10	359[c]	0.674[b]	1.661[b]	0.214[c]	0.326[c]	0.070[c]	0.786[cd]	2.464[b]	0.803[c]	9.051[d]	0.272[c]
20	320[d]	0.709[a]	1.725[b]	0.201[d]	0.291[d]	0.058[d]	0.799[bc]	2.433[b]	0.708[d]	9.671[c]	0.252[d]
30	277[e]	0.718[a]	1.811[a]	0.183[e]	0.282[e]	0.052[de]	0.817[ab]	2.522[ab]	0.711[d]	11.261[b]	0.224[e]
40	203[f]	0.721[a]	1.859[a]	0.168[f]	0.279[e]	0.047[e]	0.832[a]	2.578[a]	0.719[d]	12.769[a]	0.202[f]
C 2	467[a]	0.609[bc]	1.467[b]	0.287[a]	0.391[a]	0.112[a]	0.713[b]	2.409[a]	0.942[a]	5.984[bc]	0.403[a]
5	464[a]	0.634[b]	1.498[b]	0.277[a]	0.366[bc]	0.101[b]	0.723[b]	2.363[a]	0.865[b]	6.167[b]	0.383[a]
10	455[a]	0.645[b]	1.392[b]	0.278[a]	0.355[cd]	0.099[b]	0.722[b]	2.158[b]	0.766[cd]	5.605[cd]	0.385[a]
20	478[a]	0.652[b]	1.298[c]	0.286[a]	0.348[d]	0.100[b]	0.714[b]	1.991[c]	0.693[d]	4.970[d]	0.401[a]
30	460[a]	0.625[bc]	1.372[bc]	0.283[a]	0.375[b]	0.106[ab]	0.717[b]	2.195[b]	0.823[bc]	5.562[cd]	0.395[a]
40	412[b]	0.694[a]	1.689[a]	0.238[b]	0.306[e]	0.073[c]	0.762[a]	2.434[a]	0.745[cd]	7.792[a]	0.312[b]

Note: Means superscripted by same letter in a column are not significantly different from each other at p = 0.05; comparison was done separately for normal and tolerant strain by applying LSD.

concentrations of the insecticide. There was a rise in the fluorescence at J level in the normal strain and this increased with the increase in the insecticide concentration. Variable fluorescence and fluorescence yield (φP_0) showed a higher value in tolerance strain then that of normal one. Treatment of the cyanobacterium with 40 μM of cypermethrin caused high values of the V_J, M_0, φD_0 and DI_0/RC whereas the low values of performance parameters ψ_0, φE_0, TR_0/RC and ET_0/RC were reported indicating that at this concentration of the insecticide the cyanobacterium was at stress in both normal and tolerant strains (Table 2). No significant variation in these parameters were observed at concentrations ≤ 30 mM of cypermethrin in the tolerant strain indicating that the cyanobacterium could become able to tolerate these concentrations of the insecticide but not so in the normal strain.

Lazar (2003) has observed that J fluorescence rise (the slope of initial fluorescence rise) is caused by an increase in the accumulation of excited states that are formed when Q_A is reduced and when Q_A is reduced together with single- or double- reduced Q_B. There is also a relative increase of F_0 with 40 μM of the insecticide, indicating the increase in Q_A reduction at the beginning but improvement of multiple turn over events with adaptation (Mohapatra *et al.,* 2010; Li *et al.,* 2010). In tolerance strain there is a stabilization in the PSII-PSI electron flow indicating its well adaptation to high concentrations of insecticide compared to the normal strain.

4. Conclusion

The study showed that prolonged exposure of *Anabaena* sp. PCC 7119 to growth inhibitory concentrations of cypermethrin induced tolerance of the cyanobacterium to the insecticide. The growth and photosynthetic efficiency of the cyanobacterium was not significantly affected by the insecticide at concentrations ≤ 30 mM whereas the insecticide could strongly inhibited the growth and metabolic performance of normal strain of the cyanobacterium. The tolerant strain of *Anabaena* sp. PCC 7119 could grow well at concentrations much higher than that possible in the environment achieved after commercial application of the insecticide. Thus it can be inferred that the cyanobacterium can be a good candidate for accelerated degradation of the insecticide of contaminated fields.

References

Agosin, M. (1985). Role of microsomal oxidations in insecticide degradation, In: Kerkut, G.A. and Gilbert, L.I. (ed.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 12, Pergamon Press, New York, pp. 647–712.

Ahmad, M. (2008). Potentiation between pyrethroid and organophosphate insecticides in resistant field populations of cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Pakistan. Pestic. Biochem. Physiol. 91: 24–31.

Antal, T. and Rubin, A. (2008). In vivo analysis of chlorophyll *a* fluorescence induction. Photosynth. Res. 96: 217–226.

Barata, C., Baird, D. J., Nogueira, A. J. A., Soares, A. M. V. M. and Riva, M. C. (2006). Toxicity of binary mixtures of metals and pyrethroid insecticides to *Daphnia magna* Straus. Implications for multi-substance risks assessment. Aquat. Toxicol. 78: 1–14.

Cheikh, H. B., Ali-Haouas, B., Marquine, M. and Pasteur, N. (1998). Resistance to organophosphorous and pyrethroid insecticides in *Culex pipiens* (Diptera: Culicidae) from Tunisia. J. Med. Entomol. 35: 251–260.

Cycon, M., Wójcik, M., Borymski, S. and Piotrowska-Seget, Z. (2012). A broad-spectrum analysis of the effects of teflubenzuron exposure on the biochemical activities and microbial community structure of soil. J. Environ. Manag. 108: 27-35.

Elias, E., Marques da Silva, J., Antunes, R. and Bernardes da Silva, A. (2004). Modulated chlorophyll fluorescence and post-harvesting control of ‘‘Rocha’’ pear quality. II. Fluorescence as an indicator of maturation. In: Barreiro, G. (ed.), Actas do IV Simposio Iberico sobre Maturacoa e Pos-Colheita. Estacoa Agronomica Nocional, Oeiras, pp. 179–183.

El-Latif, A. O. A. and Subrahmanyam, B. (2010). Glutathione S–transferase in the defence against pyrethroids in insects. Pestic. Biochem. Physiol. 96: 155–159.

Elloiott, M. and Janes, N. F. (1978). Synthetic pyrethroids-a new class of insecticides. Chem. Soc. Rev. 7: 473-505.

Force, L., Critchley, C., Van Rensen, J. J. S. (2003). New fluorescence parameters for monitoring photosynthesis in plant: I. The effect of illumination on fluorescence parameters of the JIP test. Photosynth. Res. 90: 1-19.

Gomez, A. A. and Gomeez, K. A. (1984). Statistical Procedures for Agricultural Research, John Wiley and Sons, New York.

Hirschberg, J. and Chamovitz, D. (1994). Carotenoids in cyanobacteria. In. Bryant, D.A. (ed.) The Molecular Biology of Cyanobacteria, Kluwer Academic Publ., The Netherlands. pp. 559-579.

Jena, S., Acharya, S. and Mohapatra, P. K. (2012). Variation in effects of four OP insecticides on photosynthetic pigment fluorescence of *Chlorella vulgaris* Beij. Ecotoxicol. Environ. Saf. 80: 111-117.

Kallqvist, T. and Romstad, R. (1994). Effects of agricultural pesticides on planktonic algae and cyanobacteria -

examples of interspecies variations. Norw. J. Agric. Sci. 13 (Suppl.): 117-/131.

Kostaropoulos, I., Papadopoulos, A. I., Metaxakis, A., Boukouvala, E. and Mourkidou, E. P. (2001). Pyrethroid resistance and esterase activity in three strains of the cotton bollworm, *Helicoverpa armigera* (Hübner). Insect Biochem. Molec. Biol. 31: 313–319.

Lazar, D. (2003). Chlorophyll *a* fluorescence rise induced by high light illumination of dark adapted plant issues tudied by means of a model of photosystem II and considering photosystem II heterogeneity. J. Theor. Biol. 220: 469–503.

Li, L., Chen, X., Zhang, D. and Pan, X. (2010). Effects of insecticide acetamiprid on photosystem II (PSII) activity of *Synechocystis* sp. (FACHB-898). Pestic. Biochem. Physiol., 98: 300-304.

Lowry, O. H., Rosebrough, N. J., Farr, A. B. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagents, J. Bio. Chem. 193: 265 - 269.

Marques da Silva, J. (2007) Chlorophyll fluorescence parameters of three Mediterranean shrubs in a summer–autumn period in central Portugal. Biol. Plant. 51 (4): 741–745.

Mohapatra, P. K. and Mohanty, R. C. (1992). Growth pattern changes of *Chlorella vulgaris* and *Anabaena doliolum* due to toxicity of dimethoate and endosulfan. Bull. Environ. Contam. Toxicol. 49: 576-581.

Mohapatra, P. K. and Schiewer, U. (1996). Influence of dimethoate on structure and function of the natural phytoplankton assemblage of the Darss-Zingst Bodden Chain reared in a laboratory. Pol. J. Environ. Stud. 5: 31 - 36.

Mohapatra, P. K. and Schiewer, U. (2000). Dimethoate and quinalphos toxicity: Pattern of photosynthetic pigment degradation and recovery in *Synechocystis* sp. PCC 6803. Algological Stud. 99: 74 - 94.

Mohapatra, P. K., Khillar, R., Hansdah, B. and Mohanty, R. C. (2010). Photosynthetic and fluorescence responses of *Solanum melongena* L. to field application of dimethoate. Ecotoxicol. Environ. Saf. 73: 78 - 83.

Mohapatra, P. K., Patra, S., Samantaray, P. K. and Mohanty, R. C. (2003). Effect of the pyrethroid insecticide cypermethrin on photosynthetic pigments of the cyanobacterium *Anabaena doliolum* Bhar. Pol. J. Environ. Stud. 12: 207-212.

Qu X.M., Tang, D.X. and Lin X. M. (2000). Toxicity and synergistic mechanism of parathion-methyl and fenvalerate mixture Jiaqing against three agricultural pest insects, J. Huazhong Agric. Uni. 19: 424–429.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. and Stanier, R.Y. (1979). Genetic assignments, strain histories and properties of pure culture of cyanobacteria. J. Gen. Microbiol. 1: 1-61.

Rodriguez, M., Ortiz, E., Bisset, J.A., Hemingway, J. and Saledo, E. (1993). Changes in malathion and pyrethroid resistance after cypermethrin selection of *Culex quinquefasciatus* field populations of Cuba. Med. Vet. Entomol. 7: 117–121.

Roe, J. H. (1955). The determination of sugar in blood and spinal fluid with anthrone reagent. J. Biol.Chem. 212: 335 - 343.

Sogorb, M. A. and Vilanova, E. (2002). Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. Toxicol. Lett. 128: 215–228.

Sogorb, M.A., Dý´az-Alejo, N., Escudero, M.A. and Vilanova, E. (1998). Phosphotriesterase activity identified in purified serum albumins. Arch. Toxicol. 72: 219–226.

Strasser, R. J., Srivastava, A. and Govindjee (1995). Polyphasic chlorophyll *a* fluorescence transient in plants and cyanobacteria. Photochem. Photobiol. 61, 32–42.

Strasser, R.J., Tsimilli-Michael, M. and Srivastava, A. (2005) Analysis of the chlorophyll a fluorescence transient. In: Papageorgiou, G. C., Govindjee (Eds.), Chlorophyll Fluorescence: A Signature of Photosynthesis. Kluwer Academic Publishers, Dordrecht-Boston-London, pp. 1–50.

Tandon, S., Pujari, A. and Sand, N. K. (2012). Degradation of fentrazamide herbicide in soil under aerobic condition. Bull. Environ. Contam. Toxicol. 89: 312–315.

Trovaslet-Leroy, M., Musilov, L., Renault, F., Brazzolotto, X., Misik, J., Novotny, L., Froment, M.-T., Gillon, E., Loiodic, M., Verdier, L., Masson, P., Rochu, D., Jun, D. and Nachon, F. (2011). Organophosphate hydrolases as catalytic bioscavengers of organophosphorus nerve agents. Toxicol. Lett. 206: 14– 23.

Tsimilli-Michael, M., Eggenberg, P., Biro, B., Köres-Pechy, K., Vörös, I. and Strasser, R. J. (2000). Synergistic and antagonistic effects of arbuscular mycorrhizal fungi and *Azospirillum* and *Rhizobium* nitrogen fixers on the photosynthetic activity of alfalfa, probed by a chlorophyll *a* polyphasic fluorescence transient O–J–I–P. Appl. Soil Ecol. 15: 169–182.

Wendt-Rasch, L., Friberg-Jensen, U., Woin, P. and Christoffersen, K. (2003). Effects of the pyrethroid insecticide cypermethrin on a freshwater community studied under field conditions. II. Direct and indirect effects on the species composition. Aquat. Toxicol. 63: 373-389.

Plant Science Research 34 (1&2) : 54-59, 2012

Plant Science Research

ISSN 0972-8546

Human ecology of a village in Similipal Biosphere Reserve, Odisha, India

S. Upadhyay[1], S. K. Sahu[1], G. K. Panda[2] and V. P. Upadhyay[3Ψ]

[1] Department of Environmental Science, Sambalpur University, Sambalpur, India

[2] Department of Geography, Utkal University, Bhubaneshwar, India

[3] Eastern Regional office, Ministry of Environment and Forests, Government of India,Bhubaneswar-751 023, India

ARTICLE INFO

Article history:
Received : 13 July 2012
Received in revised form : 19 September 2012
Accepted : 25 September 2012

Keywords:
forests
land use
livelihood
minor forest products
human population
Similipal

ABSTRACT

The present paper is an attempt to study the village-forest interface in the Similipal Biosphere Reserve. Many villages of Jashipur Block are located within Buffer Zone of Reserve. Study of the ecosystem linkage of Gudgudia village of this block indicates that the village have total area of about 1.6 Sq. km with majority of land coming under un-irrigated and culturable wastes categories. The village is dominated by Adivasi and SC *and* ST (73%) and Non Adivasi (27%) populations. Kharia and Kohl together constitute about 71.4% of houses and Mahakud, Teli, Mahali and Kamar etc. around 10%. Population of 180 cattle of the village are looked after by two Baramashias. The population depends on rain fed cultivation for livelihood as they grow only single crop per year. To improve the soil quality, some work to check soil erosion by making check dam and water harvesting structures has been done in the village. During Summer, villagers face problem of water shortage. Ground water level is around 300 feet. The people construct trenches to ward off the wild animals from the villages. Elephant herds usually enter in the village and destroy the paddy crop. The anthropogenic disturbances in wild habitats of core area force the wild animals to invade the human settlements. The Minor Forest Products (MFP) collection continues to be their main occupation as they collect wild vegetables, wild seeds, wild fruits, wild tubers and roots and wild flower in different months. The medicinal plants are also collected by the villagers as they consider the traditional medicines very effective over allopathic ones. It appears with this preliminary investigation that there is a close link between the inhabitants and the nature. Further study shall be of much help for revalidating the local knowledge for sustainable livelihood and for eco-sustainable management.

1. Introduction

Similipahar or Similipal is a compact patch of tropical forests located between 21^0 30' and 22^0 08' No lat and 86^0 05' and 86^0 37' E long. Similipal Biosphere Reserve (SBR) is a typical example of Mahanadian Biogeographic Zone located in the Mayurbhanj district of state Odisha. The district accounts for 10,418 sq. km. area of which forest area is of about 439000 ha, next to Kandhmal and Sundergarh districts. Mayurbhanj has the highest number of inhabited villages (3718) among all districts in Odisha. SBR also retains about 65 villages of which 4 are in core zone and 59 in buffer zone. With all efforts of planners and policy makers, these villages could not be relocated. 65 revenue villages are under three Grampanchyats. viz. Astakumar, Gudgudia and Barehipani. In hill regions of SBR, most of the settlements are established on southern and south-eastern aspects. The Tribal Research Bureau carried out study of villages in Similipal, describing ethnic, social and ecological aspects of the villages.

Similipal was declared biosphere reserve on June 22 1994 under UNESCO's Man and Biosphere programme (MAB). The forests of Similipal are highly biodiverse providing a good habitat for wild animals and various indigenous tribal populations. The destruction of Similipal started when British began influencing the management of Similipal forests for business interests by assigning long-

Ψ *Corresponding author; E-mail:* vpupadhyay@gmail.com

term leases to timber companies to supply slippers for railway lines. The Tribals from Ranchi, Singhbhum, Midnapore and other places of Jharkhand and West Bengal were brought to carry out forest operations. A few Tribals settled here for agriculture in Similipal forests, and today there exists 4 villages in core zone, 65 villages in buffer zone and about 1100 villages in peripheral zone. SBR has a total area of about 4,374 Sq Km of which 845 Sq Km is designated as core zone of Similipal Tiger Reserve (STR) and 2,129 Sq Km is buffer zone (1905 Sq Km of STR buffer + 77 Sq Km of Nato reserve forest + 147 Sq Km of Satkoshia reserve forest) and remaining about 1400 Sq Km in transitional zone or peripheral zone. The high biodiversity in wilderness habitat in Odisha has benefited the local people to seek for herbal remedies in the treatment of various diseases (Behera *et al.,* 2006; Singh and Sureja, 2007; Rout and Panda, 2010; Shiddamallayya *et al.,* 2010). The traditional health service providers like Vaidyas and Kabirajs or the knowledgeable persons know about huge potential of traditional knowledge for curing the people with the help of vast biodiversity resources. Several authors have highlighted the indigenous knowledge used by Tribals for medicine (Dey and De, 2011) and need for conservation of such resources (Pandey *et al.,* 2007; Ilahi *et al.,* 2007).

2. Materials and methods

One tribal village, Gudgudia located in buffer zone of biosphere reserve was selected to collect ecological data on human-forest interface for agro ecosystem study. Gudgudia is 29 km away from the block Headquarter of Jasipur. It is a small village under Gudgudia Grampanchayat located at 21^0 52' N lat and 86^0 15'E long. The village is connected to Jasipur by a forest road which leads to the core zone of the Biosphere Reserve. It is situated in a river valley of Khairi, with valley region located 600m elevation and the nearest hill top on south to south-east slopes of a hill is at an elevation of 921m above mean sea level. Two rivers namely Jamuna and Khairi meet each other at Kumari village of Gudgudia Grampanchayat; it flows down further to Gudgudia in a North to North-West direction.

The participatory rural appraisal method was applied through well structured questionnaire so as to collect data on the important elements affecting the human life, forests, and economy of the village. A household survey was conducted on a well-structured questionnaire between July 2003 and June 2004. Each Household was studied and data on livestock, house type, house structure, infrastructural amenities, agricultural land, seeds, fertilizer, pesticides, cow dung, human and animal labour and fence wood applied as agricultural input and crop output, kerosene, fuel wood and food consumption in the household and social traditions were collected. The census data of 2001 was used for calculation of demographic parameters. All agricultural and village data collected during study have been discussed in this paper. Group discussions were also held to find out some additional details regarding traditions and practices performed by the inhabitants of the village. All the data gathered was tabulated and analysed. The secondary data collected from census and Tribal Research Bureau were used to make a comparison with respect to changes in the land use, population and other facilities.

3. Results and Discussion

3.1 Population and Communities

The village has a total population of 526 (299 male and 227 female). The schedule tribe and schedule castes constitute about 73% (383 individuals) while general category population is represented by only 27% (143 Individuals) of the total population. There are 112 households in Gudgudia, of which 44 households are Kols and 34 are Kharia (71.4%). 11% households together comprise of Mahakud, Teli, Mahali, Bhatudi and Kamar communities. Some other tribes are also present as minority (Table 1). The records of past three decade show that population of the village is continuously increasing (Table 2). The total population increased by 29.2% in decade 1971 to 1981, while the total area decreased by 59.5% in the same decade. The sex ratio is also increasing from 1971 (803 females/thousand males) to 2002 (983 females/ thousand males). The number of households increased from 54 in 1971, to 112 in 2002. 80% households are below poverty line while 20% are above poverty line.

3.2 Population and social structure

In Gudgudia, there are 5 persons per household on an average basis: 2 males, 2 females and 1 child. The poor

Table 1
Community structure of Gudgudia village in SBR.

Sl. No.	Name of tribe	Households (No.)	Total household(%)
1.	Kharia	36	32.14
2.	Kol	44	39.29
3.	Bhathudi	2	1.79
4.	Mahali	3	2.68
5.	Teli	3	2.68
6.	Mahakud	12	10.71
7.	Kamar	2	1.79
8.	Others	10	8.93
	Total	112	100

Table 2
Demographic Structure of Gudgudia village in SBR.

Sl. No	Year	Total population	No of households	Male	Female	Sex ratio
1.	1971	274	56	152	122	803
2.	1981	354 (29.19)	93	181	173	956
3.	1991	453 (27.96)	94	243	210	864
4.	2001	526 (16.11)	112	299	227	759

Note: Values in parenthesis are decadal % growth

families have 4 to 5 persons while richer families have 10 to 12 persons. Usually after marriage or after the death of one of the parents, the son gets separated from the parent to look after his own family. The Bathudi, Mahakud, Mahali and Ho are main agricultural communities. They also work for various activities of Forest department to supplement their income. Kol and Kharia are in their very beginning stage to adopt agriculture as their main occupation is still the collection of Minor Forest Products. In Gudgudia, the age of house is form 6 years to 100 years (some more than 100 years). Their houses are made up of clay, bricks, Sal and Bamboo woods. The walls are mainly made up of wood or stone. Bricks have also been used in recently made houses. Use of wood from the forest on the basis of naturally acquired indigenous knowledge for different household purposes has been reported from various locations occupied by indigenous people in various parts of Odisha (Mohanty *et al.,* 2011). Roof is constructed mainly of Bamboo, Sal and Asan wood, thatched with paddy straws or Khappral (a type of tile made up of clay)/cement tiles. Floor is mostly kuchha, usually gabbed with a mixture of clay and cow dung. The number of rooms and its size and number of doors and windows depends upon the economic condition of the household. The poor families possess house of one room of 5x6 feet size, with one door and no window, while the rich households possess house of 4-5 rooms of 12x8 or 12x15 feet size with 4-5 doors and 3-4 windows. Most of the inhabitants make open yard of 2 to 3 feet wide on all the three sides of the house sparing the back side, covered by a projected part of the roofs. This protects the wall and yard during rainy season.

Most of the settlements inside Similipal follow the law of nature as their social and cultural life is intricately intermingled with nature. The settlements in Similipal are mainly of two types (open and closed). In open or the linear settlements, the houses are built along a river or pathway. The closed settlements are formed when the houses form a closed loop encircling a central yard. The central yard is used for common agricultural operations such as drying of grains, processing of agro-products, etc. A dhenki is commonly fixed on the front yard which is covered by projected part of roof.

3.3 Agriculture and animal husbandry

Agriculture is at its beginning stage in this village as majority of the households viz. Kharia and Kols still consider the minor forest products collection as their main occupation. They are still not mentally and financially prepared to adopt agriculture as their main occupation. They are also under the influence of super natural powers/ superstitions. The villagers of Gudgudia rear good number of animals as a source of additional income. The village has 180 animals of which 131 are cows, 38 goats and 11 sheep. The animals are looked after by two Baramashias. Animals are reared only for their meat and manure but not for milk and there is no market to sell milk.

Every household rears 5 to 6 cattle including two to three cows but buffalo is conspicuously absent from Similipal. The cow rearing is comparatively cheaper than buffalo. This might be the factor that only very few households are rearing buffalo. They rear bullocks for their ploughing requirements and for bullock carts. The presence of bullocks is also a status symbol in village. The poor households deploy cows also to plough their agricultural fields in case they have no bullocks, while in some villages they also get helped by their neighbours. Goats are reared chiefly for meat. They seldom drink milk of goat or cow, instead they leave it for the young calves. The cow dung is thrown directly to the fields, however they also do composting before applying to the fields. They rear hens and ducks also for their eggs and chickens to fulfill only their household requirements. Cattle are reared as per their requirements in agriculture and to supplement their resources. There is no animal husbandry facility available in all three Grampanchyats. Veterinary attendants also make frequent visits to the villages. The cattle are under direct supervision of one or two persons called Baramashias who take them for grazing inside the reserve forest and village

Table 3
Minor forest products collected by people of Gudgudia village in SBR.

Sl. No.	Items*	Period of Collection	Average collection (gm/day)	Sl. No.	Items*	Period of Collection	Average daily collection
Wild vegetables				Wild sap and Gum			
1	Sanka saga (*Pachyrrhizus sp.*)	Jan.-Mar.	600	1	Panaria (*Elaeocarpus wallichii*)	Mar.-Apr.	1 liter
2	Gadri saga (*Alternenthera sessilis*)	Jan.-Mar.	100	1	Simili gum (*Bombax ceiba*)	Mar.-Apr.	1 kg
3	Pita saga (*Trewia nudiflora*)	Jan.-June	267	Wild Fruits		Gram	
4	Zinka saga	Jan.-June	267	1	Chara (*Buchamania lanzan*)	June	200
5	Kulari saga (*Bahunia purpurea*)	Apr. -May	333	2	Chunkari (*Ziziphus rugosa*)	June	200
6	Sajana (*Moringa oleifera*)	Whole year	100	3	Guehra (*Acacia bucopholaea*)	June	200
7	Mati saga (*Pachyrrhizus erosus*)	Jan. -June	167	4	Rajara	November	200
Wild Seeds				5	Mahua (*Madhuca latifolia*)	March	1 000
1	Rimiri (*Protium serratum*)	June	67	6	Anola (*Emblica officinalis*)	February	1 000
2	Siali (*Bauhinia valichii*)	Feb.	233	Wild Tuber's & roots			
3	Khandia	Dec.	67	1	Muandai	April	500
4	Jambiro (*Citrus medica*)	Dec.	67	2	Pinkara	December	200
5	Zellrli	Whole Yr.	67	3	Rasa	December	200
6	Ghurdu (*Gardenia gummifera*)	Feb.	167	4	Ladu	December	200
7	Kusum (*Schleichera oleosa*)	Aug.	167	5	Pitaddu	December	200
8	Kendu (*Diospyros melanoxylon*)	April	167	6	Jungle alu (*Curcuma species*)	Feb.- Mar.	7000
9	Sal (*Shorea robusta*)	June	5000	7	Karu alu	July	500
10	Jamun (*Syzygium cumini*)	August	167	Wild Grasses			
11	Bela (*Aegle marmelos*)	August	167	1	Chana grass (*Imperata cylindrica*)	January	10000

forests, that are partially to fully degraded. Most of the villages take one crop per year; main crops grown are paddy (three varieties viz- Ashu, Aman *and* Dalua), Maize, Mustard and Rasi. Land holding is very unequal, the agriculture land per household ranges from 0.5 to 10 acres, while average land per household is 1.42 acres. The paddy production per household ranges from 2 to 40 quintals, while average paddy production/acre is 3.62 quintals. Big farmers sell excess paddy in market at Jashipur. For most of the household, the paddy crop does not fulfill their annual requirements. In ploughing operations, 2 to 13 persons are engaged depending upon the economic ability of the household. Number of bullocks per households ranges from 0 to 6. In sowing operation, the manpower requirement is 2 to 10 persons. The wage rates range from Rs.30 to Rs.40 per day for a 5 hours hard labour (data collected in 2003-04). Most of the villagers use previous crop seeds for sowing due to their inability to access the new improved crops. The fertilizers are used only by big farmers. The rich households apply cow-dung thrice in a year while the poor households apply only once a year. The modern improved seeds being used in the village are Sarana, Bhargavi, Ketki, Ratana, Annapurna, Khandagiri, Udayagiri, Konark, Kanchan, Samnata and Sarangi. Rest of the crops like Corn, Rasi, Mustrad, Kulthi are grown with seeds of indigenous varieties.

3.4 Change in Land use

In 1961, the Gudgudia was recorded as an uninhabited village. Forest area recorded in Gudgudia in 1971 was 28% of the total area, while in 1991it was reduced to 26%. The culturable waste land or the area of settlements, market and other community facilities increased following the increase in population. The culturable waste land was only 4% of the total village area in 1971 while in 1991 it was recorded as 21% of the total village area. No irrigation was recorded during the period. Only the low lying areas near the streams get some irrigation water by lifting the water. Due to rains, these areas suffer from gully erosion during rainy season. So villagers usually leave the river side area as fallow. These areas are classified as “unsuitable for cultivation”, degraded land by erosion and other anthropogenic factors.

3.5 Minor Forest Products

Fishes are relished by all the communities of the village. The major group of fishes being captured in the village are Silua, Khezara, Kari, Chengo, Baliputra, Genthu, Diazihiri, Birbal, Sala, Pitark, Turi, Kuahki and Gobhi. These freshwater fishes are captured only for domestic consumption as no transportation facilities or methods to preserve them is available. They also buy the Sukua Machha or dried fish from local Haat helds on every Sunday. Various types of green leafy vegetables (*saga*) are also collected by them from 100 to 600 grams per day, mostly for domestic consumption. The principal *saga* are: *Sanka saga, Gardi saga, Pita saga, Zinka saga, Kulari saga, Sajana saga* and *Mati saga*. All of these sagas are collected during January to June as no agricultural crop is available to them during this period. The principal wild seeds collected by them are Rimiri, Siali, Khandia, Jambiro, Ghurdu, Kusum, Sal, Kendu, Jamun and Bel in quantities of 67 to 233 grams per day except Sal, which have some market value. All wild seeds are collected during June to February.

Panaria, the main wild sap and Siali Gum are collected during March and April. On an average Panaria sap is collected 1 liter daily. Among wild dry fruits, Chara, Chunkari, Guehra, Rajara, Mahua, Anolaa and Harada are colleted 200 grams daily in the month of June except Rajara in November for domestic consumption. Seeds of Mahua and Harada are collected 1 kg per day in February to March also for domestic consumption. Muandai, Pinkara, Rasa, Ladu, Pitadu, Jungle Alu, Karu Alu are main wild tubers and roots collected by Tribals for domestic consumption at the rate of 200 to 500 grams per day, except Jungle Alu (5 to 7 kg daily). All these roots and tubers are collected during December to July. Gilliri Champa and Simili are main wild flowers collected by Tribals. The medicinal Plants collected by them includes Sarpa Gandha (*Rauvolfia serpentina*), Patal garuda (*Rauvolfia serpentina*), Murica ghasa, Kanbindi, Kasanapani, Salupani, Parasapani, Gangasiali (*Nyctanthes arboritritis*), Bhunineeba (*Andrographis paniculata*), Neem (*Azadirachta indica*), Karanja (*Pongamia pinnata*) and Ocalbinda. These forest product based medicines are used in malaria, stomach ache and other diseases which are considered endemic to the Similipal and its adjoining areas. Several researchers have reported use of 33 fern species from 21 families for medicines by the people residing inside Similipal Biosphere Reserve (Mishra *et al.,* 2001; Rout *et al.,* 2009). A good amount of firewood are also extracted form the forests besides these minor forest products. 45 kg of fire wood is utilized in one month on an average basis per household. The range of firewood utilization varies with economic ability of the households maximum being 70 kg and minimum 40 kg per month. Firewood is mainly collected from the village forests and some parts form the protected forests also. The main fire wood species are Sal, Asan, Gambhari, Piasal, Beguni, Neural, Jia, Dala and Vhakra etc.

4. Conclusion

The hill village ecosystem is highly dependant on forests. 71% of household belongs to primitive group of tribes. They are less developed to accept agriculture. In fact

they are in a transitional stage and not ready to give up their traditional occupation of minor forest products collection. The village economy largely revolves around minor forest products and agriculture in a less developed form. The unavailability of various agro-development measures further increase their dependency on forest products. The pressure on forest is continuously increasing with increase in population, though the decreased population growth rate percentage shows that there is awakening about problems due to large number of children. The minor forest products are collected mainly for domestic consumption only as there is no marketing facility available. Sustainable utilization of resources is a key to development of community and forests. Any form of disturbance in the ecosystem stability will have its direct impact on the natives. All the agriculture dependant human beings will indirectly get affected from it as the Similipal hills work as water tower of the region. The participatory resource management seems to be solution for Similipal. We recommend further systematic study by competent institutions as collaborative projects for revalidating the local knowledge for sustainable livelihood and for eco-sustainable management.

References

Behera, S. K., Panda, A., Behera. S. K., Misra, M. K. (2006). Medicinal plants used by the Kandhas of Kandhamal district of Orissa. Indian J. Trad. Knowl. 5(4): 519-528.

Census of India (2001). District Census Handbook, Mayurbhanj, and land released village level population data of 2001, Odisha, Census of India, New Delhi.

Chandola, L. P. (1976). A pilot survey of fuel consumption in rural areas. Indian Forester 102: 692-699.

Das, S. and Das, B. P. (1997). Similipal Biosphere Reserve: Genesis of a Historical Reality, Similipal: A Natural habitat of Unique Biodiversity. Odisha Environmental Society, Bhubaneswar.

Gopalan, C., Ramasastri, B.V. and Balasubramanian, S.C. (1982). Nutritive Value of Indian Foods. National institute of Nutrition, ICMR, Hyderabad, India.

Ilahi, I, Rahim, F. and Jabeen, M. (2007). Enhanced clonal propagation and alkaloid biosynthesis in cultures of *Rauwolfia*. Pakistan J. Plant Sci. 13(1): 45-56.

Mishra, R. C., Panda, P. C. and Das, P. A. (2001). Taxonomic study of the ferns and ferns allies of Gandhamardan hills, Orissa. J. Econ. Taxon. Bot. 25 (3): 577- 579.

Mitchell, R. (1976). An Analysis of Indian Agroecosystems. INTERPRINT. New Delhi.

Mohanty, N., Das, P. K. and Panda, T. (2011). Use of plant diversity in household and rituals by tribal people of Dhenkanal district, Odisha, India. J. Appl. Pharma. Sci. 1:79-82

Pandey, V. P., Kudakasseril, J., Cheriaand, E. and Patani, G. (2007). Comparison of two methods for in vitro propagation of *Rauwolfia serpentina* from nodal explants. Indian Drugs 44: 514-519.

Rout, S. D. Panda, and S. K. (2010. Ethnomedicinal plant resources of Mayurbhanj district, Odisha. Indian J. Trad. Knowl. 9: 68-72.

Rout, S. D., Panda, T. and Mishra, N. (2009). Ethnomedicinal studies on some pteridophytes of Similipal Biosphere Reserve, Orissa, India. Internatl. J. Med. Medic. Sci. 1: 192-197 .

Shiddamallayya, N., Yasmeen, A. and Gopakumar, K. (2010). Hundred common forest medicinal plants of Karnataka in primary healthcare. Indian J. Trad. Knowl. 9(1): 90-95.

Singh, R. K. and Sureja, A. K. (2007). Dynamics of sustainable livestock and natural resources management. Indian J. Trad. Knowl. 6(4): 619-629.

Plant Science Research 34 (1&2) : 60-64, 2012

Plant Science Research

ISSN 0972-8546

Tree diversity in moist deciduous forests of Similipal biosphere reserve, Odisha, India

T. Kar[1Ψ], K. K. Mandal[1], C. S. Reddy[2] and A. K. Biswal[1]

[1] P. G. Department of Botany, North Orissa University, Baripada, Odisha -757003

[2] Forestry & Ecology Division, National Remote Sensing Centre, ISRO, Balanagar, Hyderabad-500625

ARTICLE INFO

Article history:
Received : 10 October 2012
Received in revised form : 08 December 2012
Accepted : 09 December 2012

Keywords:
biodiversity
conservation
phytosociology
Similipal

ABSTRACT

The phytosociological study was conducted in moist deciduous forests of Similipal Biosphere Reserve of Mayurbhanj, Odisha to assess species structure, distribution, diversity and dominance of trees. Enumeration of all tree species ≥ 30 cm GBH yielded a total of 3214 individuals belonging to 141 species, under 105 genera and 41 families. The most dominant families were Euphorbiaceae, Rubiaceae, Moraceae, Caesalpiniaceae and Fabaceae. Shanon-Weiner Index (H') was calculated as 3.46 with Simpson Index of dominance of 0.90. Analysis of population density of trees across the girth class interval showed that nearly 31.83 % of individuals belong to 30-50 cm GBH. The result of the study renders a helping hand to the forest managers in preparing a conservation plan for the highly species diverse and threatened ecosystem of moist deciduous forests of Similipal Biosphere Reserve, Mayurbhanj, Odisha.

1. Introduction

The analysis of forest structure and composition is prerequisite for formulating appropriate conservation strategies and management of protected areas. Phytosociological study indicates species diversity and distribution of individuals in a plant community of a habitat (Kar *et al.,* 2009). The depletion of plant genetic resources is mainly due to loss and fragmentation of habitats. The conversion of forest land for agriculture, human settlement and over exploitation of forest resources for timber, fuel wood, non-timber forest products, etc. are some of the anthropogenic causes which threatens the loss of biodiversity.

Similipal Biosphere Reserve (SBR) is one of the seventeen biosphere reserves of India. Due to its rich biodiversity and cultural significance it has been included in World Network of Biosphere Reserve (WNBR) since 6th may, 2009. It lies between 20^0 17' to 22^0 34'N latitude and 85^0 40' to 87^0 10' E longitude, located in the central part of Mayurbhanj district, Odisha. It extends over an area of 5569.00 km^2 with a core area of *ca* 1194.75 km^2, buffer area *ca* 1335.88 km^2 and transition area *ca* 3038.39 km^2 Similipal is considered as Himalayas of Odisha, because of its enormous influence over the climate of the state and its neighbourhood and is a treasure house of floristic diversity of Odisha.

Moist deciduous forests are predominantly composed of taxa namely *Shorea robusta*, *Terminalia alata, Haldinia cordifolia, Anogeissus latifolia, Schleichera oleosa, Pterocarpus marsupium, Syzygium cumini, Dillenia pentagyna, Terminalia bellirica, Kydia calycina, Mitragyna parvifolia, Ardisia solanacea,* etc.The shrubby elements are mostly evergreen. *Bauhinia vahlii, Butea superba* and *Millettia extensa* are worth mentioning among the climbers.

Earlier few quantitative estimation of the vegetation of Similipal were carried out by some workers (Reddy *et al.,* 2000; Mishra *et al.,* 2000, 2003, 2006, 2008). But phytosociological studies on tree species of moist deciduous forest in Similipal Biosphere Reserve is scanty. Keeping in view the above fact the present study is an attempt to

Ψ *Corresponding author; Email:* truptirekha86@gmail.com

record structural composition of moist deciduous forest types in Similipal, Mayurbhanj, Odisha, India.

2. Materials and methods

2.1 Ground data

Phytosociological study was carried out in moist deciduous forest types of Similipal Biosphere Reserve using random quadrat (20 m × 20 m). Trees greater than or equal to 30 cm GBH were recorded from all the sample plots. The data were collected from randomly selected 140 sample plots (5.6 ha) from different forest ranges of Similipal Biosphere Reserve. The elevation, aspect, latitude, longitude and level of biotic interference etc. are given in Table 1. The size and number of quadrats needed were determined using the species area curve (Misra, 1968). Voucher specimens were collected for preparation of Herbarium adopting proper scientific methodology. The species were identified in consultation with taxonomic literature, viz., Flora of Presidency of Madras (Gamble, 1915-1935), Botany of Bihar and Orissa (Haines, 1921-1925), Supplement to the Botany of Bihar and Orissa (Mooney, 1950), Flora of Orissa (Saxena and Brahmam, 1994-1996), Flora of Bilaspur district (Murti and Panigrahi, 1989-1999), and Flora of Bihar b(Singh *et al.,* 2001). The information regarding latitude, longitude and altitude of each quadrat in sample area were collected using a Global Positioning System (GPS).

2.2 Data analysis

The main purpose of phytosociological analysis is carried out to understand vegetation characteristics and to estimate the species richness and species diversity existing in the study area. In order to express the dominance and ecological success of any species with a single value, the concept of important value index has been utilized. This index utilizes three characteristics, viz., relative frequency, relative density and relative dominance. The analysis was carried out for each vegetation type for computing important value index.

Relative density (RD) = (Density of sp. /Total density of all spp.) x 100.

Relative frequency (RF) = (Frequency of sp./Total frequency of all spp.) x 100.

Relative dominance (ReD) = (Basal area of sp./Total basal area for all spp.) x 100.

Importance Value Index (IVI) = RD + RF + ReD

Species diversity of this forest type was determined as explained by Shannon and Wiener (1963) (*H'*). Concentration of dominance was also measured as Simpson's index (C) (Simpson, 1949).

3. Results and discussion

3.1 Floristic composition

During the present study a total of 3214 trees belonging to 41 families from 140 sample plots were enumerated. Among the families, Euphorbiaceae (15 species), Rubiaceae (14 species) and Moraceae (8 species) were most diverse. Fabaceae and Caesalpiniaceae were represented by 7 species each.

The dominant tree elements are *Shorea robusta, Terminalia alata, Anogeissus latifolia, Syzygium cumini, Protium serratum, Xylia xylocarpa,* etc. The tree species with their IVI values are given in Table 2. The top most 20 species represented 73.55 % of individuals in the forests. The Shannon-Weiner Index (H') was 3.46 with Simpson index of 0.9.

3.2 Forest structure

Stem density was found decreased with increasing girth class of tree species from 50 cm girth (Fig. 2). The

Table 1
Characteristic features of the study area.

Forest Range of Similipal	Elevation (m)	Aspect	Latitude	Longitude	Level of biotic interference
Pithabata	200 - 830	East	21° 55' - 21° 59'	86° 32' - 86° 36'	HB
Nawana	650 - 810	North	21° 52' - 21° 56'	86° 18' - 86° 25'	MB
Chahala	780 - 850	North	21° 56' - 21° 59'	86° 17' - 86° 22'	NB
Jenabil	740 - 890	South	21° 25' - 21° 52'	86° 21' - 86° 52'	NB
Upper barakamuda	815 - 1165	South	21° 02' - 21° 50'	86° 16' - 86° 50'	NB
National Park	444 - 920	North-west	21° 09' - 21° 52'	86° 14' - 86° 54'	MB

Abbreviations: HB – High biotic interference; MB – Moderate biotic interference; NB – No biotic interference.

Table 2
The top twenty predominant species with their IVI values.

Sl. No.	Name	Relative Density	Relative Frequency	Relative Dominance	IVI
1	*Shorea robusta* Gaertn.f.	27.785	7.749	35.773	71.307
2	*Terminalia alata* Heyne ex Roth	8.525	7.159	8.247	23.931
3	*Anogeissus latifolia* (Roxb.ex DC) Wall.ex Guill.&Perr.	4.636	4.797	4.634	14.067
4	*Syzygium cumini* (L.) Skeels	4.449	4.502	3.642	12.593
5	*Protium serratum* (Wall.ex Colebr.) Engl.	3.205	3.247	3.574	10.026
6	*Xylia xylocarp*a (Roxb.) Taub.	2.862	2.435	3.716	9.014
7	*Dillenia pentagyna* Roxb.	2.302	3.173	2.674	8.150
8	*Haldinia cordifolia* (Roxb.) Ridsd.	2.085	3.100	2.646	7.831
9	*Scheleichera oleosa* (Lour.) Oken	1.836	2.657	3.003	7.495
10	*Madhuca indica* Gmel.	1.773	2.140	2.843	6.756
11	*Croton roxburghii* Balak.	2.427	2.583	0.785	5.795
12	*Pterocarpus marsupium* Roxb.	1.680	1.993	1.620	5.293
13	*Syzygium cerasoides* (Roxb.) Chatt.& Kanjilal	1.805	1.771	1.142	4.718
14	*Bridelia retusa* (L.) Spreng	0.965	1.476	1.435	3.876
15	*Mangifera indica* L.	0.498	0.664	2.708	3.870
16	*Bombax ceiba* L.	0.933	1.771	1.154	3.858
17	*Buchanania lanzan* Spreng.	1.369	1.919	0.555	3.843
18	*Nyctanthes arbor-tristis* L.	1.836	1.624	0.293	3.752
19	*Terminalia chebula* Retz.	1.089	1.697	0.936	3.722
20	*Cleistanthus collinus* (Roxb.) Benth.ex Hook.f.	1.493	1.697	0.465	3.656

Fig. 1. Location map of Study area.

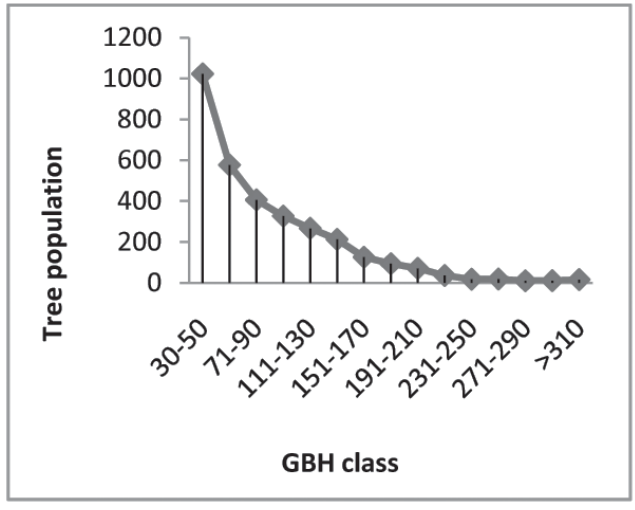

Fig. 2: Relation between girth class and tree population.

Table 3
Population density of tree elements across girth class intervals.

GBH class (cm)	No. of Species	% of Species	No. of individuals	% of individuals
30-50	122	86.52	1023	31.83
51-70	87	61.70	577	17.95
71-90	71	50.35	406	12.63
91-110	110	78.01	327	10.17
111-130	46	32.62	267	8.31
131-150	38	26.95	213	6.63
151-170	28	19.86	126	3.92
171-190	23	16.31	94	2.92
191-210	21	14.89	71	2.21
211-230	18	12.77	36	1.12
231-250	8	5.67	18	0.56
251-270	12	8.51	19	0.59
271-290	7	4.96	10	0.31
291-310	6	4.26	11	0.34
>310	10	7.09	16	0.50
Grand Total	141	100	3214	100

distribution of the basal area across different GBH interval showed that the GBH class having 30-50 cm contributed 86.52 % of species richness (Table 3). The highest GBH was measured case of *Ficus rumphii* (672 cm), *Mangifera indica* (455 cm), *Shorea robusta* (430 cm), *Terminalia chebula* (390 cm), etc. Among the tree species the relative density was highest in *Shorea robusta* (27.78) followed by *Terminalia alata* (8.52), *Anogeissus latifolia* (4.63), *Syzygium cumini* (4.44) and *Protium serratum* (3.20). Relative dominance was highest in *Shorea robusta* (35.77) followed by *Terminalia alata* (8.24), *Anogeissus latifolia* (4.63), *Xylia xylocarpa* (3.71) and *Syzygium cumini* (3.64).

The IVI of most dominant tree *i.e*. *Shorea robusta* is 71.307, which is comparable with the IVI value of Mishra *et al.* (2006, 2008) reported in Site 10 as 53.75 for the same species. The change in IVI of *Shorea robusta* is due to the change in species composition, disturbance and altitude. The tree species with higher IVI values indicate their good regeneration capacity and more adaptability.

Many researchers have reported the diversity value for Indian forests in the range of 0.8 to 4.1 (Parthasarathy *et al.,* 1992; Visalakshi, 1995).The diversity value of the tree species obtained in the present study is within the reported range of tropical forests which is significant. However, our value is lower than some other tropical forest (Knight, 1975) due to anthropogenic disturbances.

4. Conclusion

The quantitative characters with reference to density, diversity and frequency distribution could well act as indicators of anthropogenic disturbances that are affecting various forest types and such studies would help in understanding the threats that are being faced by the tropical forests and would help in deriving conservation policies. An understanding of the distribution of tree species and their assemblages must play an important role in elucidating the larger patterns of distribution of biodiversity.

Acknowledgements

The authors are thankful to Head, P.G. Department of Botany, North Orissa University, Takatpur, Baripada for providing necessary facilities, valuable suggestions and constant encouragement. Special thanks to local community and Forest deptt. Staff who have rendered their co-operation to carry out the field work.

References

Gamble, J. S. (1915-1935). Flora of Presidency of Madras. London; reprinted 1967. Calcutta.

Haines, H. H. (1921-1925). The Botany of Bihar and Orissa. Adlard & Son Ltd., London.

Kar, A., Reddy, C. S. and Biswal, A. K. (2009). Structure, composition and diversity of tree species in tropical deciduous forests of Keonjhar district, Orissa. Plant Sci. Res. 31(1&2): 66-70.

Knight, D.H. (1975). A phytosociological analysis of species rich tropical forest on Barro Colorado Island, Panama. Ecol. Monogr. 45: 259-289.

Mishra, R. K., Upadhyay, V. P. and Mohanty, R. C. (2000). Niche width, diversity and distribution pattern of tree species in Similipal Biosphere Reserve. J. Indian Bot. Soc. 79: 61-65.

Mishra, R. K., Upadhyay, V. P. and Mohanty, R. C. (2003). Vegetation diversity of Similipal biosphere reserve. E-Planet 1:4-9.

Mishra, R. K., Upadhyay, V. P., Mohapatra, P. K., Bal, S. and Mohanty, R. C. (2006). Phenology of species of moist deciduous forest sites of Similipal biosphere reserve. Lyonia 11:5-17.

Mishra, R. K., Upadhyay, V. P. and Mohanty, R. C. (2008). Vegetation ecology of the Similipal Biosphere Reserve. Appl. Eco. Env. Res. 6(2): 89-99.

Misra, R. (1968). Ecology Workbook. Oxford and IBH Publishing Co., New Delhi, India.

Mohanty, R. C., Mishra, R. K., Bal, S. and Upadhyaya, V. P. (2005). Plant diversity assessment of *Shorea robusta* dominated forest stands of Similipal Biosphere Reserve. J. Ind. Bot. Soc. 84: 21-29.

Mooney, H. F. (1950). Supplement to the Botany of Bihar and Orissa, Catholic Press, Ranchi.

Murty, S. K. and Panigrahi, G. (1989-1999). Flora of Bilaspur district, M.P. BSI Publication, Calcutta.

Parthasarathy, N., Kinhal, V., and Praveen Kumar, L. (1992). Plant species diversity & human impact in the tropical wet evergreen forests of Southern Western Ghats. In: Indo-French Workshop on Tropical Forests Ecosystem: Natural functioning & Anthropogenic Impact. French Institute, Pondichery.

Reddy, C. S., Pattanaik, C., Mohapatra, A. and Biswal, A. K. (2007). Phytosociological observation on tree diversity of tropical forest of Similipal Biosphere Reserve, Orissa, India. Taiwania 52(4): 352-359.

Saxena, H. O. and Brahmam M. (1994-1996). The Flora of Orissa. Vol. I-IV. Orissa Forest Development Corporation Ltd, Bhuabneswar, India.

Shannon, C. E. and Wiener, W. (1963). The mathematical theory of communication. University of Illinois press, Urbana, USA.

Simpson, E. M. (1949). Measurement of diversity. Nature. 163: 688-670.

Singh, N. P., Mudgal, V., Khanna, K. K., Srivastava, S. C., Sahoo, A. K., Bandopadhyay, S., Aziz, N., Das, M., Bhattacharya, R. P. and Hajara, P. K. (2001). Flora of Bihar. Analysis. BSI publication, Culcutta.

Visalakshi, N. (1995).Vegetation analysis of two tropical dry evergreen forests in Southern India. Trop. Ecol. 36: 117-127.

Plant Science Research 34 (1&2) : 65-71, 2012

Plant Science Research

ISSN 0972-8546

Distribution of Rhizophoraceae mangroves of intertidal regions of Odisha coast, India

S. Acharya[1] and P. K. Mohapatra[2Ψ]

[1] P.G. Department of Botany, Utkal University, Bhubaneswar-751004, India

[2] P.G. Department of Botany, Ravenshaw University, Cuttack-753003, India

ARTICLE INFO

Article history:
Received : 9 September 2012
Accepted : 22 October 2012

Keywords:
Bhitarkanika
Rhizophoraceae
mangroves
phytosociology
IVI

ABSTRACT

Phytosociological analysis of the Rhizophoraceae mangroves was carried out in Bhitarkanika mangrove division, Odisha to describe the forest composition and structure, and to assess the distribution of these species. Ten transect plots of 10 m x 100 m with a total area of one hectare were established perpendicular to the coast line in five different mangrove blocks of the study site. A total of 8 species of trees and saplings were recorded. Three community types were recognized namely Rhizophora apiculata - *R. mucronata*, *Bruguiera-Kandelia candel* and *Ceriops* reflecting the zonation in this forest. The maximum number of trees was 6780/ha found in Kadua block. *R. apiculata* was most successful in distribution with 6440 saplings and trees/ha followed by *R. mucronata* (3600/ha). Trees of both the *Rhizophora* species were mostly growing under Low Tide Lines (LTL) while the *Bruguiera* and *Ceriops* species occurred around the High Tide Lines (HTL). The IVI values of *R. apiculata* indicates maximum in Mahanadi deltaic region whereas *R. mucronata* exhibits maximum in core areas of Bhitarkanika. The present study also reveals the fact that *Bruguiera gymnorrhiza* is well-adapted in the varying salinities between LTL and HTL.

1. Introduction

Mangroves are typically tropical and subtropical coastal vegetations found in inter-tidal zones of river deltas and backwater areas. Mangrove has been defined as "any woody, tropical facultative halophyte that is an obligate inhabitant of 'mangal' (wetland community) (Tomlinson, 1986; Krauss and Ball, 2012). They from a dynamic ecosystem, which flourishes only in the region where there is influx of both fresh and tidal water. Mangrove forests dominate one-quarter of the world's tropical coastline. Mangroves have been defined by Hamilton and Snedaker (1984) as salt tolerant ecosystems of the intertidal regions along coastlines. The total area of Indian mangroves is estimated to be 4827 km^2 distributed along the coastal States/Union Territories, which account for about 5% percent of the World's mangrove vegetation and 0.4 percent of the geographical area. Mangroves forests are widely distributed throughout the tropics where they grow abundantly along the coasts (Choong *et al.,* 1990). Mangroves are fast disappearing at a time when there are clear indications of potential changes in climate, sea level and the levels of UV-B radiation. Indiscriminate exploitation of mangrove resources without any land use plan has degraded mangrove ecosystems in India. India has lost 40 percent of its mangrove area cover during the last 100 years (Anonymous, 1987).

The state of Odisha has an area of 195 km^2 under Mangrove forests, which comes to 0.125 % of geographical area and 0.414 % of actual forest cover (Daniels and Acharjyo, 1997). Worldwide concern to conserve mangroves necessitated propagation of mangroves to re-establish them on barren and swampy land along tidal creeks around degraded salt-marshy wetlands of Mahanadi delta where mixed stands of Rhizophoraceae mangroves depending on the intensity and the frequency of tidal inundation at the experimental site. The present paper highlights ecological structures of mangroves ecosystem of Odisha coast with a

Ψ *Corresponding author; Email:* pradiptamoha@yahoo.com

special reference to Rhizophoraceae family based on phytosociological studies.

2. Materials and methods

2.1 The study site

The study site is located at 20^0 4'- 20^0 8' N Latitude and 86^0 45'- 87^0 5' E Longitude in Bhitarkanika sanctuary (19^0 N - 22^0 N and longitude 85^0 E - 87^0 E) and reserve forest of Mahanadi delta area in the north-eastern coastal plain of Kendrapara district. This area receives water from three rivers, known to be rich in species diversity and trees are dense and tall like those of Sunderbans (Selvam, 2003). Four forest blocks in the Bhitarkanika wildlife sanctuary were selected for carrying out vegetation survey. The area of Bhitarkanika forest block is 1712 ha, Dangmal 636 ha, Kakranasi 310 ha, and Thakurdia 272 ha (Chadha and Kar, 1999). Bhitarkanika and Dangmal blocks constitute the core area. These sites experience tide of semi diurnal type. The mean sea level in the region is about 1. 66 m. The Bhitarkanika sanctuary is bounded by river Dhamra in the north, the river Hansua to the west and Bay of Bengal on the eastern and southern sides. The sanctuary encompasses 35 km sea coast known as 'Gahirmatha Coast' from Dhamra mouth to Barunei, the mouth of river Hansua. The area has about 200 km of water body inside the sanctuary and falls in the deltaic region of the river Brahmani, Baitarani, and their distributaries. The estuarine rivers- Brahmani, Baitarani, Kharasrota, Dhamra, Pathasala, Maipura, Hansua and Hansina during their course flow into the Bay of Bengal are further criss crossed by numerous creeks, channels, and nallahs, thus providing the peculiar ecological niche for the growth, development of rich and varied mangrove life forms, both flora and fauna along with their associates. There are many villages within the sanctuary as well as surrounding it. The population in these villages has been growing very fast. Part of the population rise is because of the heavy influx of refugees from East and West Bengal and habitations are reported to have been started by clearing mangrove forests. A total of 81 villages are adjacent to the mangrove forests.

2.2 Collection and preparation of herbarium specimen

Plants were collected from the study sites at regular intervals and detailed field notes were recorded on the spot which included field number, date of collection, locality, habit, habitat, associated species. The specimens were identified with the help of regional flora viz., the flora of Orissa (Saxena and Brahmam, 1994-96), the botany of Bihar and Orissa (Haines, 1921-25) and its supplement (Mooney, 1950) and different monographs and revisionary works. After collection, the specimens were carefully pruned to convenient size and poisoned in formaldehyde to prevent defoliation. Care was taken to arrange them properly so that all parts are well displayed. The collected species were pressed in the blotters and to avoid infection, the blotters were frequently changed. The specimens were sprinkled with naphthalene powder and when fully dried these were poisoned with 4% solution of $HgCl_2$ in dehydrated alcohol and kept under pressure for about a week. After these processes, the specimens were mounted on to the herbarium sheets (42 × 28 cm) by the application of animal glue. For better adhesion, the mounted materials were again kept under pressure for two days. Then field data were transferred to the herbarium labels from the field note book after which the mount boards were kept as herbarium specimen following standard methods (Lawrence, 1951; Jain and Rao, 1977).

2.3 Phytosociological analysis

Phytosociological studies were carried out during 2010-2011 by laying random quadrats (10 x10 m^2). Ten transect plots of 10 x 100 m^2 (1 ha) were taken perpendicular to the coast line. Each transect was divided into 10-12 subplots of 10 x 10 m^2 from Low Tide Line (LTL) towards High Tide Line (HTL). The total study area was divided into five blocks, viz., Kadua, Kansaridia and Kharnasi forest blocks of Mahanadi deltaic area and Bhitarkanika and Khola forest blocks of National Park region. Every tree (DBH >10cm) and sapling (DBH 2-10 cm) present within each plot was counted. The diameter of trees and saplings was also measured. The canopy of each tree was mapped to construct the forest profile. Phytosociological analysis in five study sites was carried out by following Mishra (1968), Kershaw (1973), Cintron and Novelli (1984) and Snedaker and Snedaker (1984). The structural parameters viz., abundance, density, frequency, basal area, relative parameters and IVI were calculated from the data obtained from quadrats.

3. Results and discussion

3.1 Phytosociological analysis

The Kansaridia forest block contain highest number of tree species followed by Bhitarkanika, Kadua, Khola and Kharnasi blocks. Bhitarkanika is a part of core area of the Bhitarkanika wildlife sanctuary. Among the five forest blocks studied in the coastal stretches of Odisha, Kansaridia protected forest block of the Mahanadi deltaic region exhibit maximum diversity and abundance of Rhizophoraceae mangroves (Table 1). Availability of fresh water through Bhitarkanika (Maipura river) and Brahmani rivers and saline water from sea in core area helped wide range of niches for different species to occur and, thus, species diversity is the highest. Structural analysis of vegetation of different study sites showed that *R. apiculata* and *R. mucronata* exhibited

greater density, frequency and IVI values across all sites (Table 1). The species with lower density and IVI were found different from one site to the other. All the species showed contagious distribution. Odum (1971) has mentioned that contagious distribution is commonest in nature, random distribution is found only in very uniform environment and regular distribution occurs where severe competition exists between individuals. A/F ratio range in Kharnasi block was proportionately less wide compared to other blocks.

3.2 Community distribution along tidal lines

Eight species were recorded within the ten sampling plots in five different forest blocks. These consisted of 8 tree species (Fig. 1) and two shrubs. It can be seen that 2 Rhizophoraceae species (25 %) of the genus Rhizophora are true mangrove species, and these represent 49.8% of the true mangrove species found in Odisha Coast (Upadhyay and Mishra, 2008). Cluster analysis using a Bray-Curtis dissimilarity measure and group average sorting strategy showed three groups in the sample plots, reflecting the zonation within the mangrove forest (Clifford and Stephenson, 1975). The three zones, extending from the seaward to the landward side were characterized by the following three community types (Fig. 2): 1. *Rhizophora apiculata-Rhizophora mucronata* community, 2. *Bruguiera-Kandelia candel* community and, 3. *Ceriops* community.

The *Rhizophora apiculata-Rhizophora mucronata* community occupied the seaward fringe and had a width of

Fig. 1. Distribution of Rhizophoraceae family mangroves in Bhitarkanika and Mahanadi delta Mangrove Division. a. creek showing Rhizophoraceae plants along the bank; b. *Rhizophora apiculata*; c. *Rhizophora mucronata*; d. *Bruguiera gymnorrhiza*; e. *Bruguiera cylindrica*; f. *Bruguiera parviflora*; g. *Ceriops tagal*; h. *Ceriops decandra*; i. *Kandelia candel*.

Table 1
Phytosociological parameters of Rhizophoraceae mangroves.

Sl. No.	Species	Dn (%)	F (%)	A (%)	A/F	RD (/1000)	RF	RDn	IVI
				Kadua block					
1	*Rhizophora apiculata* Bl.	15.70	90.00	17.44	0.19	870	25.71	0.23	26.82
2	*Rhizophora mucronata* Lam.	3.60	50.00	7.20	0.14	128	14.29	0.05	14.47
3	*Brugiera gymnorrhiza* L.	5.10	50.00	10.20	0.20	0.58	14.29	0.08	14.36
4	*Brugiera paviflora* Roxb.	0.70	30.00	2.33	0.08	0.03	8.57	0.01	8.58
5	*Brugiera cylindrica* Bl.	15.40	60.00	25.67	0.43	0.62	17.14	0.23	17.37
6	*Ceriops decandra* Griff.	26.60	70.00	38.00	0.54	0.41	20	0.40	20.40
7	*Ceriops tagal* Perr.	1.60	40.00	4.00	0.10	0.02	11.43	0.02	11.45
8	*Kandelia candel* L.	-	-	-	-	-	-	-	-
				Kansaridia block					
1	*Rhizophora apiculata* Bl.	17.5	60	29.17	0.49	830	20.69	0.32	21.84
2	*Rhizophora mucronata* Lam.	4.95	45	11.40	0.27	168	15.52	0.09	15.78
3	*Brugiera gymnorrhiza* L.	4.5	20	22.50	1.13	0.8	6.90	0.10	7.00
4	*Brugiera paviflora* Roxb.	2.1	25	7.75	0.30	0.1	8.62	0.04	8.67
5	*Brugiera cylindrica* Bl.	9.1	55	15.92	0.28	0.5	18.97	0.19	19.16
6	*Ceriops decandra* Griff.	12.2	65	19.19	0.30	0.2	22.41	0.23	22.65
7	*Ceriops tagal* Perr.	4.45	60	7.53	0.13	0.1	20.69	0.09	20.78
8	*Kandelia candel* L.	7.5	60	12.50	0.21	0.4	20.69	0.13	20.82
				Kharnasi block					
1	*Rhizophora apiculata* Bl.	-	-	-	-	-	-	-	-
2	*Rhizophora mucronata* Lam.	-	-	-	-	-	-	-	-
3	*Brugiera gymnorrhiza* L.	3.1	40	7.75	0.19	210	33.33	0.16	33.70
4	*Brugiera paviflora* Roxb.	-	-	-	-	-	-	-	-
5	*Brugiera cylindrica* Bl.	0.3	20	1.50	0.08	40	12.50	0.02	12.55
6	*Ceriops decandra* Griff.	8.5	60	10.90	0.23	370	39.58	0.50	40.45
7	*Ceriops tagal* Perr.	1.9	40	4.67	0.15	50	27.08	0.11	27.24
8	*Kandelia candel* L.	8	50	13.71	0.24	510	37.50	0.42	38.43
				Bhitarkanika block					
1	*Rhizophora apiculata* Bl.	19.3	60	32.17	0.54	662	20.69	0.33	21.68
2	*Rhizophora mucronata* Lam.	28.5	100	28.50	0.29	626	34.48	0.48	35.59
3	*Brugiera gymnorrhiza* L.	4.2	70	6.00	0.09	0.29	24.14	0.07	24.21
4	*Brugiera paviflora* Roxb.	2	50	4.00	0.08	0.06	17.24	0.03	17.28
5	*Brugiera cylindrica* Bl.	4.7	60	7.83	0.13	0.12	20.69	0.08	20.77
6	*Ceriops decandra* Griff.	2.8	50	5.60	0.11	0.03	17.24	0.05	17.29
7	*Ceriops tagal* Perr.	1.1	40	2.75	0.07	0.01	13.79	0.02	13.81
8	*Kandelia candel* L.	2.9	50	5.8	0.12	0.16	17.24	0.05	17.29

Sl. No.	Species	Dn (%)	F (%)	A (%)	A/F	RD (/1000)	RF	RDn	IVI
				Khola block					
1	*Rhizophora apiculata* Bl.	4.1	50	8.20	0.16	141	17.24	0.07	17.45
2	*Rhizophora mucronata* Lam.	9.9	60	16.50	0.28	217	20.69	0.17	21.07
3	*Brugiera gymnorrhiza* L.	2.4	40	6.00	0.15	0.17	13.79	0.04	13.83
4	*Brugiera paviflora* Roxb.	1.5	50	3.00	0.06	0.04	17.24	0.03	17.27
5	*Brugiera cylindrica* Bl.	8.2	80	10.25	0.13	0.20	27.59	0.14	27.73
6	*Ceriops decandra* Griff.	2.8	50	5.60	0.11	0.03	17.24	0.05	17.29
7	*Ceriops tagal* Perr.	-	-	-	-	-	-	-	-
8	*Kandelia candel* L.	2.6	60	4.33	0.07	0.14	20.69	0.04	20.73

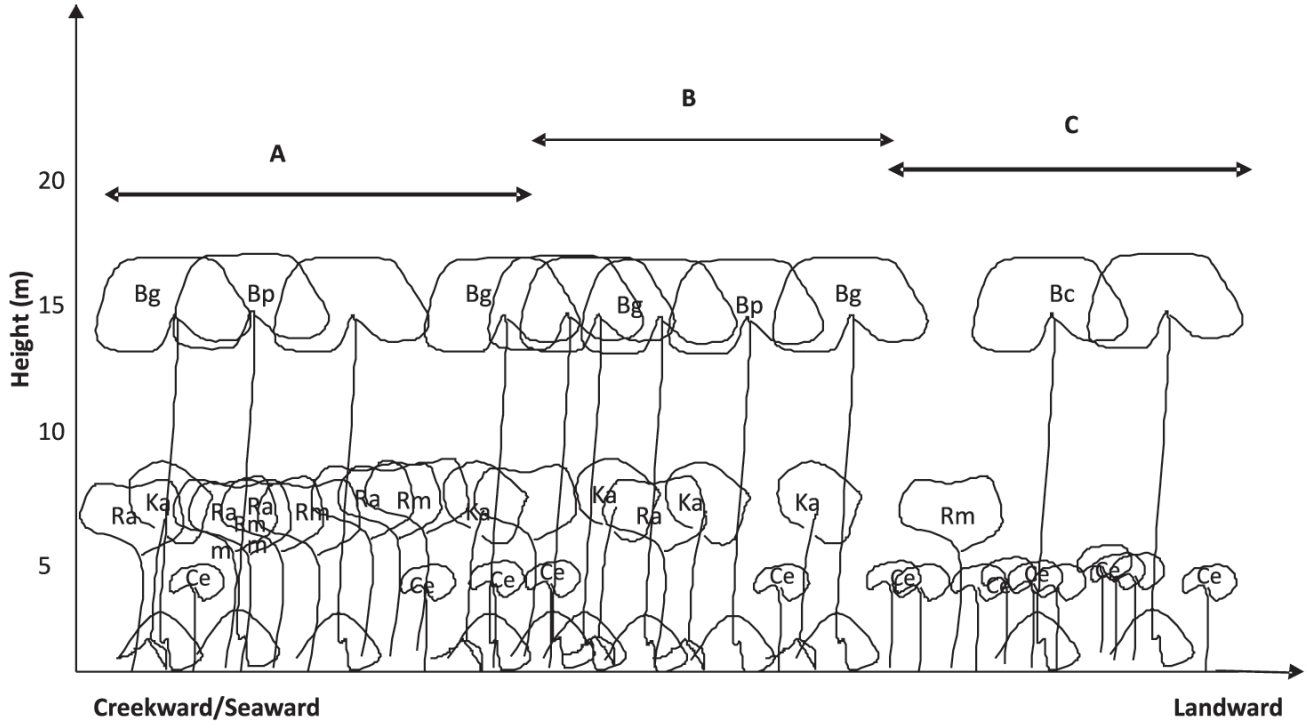

Fig. 2. Profile diagram of Rhizophoraceae mangroves, showing the community changes from beach toward inland. (A) *Rhizophora apiculata-R. mucronata* community, (B) *Bruguiera-Kandelia* community and (C) *C. decandra* community. Abbreviations in canopy: Ra = *Rhizophora apiculata*; Rm =*Rhizophora mucronata*; Bp = *Bruguiera parviflora*; Bg = *Bruguiera gymnorrhiza*; Bc = *Bruguiera cylindrical*; Ka = *Kandelia candel*; Ce = *Ceriops decandra/tagal.*

40 m. The substrate of the region consisted of sandy mud with a pH of 5.6 ± 0.2 and electrical conductivity 9.8 ± 0.5 mS/cm along the LTLs. The community was dominated by pure stands (80%) of *Rhizophora apiculata* and *R. mucronata*. The other species occurring in the community were *Bruguiera gymnorrhiza* and *Kandelia candel* (Fig. 2). This community is still young, with a homogenous structure with respect to plant distribution. The density and the basal area were very high in comparison to other communities. Trees were generally small with an average diameter of 27 cm. Trees of 6 m height formed one layer A (Fig. 2). Saplings were also dense and composed mostly of *Rhizophora apiculata* (31.3%) and *Rhizophora mucronata* (24.2 %).

Bruguiera-Kandelia candel community occurred behind the *Rhizophora apiculata- Rhizophora mucronata* community. It comprised a community with a more heterogenous floristic composition in B region (Fig. 2), in which 6 species of trees were recorded. The two dominant

species were *Bruguiera gymnorrhiza* and *Bruguiera cylindrica* with optimal importance values (IVI) of 33.70 and 20.77 respectively. The density of this community was slightly lower than that of the *Rhizophora* community. The basal area, however, was half of the *Rhizophora* community. Trees in this community were bigger, with an average diameter of 32 cm. The big trees were mostly *Kandelia candel* and *Bruguiera gymnorrhiza* with average diameters of 54 cm and 31 cm respectively (Table 1). The largest tree was Kandelia candel with a diameter of 78 cm. It also showed the highest DBH at the study sites (Table 2).

Table 2
Average DBH of different species of Rhizophoraceae mangroves.

Sl. No.	Name	DBH (cm)
1	*Rhizophora apiculata* Bl.	26.52 ± 1.12
2	*Rhizophora mucronata* Lam.	27.33 ± 2.11
3	*Brugiera gymnorrhiza* L.	31.00 ± 1.58
4	*Brugiera paviflora* Roxb.	21.35 ± 1.47
5	*Brugiera cylindrica* Bl.	29.19 ± 1.12
6	*Ceriops decandra* Griff.	17.11 ± 0.98
7	*Ceriops tagal* Perr.	14.28 ± 0.84
8	*Kandelia candel* L.	54.00 ± 2.87

Ceriops community developed on better drained and firm soils. The soil pH was low i.e., 4.3 ± 0.6 and electrical conductivity 6.23 ± 0.51 mS/cm along HTLs. *Ceriops decandra* was the most dominant species with the highest IVI of 40.45 (Table 1). Its density was 70.2% of the total density and the basal area was 78.6 % of the total basal area within this community. This indicated the dominance of the species in the community becoming the determining species of the habitat. At least two canopy layers were recognized in C region (Fig. 2). The top layer was dominated by *Bruguiera gymnorrhiza* which reached a height of 16 m. The second layer consisted of *Ceriops decandra* and *Ceriops tagal* of height around 3 m. Such distribution is favourable for optimum utilization of the light intensity thus making the forest highly productive in comparison to the homogeneous community with single layered canopy structure.

4. Conclusion

The Rhizophoraceae mangroves of Mahanadi delta and Bhitarkanika, Odisha contained a good number of species which showed good survival and regenerative ability. *Bruguiera gymnorrhiza* L. plants exhibits better adaptability to varying levels of salinities along the tide lines whereas *Rhizophora apiculata* Bl. and *Rhizophora mucronata* L. resemble higher tolerance to salinity as its ecological occurrences. The functional importance of this mangrove forest to the coastal environment should be recognized and efforts should be made to conserve this remnant forest.

Acknowldgement

Authors are highly thankful to SAP-DRS, UGC, New Delhi for financial support for smooth execution of this present work. We also thankful to Mr. Nitesh Mund for valuable cooperation and support during the field survey.

References

Anonymous (1987). Mangroves in India - Status report. Govt, of India. Ministry of Environment and Forests, New Delhi.

Chadha, S. and Kar, C. S. (1999). Bhitarkanika: Myth and Reality. Natraj Publishers, Dehradun.

Choong, E. T., Wirakusumah, R. S. and Achmadi, S. (1990). Mangrove forest resources in Indonesia. Forest Ecol. Manag. 34: 45-57.

Cintron, G. and Novelli, Y. S. (1984). Methods for studying mangrove structure. In: Samuel Snedaker C. and Snedaker J.G. (ed.) The mangrove ecosystem: research methods. UNESCO, p. 251.

Clifford, H. T. and Stephenson, W. (1975). An introduction to numerical classification. Academic Press, London.

Daniels, R. J. R. and Acharjyo, L.N. (1997). Rejuvenation of degraded mangroves and development of seed banks at Bhitarkanika. Final Technical Report Submitted to NORAD by M.S. Swaminathan Research Foundation, Chennai.

Haines H. H. (1921-25). The Botany of Bihar and Orissa. Vol. 1-4, Allard and Sons Ltd., London.

Hamilton, L. S. and Snedaker, S. C. (1984). Handbook of Mangrove Area Management. United Nations Environment Programme, and East West Center Environment and Policy Institute. COE/IUCN, Gland-Switzerland.

Jain, S. K. and Rao, R. R. (1977). A Handbook of Field and Herbarium Methods. Today & Tomorrow's Printers and Publishers, New Delhi.

Kershaw, K. A. (1973). Quantitative and Dynamic Plant Ecology (second edition), Edward Arnold, London.

Krauss, K. W. and Ball, M.C. (2012). On the halophytic nature of mangroves. Trees. DOI 10.1007/s00468-012-0767-7.

Lawrence, G. H. M. (1951). Taxonomy of Vascular Plants. The Macmillan Company, New York.

Mishra, R. (1968). Ecology Work Book. Oxford and IBH Publishing Co., New Delhi.

Mooney, H. F. (1950). Supplement to the Botany of Bihar & Orissa. International Book Distributors, Dehra Dun.

Odum, E. P. (1971). Fundamentals of ecology. W. B. Saunders, Philadelphia.

Saxena, H. O. and Brahmam, M. (1996). The Flora of Orissa. Vol. I-IV, Orissa Forest Development Corporation, Bhubaneswar, India.

Selvam, V., Ravichandran, K. K., Gnanappazham, L. and Navamuniyammal, M. (2003). Assessment of community-based restoration of Pichavaram mangrove wetland using remote sensing data. Curr. Sci. 85(6): 794-798.

Snedaker, S. C. and Snedaker, J. G. (1984). The Mangrove Ecosystem: Research Methods. UNESCO, Paris.

Tomlinson, P. B. (1986). The botany of mangroves. Cambridge Tropical Biology Series, Cambridge University Press, London.

Upadhayay, V. P. and Mishra P. K. (2008). Population status of mangrove species in estuarine regions of Orissa coast, India. Trop. Ecol. 49(2): 183-188.

Plant Science Research 34 (1&2) : 72-78, 2012

Plant Science Research

ISSN 0972-8546

Variation in karyotype and DNA markers in different ecotypes of a mangrove associate, *Suaeda nudiflora* (Willd.) Moq. from Bhitarkanika, Odisha

C. Pradhan[1Ψ] and A. B. Das[2]

[1] P. G. Department of Botany, Utkal University, Vani Vihar, Bhubaneswar 751004, Odisha, India.

[2] Depatrment of Agricultural Biotechnology, Orissa University of Agriculture and Technology, Bhubaneswar-751003, Odisha, India.

ARTICLE INFO

Article history:
Received : 15 November 2012
Received in revised form : 03 December 2012
Accepted : 04 December 2012

Keywords:
chromosome
karyotype
mangrove
RAPD
Suaeda nudiflora

ABSTRACT

Karyotype and chromosome number variation was observed in different ecotypes of *Suaeda nudiflora*, a mangrove associate collected from Bhitarkanika mangrove forest of Odisha, India. The chromosome number varied from 2n=36 to 54 and Ecopye IV from Gupti (2n=54) and Ecotype V from Ekakula (2n=40) with changes in karyotype was observed beside the normal diploid number 2n=23 in Ecotype-I (Dhamra), -II (Rajnagar) and –III (Dangmal). Investigation of RAPD profile among these ecotypes revealed genetic variation among the ecotypes. Some of the ecotype specific bands are obtained in Ecotype-IV like 100 bp in OPD-12, 300bp in OPN15 and 1000bp in OPA05 are unique which can be used for developing SCAR markers for future use. Ecotype-V having 400bp in OPN-15, 500bp in OPA-14, 800bp in OPA-05 are found marker bands. While some of the monomorphic bands are common in all the ecotypes like 500pb in OPA-05 beside the marker bands of 600 bp and 1500 bp in OPA-14 in Ecotype-I are characteristics of the Ecotypes. Phylogenetic relationship and chromosome number and karyotype suggest the possible link of saline adaptability with ploidy changes of different ecotypes and subsequent changes in DNA profile rather than its epigenetic modifications in DNA level.

1. Introduction

Mangroves are one of the most threatened ecosystems all over the world today due to direct and anthropogenic indirect degradation (Alongi, 2002; Duke *et al.,* 2007) and has resulted in great loss of genetic diversity in the mangrove ecosystem (Maguire *et al.,* 2002; Triest *et al.,* 2008). Conservation of mangrove including genetic resources implicates not only to protect the coastal areas and communities from seawater intrusion and potential changes in sea level rise but also to ensure the availability of resources for future use through adaptation to changing environments. Information on genetic diversity of mangrove species is very important in planning for conservation of genetic resources and afforestation program (Hamrick *et al.,* 1992; Duke *et al.,* 1998). Knowledge of genetic diversity and its causes can provide insight into their ecological and evolutionary histories; thus, such information also may help in conservation and restoration. The genetic variation of a species can be assessed by different techniques from morphological and metric characters in the field to biochemical and molecular markers in the laboratory (Graudal *et al.,* 1997). Molecular markers are important tools for identifying appropriate population sources for reforestation of these unique and important habitats of mangrove forests (Schwarzbach and Ricklefs, 2001) beside their chromosome status.

Suaeda nudiflora, a tropical halophytic mangrove associate commonly known as 'Giringa' leafy vegetable that tolerates high temperature which is found on sea ward fringe which always remain waterlogged with high and low tides. This unique plant, which has a versatile form with branches spreading on the soil surface. It is very much important for its food value used by poor man in the coastal belt. Due to the hostile condition of the mangrove environment, the plant species in the mangrove forests are

Ψ *Corresponding author; E-mail:* chinmay_pr@yahoo.com

constantly under environmental stress due to high saline conditions, extreme temperature and high salt deposition on the mud flat and have adapted themselves against these frequent and fluctuating environmental changes. *Saudea* is dioeciously plant that lacks vegetative propagation. Moreover, the species is insect-pollinated and thus gene flow is expected to decrease considerably with distance. However, under altered ecological and physical conditions in mangrove ecosystem, discernible changes were reported in genetic architecture of *Suaeda nudiflora* (Jena and Das, 2006) besides its morphology (Tomlinson, 1986). The chromosome number were reported to be 2n=36 in *S. nudiflora* (Kumar and Subramanian, 1988; Jena *et al.,* 2002). Molecular markers, such as allozymes, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites (SSR), Inter-simple sequence repeats (ISSR), and DNA sequence, have proven to be a very efficient means to investigate population genetics of mangrove species (Triest *et al.,* 2008). The application of these markers in assessing intra-specific variations in mangrove species have been recently studied (Parani *et al.,* 1997; Lakshmi *et al.,* 1997). Among the various DNA marker, RAPDs have been used extensively for a variety of purposes, including ecotype studies. Although several studies on population genetic in mangoves have been reported (Nunez-Farfan *et al.,* 2002; Arbelaez *et al.*, 2007; Pil *et al.,* 2011), the extent and patterns of genetic diversity in this mangrove species remain obscure. Genetic diversity is very much critical for adaptation to environmental changes and for long-term survival of the species. The genetic variations have to be conserved before completely restoring the ecology role that has long been lost due to the mangrove ecosystem deforestation (Schwarzbach and Ricklefs, 2001). The objective of the present study was to assess the genetic variation among different ecotypes grown in various saline regime with a chromosome number, detail karyotype and RAPD profile of *S. nudiflora* distributed in East coast of India besides our earlier report with ploidy changes (Jena and Das, 2006) to acquire useful genetic information to support mangrove forest conservation.

2. Material and Methods

2.1 Plant Materials

Different ecotypes of *Suaeda nudiflora* from Bhitarkaniaka mangrove forest of Odisha, India with a latitude and longitude of 20^0 40'N, 86^0 52'E respectively were collected for the present study (Table 1). From each study site, root tips and young leaves were collected for chromosome number and detail karyotype study and DNA isolation respectively. Young leaves were stored in a –85^0C freezer for not more than two weeks before DNA extraction and roots were subjected to pretreatment and fixation.

2.2 Chromosome preparation and karyotype

Root tips were collected in the field and put them in 0.05M oxiquoline solution and kept in room temperature for 3 h and subsequently fixed in 1:3 (acetic acid: ethanol) for over night. Root-tips were transferred to 70% ethanol in the field and kept for further study. Preserved root tips were soaked in 45% acetic acid for 20 min stained in 2% acetic-orcine:1NHCl (9:1) for over night. Root tip squash was made separately for each ecotypes in 45% acetic acid and observed under microscope for chromosome count and photography. For karyotype study, each ecotypes at least 5 roots from different plants were taken following the procedure of (Das and Mallick, 1993a).

2.3 DNA isolation

Total genomic DNA was extracted from leaf tissues using modified cetyl trimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle, 1987). 5g of each of young leaf tissue was ground under liquid nitrogen and suspended in 10 ml of CTAB buffer (2% Cetyl Trimethyl Ammonium Bromide, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4M NaCl and 1% b-mercaptoethanol). The suspension was incubated at 60^0C for 1h. The DNA was extracted in chloroform:isoamyl alcohol (24:1) for 10 min with gentle shaking and centrifuged at 10,000g for 20 min. The aqueous phase was taken in a separate clean sterilized tube and DNA was precipitated with two volumes of chilled iso-propanol. The DNA was hooked out and dried with vacuum concentrator and dissolved in TE (10 mM Tris-HCl + 1mM EDTA, pH 8.0). The DNA again purified treating with RNase at 37^oC for 1h followed by phenol: chloroform:isoamyl alcohol extraction (25:24:1) followed by chloroform:isoamyl alcohol (24:1) and centrifuges. The supernatant was precipitation with chilled ethanol in presence of 0.3M sodium acetate (pH 5.2). The DNA was spooled out, washed in 70% ethanol; air dried and dissolved in TE buffer and the DNA concentration was estimated in Versafluor TM Fluorometer (Bio-Rad, USA) using Hoechst 33258 as the flurometric dye. The quality of the DNA was also evaluated using 1% agarose gels and then quantified by UV-Spectrophotometer (Shimadzu, Kyoto, Japan). The DNA was diluted to final concentration of 25ng ml^{-1} using TE buffer and used as template DNA for RAPD analysis. The material for PCR analysis were stored at -20 ^{0}C.

2.4 RAPD analysis

RAPD profiles were generated by using single decamer random oligonucleotide primers (Operon Technologies,

Alameda, USA) in polymerase chain reaction (PCR) following the standard protocol of Williams *et al.* (1990). The sequence of primer is given in Table 2. Amplification reaction mixture of 25ml for each polymerase chain reaction (PCR) contained 25ng of genomic template DNA, 200mM of each dNTP, 25ng of primer, 0.5 unit of Taq DNA Polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 10 × PCR assay buffer (50mM KCl, 10m M Tris-HCl, 1.5mM $MgCl_2$, pH 9.0). The reaction mixture was carried out in a Gene AmpPCR 2400 thermal cycler (Perkin Elmer, USA) in the following temperature cycles: holding at 94^0C for 5min at start, followed by 44 cycles of 92^0C for 1 min , 40^0C for 1min and 72^0C for 2min and a final additional extension at 72^0C for 15min. The amplified samples were stored at 4^0C and electrophoretically separated in 1.5% agarose gel in 1×TAE buffer and visualized by ethidium bromide staining. To determine the size of the polymorphic fragments, Gene Ruler 100bp DNA ladder plus (MBI Fermantas, Lithuania) was used as size standard. The gel was photographed under UV light for documentation.

2.5 RAPD data scoring and statistical analysis

In RAPD analysis, the presence or absence of the bands was taken into consideration and the difference in the intensity of the band was ignored. For all ecotypes, bands on RAPD gels were scored, as present (1) or absent (0). Jaccard's similarity coefficient values (Jaccard, 1998) were calculated for each pair wise comparison between ecotypes and similarity matrix was constructed. This matrix was subjected to unweighted pair group method for arithmetic average analysis (UPGMA) to generate a dendrogram using average linkage procedure. All computing were carried out using NTSYS-pc (Rohlf, 1993). The RAPD data were further subjected to analysis of molecular varience (AMOVA) as described by Excoffier *et al.* (1992) using three hierarchical levels: individual, population and their regions with the GenALEX software (Peakall and Smouse, 2001), and also used for principal coordinate analysis (PCA) of the relationship between the distance matrix and elements based on the first two principal coordinates.

3. Result

3.1 Chromosome and karyotype analysis

Somatic chromosomes were counted from all the ecotypes collected from different saline zones showed chromosome number variation from 2n=36 to 54. Ecotype-I, II and III showed 2n=36 chromosome while Ecotype-IV with 2n=54 and Ecotype-V with 2n=40 were recorded (Table 1, Figs 1a-1c). On the basis of the size and position of the constrictions on the chromosome, a number of chromosome type were found to be common within the ecotype studied, though there were minute differences of the karyotype. A general description of the representative types of chromosomes is given bellow.

Type A: Medium sized chromosome with primary and secondary constrictions at sub-medium and subterminal in position respectively.

Type B: Medium sized chromosome with two constrictions one in the median to sub-median position and other in the sub-terminal position.

Type C: Medium to small sized chromosome with median primary constrictions.

Type D: Medium to small sized chromosome with sub-median primary constrictions.

There are no much of variation in karyotype formula of Ecotype-I,II and III was found with about same number of median and sub-median chromosomes. Ecotype-IV showed 4 each of Type A and Type B chromosomes with secondary constrictions with 33 number of medina chromosome (Type C) and 15 number of sub-median chromosome (Type D). However, Ecotype-IV showed less numbers of sub-median chromosomes (Type D) as compared to the number of median chromosome (Table1, Fig.1).

Table 1
Ecotypes of *S. nudiflora* from different places of Bhitarkanika, Odisha, India, with their somatic chromosomenumber and karyotype.

Ecotypes	Place of Collection	Soil type	pH	2n	Karyotype formula
I	Dhamara	Heavy clayey	6.2-6.5	36	2B+18C+16C
II	Rajnagar	Silt mixed with clay	6.5-7.0	36	2B+18C+16C
III	Dangamal forest	Heavy silt clay	5.5-7.2	36	2B+18C+16C
IV	Gupti	Sandy silt	6.8-7.6	54	4A+4B+33C+15D
V	Ekakula	Sandy	6.5-7.3	40	4A+4B+24C+8D

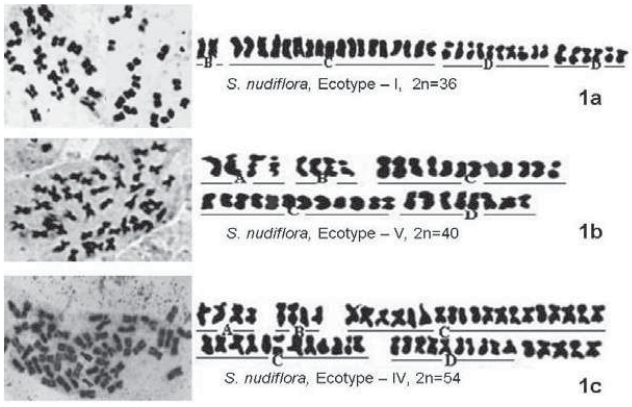

Fig. 1. Somatic chromosome numbers with corresponding karyotypes of different respective of *S. nudiflora* collected from Bhitarkanika forest mangrove of Orissa. Ecotype-I showing 2n=36 (1a), Ecotype-V showing 2n=40 (1b), Ecotype-IV with 2n=54.

3.2 RAPD analysis

The agarose gel electrophoresis derived PCR amplification photographs were analyzed and it was found that a total of 313 amplicons were amplified using 14 Operan primers. The fragment size varied from 100-2800bp and the lowest range (100-950bp) was observed OPA-10 primer. The total polymorphic percentage was 49.20% were as it varied among the Ecotypes ranging from 6.389% (Ecotype-I) to 15.015% (Ecotype-IV). The number of amplification products ranged from 20 to 35 for different ecotypes. RAPD profiles of five ecotypes shared a number of common bands for all primers. Ecotype specific polymorphic bands varied from 2 to 5 among the ecotype (Table 2). RAPD profile of five ecotypes showed variations in banding pattern when amplified by OPA-05 with a prominent marker band of ~1000bp and ~800bp found unique in Ecotype-IV and Ecotype-V respectively. OPA-14 although produced major monomorphic bands while succeed to produced two unique bands (1500bp and 600 bp) for Ecotype-I and 500bp for Ecotype-V (Figs. 2a & 2b). In OPD-12, a very low size fragment of ~100bp found as unique band for Ecotype-IV. Two marker bands of 300bp and 400bp were recorded in OPN-15 primer for Ecotype-IV and Ecotype-V respectively.

3.3 Cluster analysis

Pair wise comparisons were made for the RAPD profiles obtained through the use of 14 random primers in the representative samples of all five genotypes of different Ecotypes of Bhitarakanika. Ecotype-I. Ecotype-II and Ecotype-II clustered together with a similarity coefficient of 0.83 made one branch while the rest two Ecotype i.e. Ecotype-

Table 2
RAPD profile generated from different ecotypes of *S. nudiflora* with percentage of polymorphism.

Primer	Primer Sequence	No. of Amplicon	Eco-I	Eco-II	Eco-III	Eco-IV	Eco-V	Size range
OPD-02	5'GGACCCAACC3'	18	1	0	1	2	3	300-1200
OPA-05	5'AGGGGTCTTG3'	27	3	5	3	2	3	200-1500
OPA-07	5'GAACGGGTG3'	16	3	1	2	2	3	300-1050
OPA-08	5'GTCACGTAGG3'	22	2	3	2	3	2	300-1600
OPA-10	5'GTGATCGCAG'	27	2	3	1	6	3	100-950
OPA-11	5'CAATCGCCGT3'	19	0	0	1	5	2	200-1650
OPA-13	5'CAGCACCCAC3'	28	2	1	1	4	2	230-1200
OPA-14	5'TCTGTGCTGG3'	30	1	1	2	6	2	150-2800
OPD-02	5'GGACCCAACC3'	19	2	2	4	5	5	350-1800
OPD-08	5'GTGTCCCCCA3'	27	2	1	2	4	4	300-2300
OPD-12	5'CACCGTATCC3'	25	1	2	2	1	1	200-2500
OPN-04	5'GACCGACCCA3'	24	0	0	3	2	2	350-980
OPN-11	5'TCGCCGCAAA3'	14	2	1	2	3	1	200-950
OPN-15	5'CAGCGACTGT3'	17	1	2	3	2	2	350-1200
	Total Bands	313	20	22	30	47	35	
	Polymorphic %	49.20	6.389	7.028	9.584	15.015	11.182	

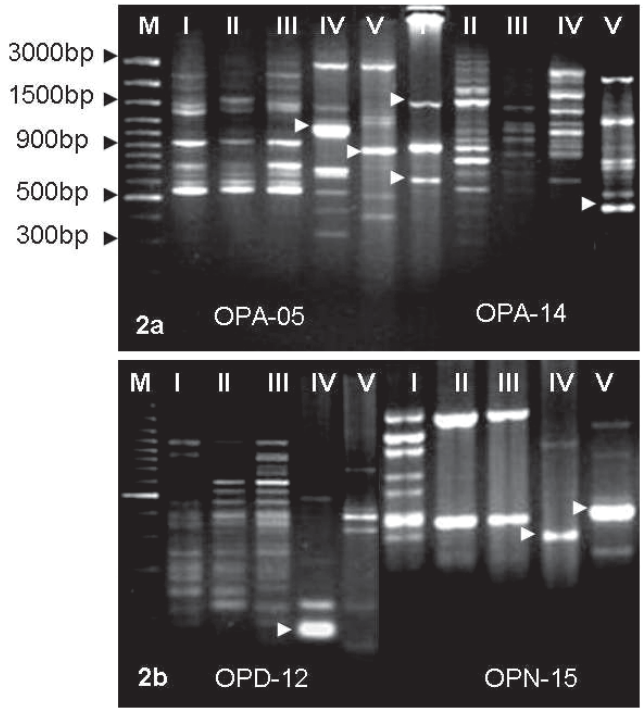

Fig. 2. RAPD amplification profiles of five ecotype of *S. nudiflora* using OPA-5 and OPA-14(2a), OPD-12 and OPN-15 (2b). M=Gene Ruler 100bp DNA ladder plus (MBI Fermantas, Lithuania), I to V = Ecotypes from left to right showing major marker RAPD fragments (arrow heads).

IV and Ecotype-V formed the other branch of the tree (Fig. 3). The highest value of mean similarity coefficient 0.57 was found in Ecotype-I and Ecotype-II followed by Ecotype-III (0.56). The lowest value of mean similarity coefficient was recorded in Ecotype-IV and Ecotype-V.

AMOVA helped to pertain the RAPD variations among different Ecotypes and among individuals within a ecotype. About 3.35% molecular variation within ecotypes and about

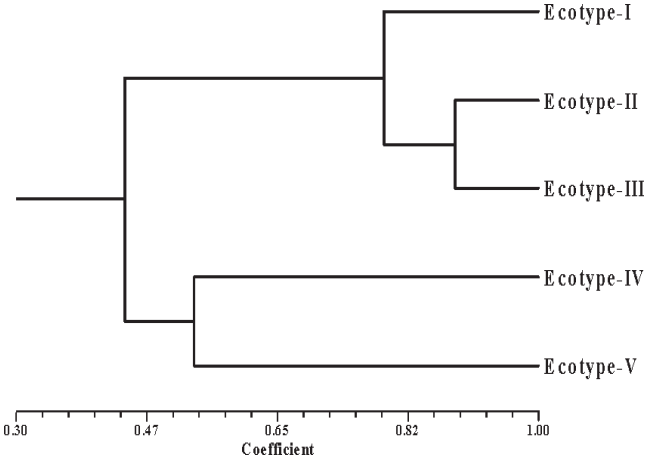

Fig. 3. Dendrogram showing genetic relationships among ecotypes of *S. nudiflora* on the basis of RAPD analysis.

~48% variation among Ecotypes were recorded. This may be useful in strategies for germplasm collection and evaluation. The PCA analysis (Fig. 4) was comparable with the cluster analysis (Fig. 3), with all similar chromosome number Ecotypes having 2n=36 chromosomes i.e. Ecotype-I, -II and –III cluster together from the rest of the Ecotypes. Gupti (Ecotype-IV) having 2n=54 and Ekakula (Ecotype-V) with 2n=40 were distinct from other genotypes in the PCA with a separate group all together.

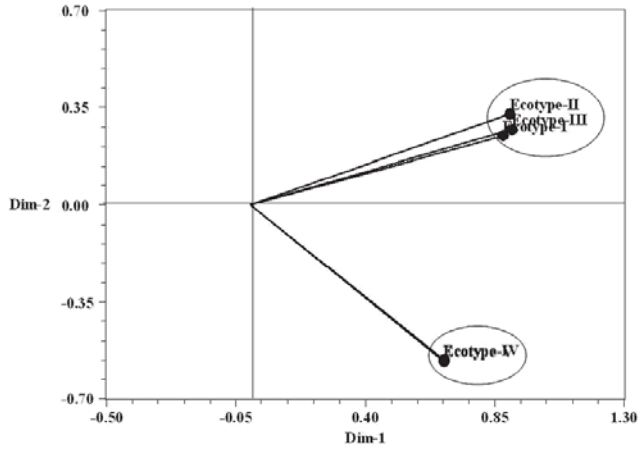

Fig. 4. Two-dimensional plot of principal component analysis of five Ecotypes (I-V) of *S. nudiflora*.

4. Discussion

Numerical variation of somatic chromosomes were recorded in ecotype level in *S. nudiflora*. The somatic chromosome 2n=36 which was found in Ecotype-I, II and III reconfirm the earlier report (Kumar and Subramanian, 1988; Jena *et al.,* 2002). However, the chromosome number for Ecotype-IV with 2n=54 from Gupti area of Bitarkanika which was confirmed earlier by us (Jena and Das, 2006). But a new Ecotype-V collected from Ekakula of Bitarkanika showed 2n=40 chromosome. Same type of cytotypes were also reported earlier from Talchua region of Bitarkaika (Jena and Das, 2006). We performed the detailed karyotype of all the Ecotypes and revealed that a high number of median constricted chromosomes as compared to sub-median chromosome were observed from Ecotype-IV which grown in high saline region of sanctuary (Table 1, Fig.1). Shifting of sub-median constricted chromosome to median chromosome in the karyotype of Ecotype-V was found which was collected from Ekakula Island of the mouth of the sea. The structural alteration of the chromosome morphology in Ecotype-IV and Ecotype-V of high saline zone might be due to partial duplication of chromosomes or translocation between the chromosomes with or without secondary constricted chromosomes during ecotype/cytotype formation for better adaptability of this plant in these hostile conditions (Das, 1991; Das and Mallick, 1993a,b; Das *et al.,* 1994).

DNA marker profile identify Ecotypes directly and therefore help to mitigate complications arising from earlier cytological and morphological studies. Between adjacent geographically defined Ecotypes of *S. nudiflora*, there was a significant polymorphism. Remarkably high individual genetic diversity was observed in Ecotype-IV and Ecotype-V with high chromosome number in high saline zone indicate the genetic changes in Ecotype level of *S. nudiflora* (Fig. 2). Since 1930, investigators have tried to associate the numerical chromosome variation found in plants with the environment and to relate the different cytotypes to the occupation of different niches in terms of temperature, luminosity, humidity etc. (Bennet, 1987). Although we have assumed earlier the existence of different cytotypes and population of *S. nudiflora* (Jena *et al.,* 2002; Jena and Das 2006) it has been now better understood with chromosomal, karyotype and RAPD data for the existence of new Ecotypes with high chromosome number in other locations of Bitarkanika like Ekakula which needs thorough investigation for further discovery of new cytotypes from this area for *S. nudiflora*. RAPD data support the existence to defined cytotypes for adaptation of different Ecotypes at various environmental conditions. RAPD profile of five ecotypes showed variations in banding pattern when amplified by OPA-05 with a prominent marker band of ~1000bp and ~800bp found unique in Ecotype-IV and Ecotype-V respectively. The marker bands of 600 bp and 1500bp in OPA-14 for Ecotype-I and 500bp for Ecotype-V might be due to genetic changes of the Ecotypes (Fig. 2). Gene diversity between Ecotypes was more prominent in the gel figures, where each genotype from each Ecotype has been amplified with the same primer. In addition to Ecotype, cytogenetic data and RAPD data at inter-ecotype levels have proved to be extremely instructive in developing a better understanding of divergence. In particular, there are highlighted differences between Ecotype and local groups within same species, which are not only genetically distinct but are confined to geographically restricted and unique plant communities. In OPD-12, a very low size fragment of ~100bp found as unique band for Ecotype-IV. Two marker bands of 300bp and 400bp were recorded in OPN-15 primer for Ecotype-IV and Ecotype-V respectively. In the present study we showed the variability of the RAPD banding pattern in *S. nudiflora,* which was evident by chromosome number and detail karyotype data with no much of morphological changes of the plant. Hence, it is suggested that there is a genetic divergence among the different ecotype as they found to belong to different biological units. Ecological comparison is also powerful method to recognize different biological units with similar morphology, especially when they are distributed. The observed inter-ecotypic divergence could be ascribed to the adaptability with the fluctuating micro-climatic conditions of different degree of temperature, light tolerance, salinity gradient for their different geo-locations (Dawson *et al.,* 1993). Examination of the UPGMA dendrogram (Fig. 3) clearly showed the isolated position of Ecotype-IV and Ecotype-V from a single cluster from the rest of the three Ecotypes i.e. Ecotype-I, Ecotype-II and Ecotype-III. The former Ecotypes have the higher chromosome number with an altered karyotype which might be a adaptive strategies for the mangrove associate to survive in high saline area with increase chromosome number with varied genetic makeup.

This is for the first time assessing a huge percentage of inter-ecotypic genetic variation of *S. nudiflora* through molecular genetic studies (RAPD). Between Ecotype, genetic variations are relatively high suggesting that the ecotype are largely isolated from each other with little mutual gene flow. Local selection and restricted gene flow between the genotypes has been contributed more to the limited genetic variability of this species. Thus, it seems likely that fragmentary process will accelerate in this species, which appear to be an inherently slow group to respond in an evolutionary sense. Since the Ecotype were physically isolated, the genetic content of the individuals that originally colonized the locations might be one of the causes of divergence. In conclusion, it is observed that though *S. nudiflora* does not show significant morphological variations, the present investigation using chromosomal data RAPD data reveals that substantial inter-ecotype variation does exist that confirm the existence of cytotypes and genotypes for better adaptation of this species in high saline area

Acknowledgement

The financial support received from Dept. of Forest and Environment, Govt. of India is duly acknowledged.

Reference

Alongi, D. M. (2002). Present state and future of the world's mangrove forests. Environ. Conserv. 29: 331–349.

Arbelaez, E., Castillo, M. E., Toro, N. and Cardenas, H. (2007). Genetic structure of the red mangrove (*Rhizophora mangle* L.) on the Colombian Pacific detected by microsatellite molecular markers. Hydrobiol. 583: 321–330

Bennet, M. D. (1987). Variation in genomic form in plants and its ecological implications. New Phytol. 106(suppl.): 177-200.

Das, A. B. (1991). Genome analysis and variation of 4C DNA con-tent in the subtribe Carinae. Cytologia 56: 627-632.

Das, A. B., Basak, U. C. and Das, P. (1994). Genome analysis and karyotype diversity in 3 species of *Heritiera*, a common mangrove of Orissa. Cytobios 80: 71-78.

Das, A. B. and Mallick, R. (1993a). Karyotype diversity and interspe-cific 4C DNA variation in *Bupleurum.* Biol. Plant. 35: 355-363.

Das, A. B. and Mallick, R. (1993b). Nuclear DNA and chromosomal changes within tribe Ammineae. Cytobios 74: 197-207.

Dawson, I. K., Chalmers, K.J., Waugh, R. and Powell, W. (1993). Detection and analysis of genetic variation in *Hordeum spontaneum* population from Israel using RAPD markers. Molec. Ecol. 3: 151-159.

Doyle, J. J. and Doyle, J. L. (1987). A Rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19: 11–15.

Duke, N. C., Benzie, J. A. H., Goodall, J. A. and Ballment, E. R. (1998). Genetic structure and evolution of species in the mangrove genus *Avicennia* (Avicenniaceae) in the Indo-West Pacific. Evolution 52: 1612–1626.

Duke, N. C., Meynecke, J. O., Dittmann, S., Ellison, A. M., Anger, K., Berger. U., Cannicci, S., Diele, K., Ewel, K. C., Field, C. D., Koedam, N., Lee, S. Y., Marchand, C., Nordhaus, I. and Dahdouh-Guebas, F. (2007). A world without mangroves? Science 317: 41–42.

Excoffier, L., Laval, G. and Schneider, S. (2005). Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol. Bioinform. Online 1: 47–50.

Graudal, L., Kjaer, E., Thomsen, A. and Larsen, A.B. (1997). Planning national programmes for conservation of forest genetic resources. Danida Forest Seed Centre. Technical Note 48: 9–-25.

Hamrick, J. L., Godt, M. J. W. and Sherman-Broyles, S. L. (1992). Factors influencing levels of genetic diversity in woody plant species. New Forest 6: 95–124.

Jaccard, P. (1998). Nouvelles researches sur la distribution forale. Bull. Soc. Sci. Nat. 44: 223-270.

Jena, S. and Das, A. B. (2006). Inter-population variation of chromosome and RAPD markers of *Saaeda nudiflora* (Willd.) Moq. A mangrove species in India. African J. Agric. Res. 1(4): 137-142.

Jena, S., Sahoo, P., Mohanty, S., Das, A.B. and Das, P. (2002). Karyotype variation and cytophotometric estimation of *in situ* DNA content in some minor and associate mangroves of India. Cytologia 67: 15-24.

Kumar, V. and Subramanian, B. (1988). Chromosome Atlas of Flowering Plants of Indian Sub-continent. Botanical Survey of India, Calcutta, India.

Lakshmi, M., Rajalakshmi, S., Parani, M., Anuratha, C.S. and Parida, A. (1997). Molecular phylogeny of mangroves I: Use of molecular markers in assessing the genetic variability in the mangrove species *Acanthus ilicifolius* Linn. (Acanthaceae). Theor. Appl. Genet. 94: 1121-1127.

Maguire, T. L., Peakall, R. and Saenger, P. (2002). Comparative analysis of genetic diversity in the mangrove species *Avicennia marina* (Forsk.) Vierh. (Avicenniaceae) detected by AFLPs and SSRs. Theor. Appl. Genet. 104: 388-398.

Nunez-Farfan, J., Dominguez, C. A., Eguiarte, L. E., Cornejo, A., Quijano, M., Vargas, J. and Dirzo, R. (2002). Genetic divergence among mexican populations of red mangrove (*Rhizophora mangle*): geographic and historic effects. Evol. Ecol. Res. 4: 1049–1064.

Parani, M., Lakshmi, M., Elango, S., Ram, N., Anuratha, C.S. and Parida, A. (1997). Molecular phylogeny of mangroves II Inter and intra specific variation in *Avicennia* revealed by RAPD and RFLP markers. Genome 40: 487-495.

Peakall, R. and Smouse, P. E. (2001). GenAIEx V5: genetic analysis in excel. Population genetic software for teaching and research[Internet], Canbera (Australia): Australian National University; [Cite 2009 Nov8]. Available from: http://anu.edu.au/BoZo/GenAlEx/.

Pil, M.W., Boeger, M.R., Muschner, V.C., Pie, M.R., Ostrensky, A. and Boeger, W.A. (2011). Postglacial north-south expansion of populations of *Rhizophora mangle* (Rhizophoraceae) along the Brazilian coast revealed by microsatellite analysis. Am. J. Bot. 98: 1031–1039

Rohlf, F.J. (1993). Ntsys-PC. Numerical taxonomy and multivariate analysis system Version I. 80-Setauket, NY, Exeter Software.

Schwarzbach, A. E. and Ricklefs, R. E. (2001). The use of molecular data in mangrove plant research. Wetlands Ecol. Manag. 9: 195–201.

Tomlinson, P. B. (1986). The Botany of Mangroves. Cambridge University Press Cambridge. London.

Triest, L. (2008). Molecular ecology and biogeography of mangrove trees towards conceptual insights on gene flow and barriers: A review. Aquat. Bot. 89: 138–154

Williams, J. G. K., Kulelik, A.R., Liver, J., Rafalski, A. and Tingey, S.V. (1990). DNA polymorphism identification by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535.

Plant Science Research 34 (1&2) : 79-92, 2012

Plant Science Research

ISSN 0972-8546

Phylogenetic relationships among pigeon pea (*Cajanus cajan*) and its wild relatives as revealed by RAPD and ISSR markers

A. K. Debata[1], A. K. Mukherjee[2] and P. C. Panda[1Ψ]

[1] Taxonomy & Conservation Division, Regional Plant Resource Centre, Bhubaneswar 751 015, Odisha, India

[2] Central Institute for Cotton Research, Nagpur 440010, Maharashtra, India

ARTICLE INFO

Article history:
Received : 14 November 2012
Received in revised form : 12 December 2012
Accepted : 14 December 2012

Keywords:
Pigeonpea
genetic diversity
molecular markers

ABSTRACT

The genetic relationships among 10 species of *Cajanus* (Fabaceae) and 11 accessions of *Cajanus cajan* (pigeonpea) were assessed using RAPD and ISSR markers. All the species and accessions had an average genetic similarity of 63% and several accessions of *Cajanus cajan* had more than 90% similarity among themselves. In the genus *Cajanus*, the clustering of species in the dendrogram based on molecular data supported the sectional classification of the genus proposed by van der Maesen (1986) to a large extent. While *C. cajan* and its wild progenitor *C. cajanifolius* belonging to the sect. Cajanus came in a cluster, *C. lineatus, C. sericeus* and *C. reticulatus* of the sect. Atylia formed a separate clade. Similarly, members of the sect. Volubilis (*C. crassus* and *C. mollis*) and sect. Cantharospermum (*C. scarabaeoides* and *C. albicans*) also formed distinct groups justifying the established infra-generic classification. The pigeonpea (*Cajanus cajan*) accessions of Indian and African origin got separated in the dendrogram and Indian genotypes formed clusters according to their geographical area of occurrence and cultivation. The genetic diversity and molecular phylogeny of the genus *Cajanus* and pigeonpea cultivars have been discussed in the paper.

1. Introduction:

The subtribe Cajaninae (tribe Phaseoleae) of the family Fabaceae contains a large number of agriculturally important crops and currently, 11 genera come under Cajaninae, including *Cajanus, Flemingia, Rhynchosia, Eriosema, Dunbaria* and *Paracalyx*. Though the species of *Atylosia* and *Cajanus* were relegated to two separate genera mainly on the basis of the presence or absence of a seed strophiole, van der Maesen (1986) while revising the group, merged the two genera under *Cajanus* following systematic analysis of morphological, cytological and chemo-taxanomical data. The revised genus *Cajanus* now comprises 32 species distributed in Asia, Australia and West Africa. These species of *Cajanus* were grouped into six sections namely, Cajanus, Atylia, Fruticosa, Cantharospermum, Volubilis and Rhynchosoides based on growth habit, leaf shape, hairiness, nature of corolla, pod size and strophiole characteristics (van der Maesen, 1986).

Ψ *Corresponding author; Email*: pcpanda2001@yahoo.co.in

Numerous morphological and alpha taxonomic studies of *Cajanus* and related genera have been undertaken (Grear, 1978; Lackey, 1978; Stirton, 1981; Pundir & Singh, 1985a, b & c; van der Maesen, 1986, 1990). The isozymes (Krishna and Reddy, 1982) and seed proteins (Jha and Ohri, 1996; Panigrahi *et al*., 2007) have also been used to establish phylogeny of different taxa. A number of workers studied the cytogenetics and breeding behaviour of *Cajanus* and related genera (Deodikar and Thakar, 1956; Reddy, 1981a & b; Ohri & Singh, 2002; Mallikarjuna *et al*., 2011). During the last two decades, molecular marker techniques such as RFLP (Nadimpalli *et al*., 1992; Sivaramakrishnan *et al*., 2002), RAPD (Ratnaparkhe *et al*., 1995; AFLP (Parani *et al*., 2000; Panguluri *et al*., 2006; Ganapathy *et al*., 2011), SSR (Odeny *et al*., 2009; Dutta *et al*., 2011) have been used for estimating genetic diversity and phylogenetic relationship among different genera of Cajaninae.

Biochemical, cytological, molecular, crossability experiments and phytogeographical studies have established that India is the country of origin of cultivated pigeonpea

and *Cajanus cajanifoius* (=*Atylosia cajanifolia*) as its wild progenitor. The later was described by Haines (1919) from Odisha and subsequently, besides it type locality, the species has been reported to occur wild in a number of localities. It is found to exhibit morphological variations in its type locality which need to be examined using morphological and molecular tools. Several traditional landraces of pigeonpea cultivated in tribal districts of Odisha are important germplasm materials for crop improvement and require in depth molecular genetic studied. In the present study, the genetic diversity and molecular phylogeny of 10 species of *Cajanus* and 11 accessions of *Cajanus cajan* (pigeonpea) have been assessed using RAPD and ISSR markers. The findings of the study will prove useful in the process of selection of species and accessions for breeding and crop improvement in pigeonpea using its wild relatives.

2. Materials and Methods

2.1 Plant materials

Seed samples of 30 accessions belonging to 10 species of *Cajanus* of the sub-tribe Cajaninae were collected from the germplasm collection of International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patencheru, Hyderabad and from different districts of Odisha. The accession number, locality of collection and abbreviation used for each of the taxon is shown in Table 1. The seed materials were geminated in pro-trays under greenhouse conditions at Regional Plant Resource Centre, Bhubaneswar and the tender leaves were used for DNA extraction for molecular analyses.

2.2 Genomic DNA extraction

Genomic DNA was extracted from the leaf tissues using the modified CTAB protocol (Doyle and Doyle, 1990) with modification. Two grams of leaf tissues from young seedlings were ground with grinding buffer composed of 100 mM sodium acetate (pH 4.8), 500 mM NaCl, 50 mM EDTA (pH 8.0); 50 mM Tris (pH 8.0); 2% polyvinyl pyrollidone (PVP) and 2% CTAB. Purification of DNA was done twice with extraction of phenol: chloroform: isoamyl alcohol (25:24:1). RNAse @ 40 µl from 1 mg/ ml was applied in the supernatant to get rid of RNA. The quality and quantity of DNA were checked through 0.8% agarose electrophoresis with standard DNA before PCR amplification.

2.3 RAPD and ISSR analyses

A total of 36 RAPD and ISSR primers (Operon Technologies, Alameda, USA) were selected for PCR analysis based upon their performance and reproducibility (Table 2, 3). PCR mixture of 25 µl contained 25 ng of genomic DNA template, 0.6 U of Taq DNA polymerase (Bangalore Genei, Bangalore, India), 0.3 µM of decamer primers, 2.5 µl of 10 x PCR assay buffer (50 mM KCI, 10 mM Tris-HCl), 1.5 mM $MgCl_2$) and 0.25 µl of pooled dNTPs. PCR condition used for ISSR amplification was: Initial denaturing step at 94^0C for 5 min followed by 42 cycles of 94^0C for 1 min, 45^0 - 55^0C for 1 min and 72^0C for 2 min, the last cycle, primer extension at 72^0C for 7 min. The PCR condition used for RAPD was: Initial denaturing step at 94^0C for 5 min followed by 42 cycles of 94^0C for 1 min, 37^0C for 1 min and 72^0C for 2 min, the last cycle, primer extension at 72^0C for 7 min. The amplified products were separated by agarose (1.5%) gel electrophoresis and documented in gel documentation system (Bio Rad XR, Biorad, USA). O'Gene Ruler™ 100 bp DNA Ladder plus (ladder range 3000 bp to 100 bp from Fermentas Life Sciences, USA) was used as molecular weight marker. Bands were scored for its presence/absence (1/0) for each primer-genotype combination. The NTSYS-pc, version 2.1 software (Rolf, 2000) was used for estimation of genetic relatedness among the genotypes using Jaccard's similarity coefficient and clustering was done with UPGMA (unweighted pair group method using arithmetic averages).

3. Results

3.1 Randomly Amplified Polymorphic DNA (RAPD) analysis

Out of 40 RAPD primers screened, 18 primers produced distinct reproducible amplifications in all the 10 species and 30 accessions of *Cajanus*. The RAPD banding pattern is shown in Fig 1. The DNA profiles obtained from RAPD analysis are presented in Table 2. A total of 128 amplified fragments were generated, which includes 87 polymorphic; 29 monomorphic and 7 unique bands. The resolving power of primers ranged from 0.58 (OPP02) to 1.84 (OPA10), whereas the primer index varied from 0.13 to 0.41 with the primers OPN15 and OPD08 respectively. OPN06 and OPD08 produced highest number of amplified bands (13 & 12 respectively), whereas OPA10 and OPP02 produced least number of loci (2). Two primers OPD08 and OPP02 showed 100% polymorphism and the polymorphism obtained using OPN06 primer was as low as 30.8%. The average number of bands and polymorphic bands per primer was 7.11 and 4.83 respectively. Jaccard's similarity coefficient analysis revealed that all the taxa were related to each other with an average similarity of 70%. The highest similarity (100%) was observed between *Cajanus cajan* (Ca-c2/1) and *Cajanus cajan* (Ca-c1) and lowest (48%) between *Cajanus platycarpus* (Ca-pl6/2) and *Cajanus albicans* (Ca-a1/1) (Table 3). The highest numbers of bands (93) were amplified in case of *Cajanus reticulatus* (Ca-rt7) and lowest (71) in *Cajanus platycarpus* (Ca-pl6/2), *Cajanus crassus* (Ca-sc3/1) and *Cajanus mollis* (Ca-mo5/2).

Table 1
Details of plant samples used for study of genetic diversity and phylogeny

Sl.No.	Accession No.	Origin	Species	Code used
1	RPRC-C/4	India (Odisha)	*Cajanus scarabaeoides*	Caj-sca
2	RPRC-C/3	India (Odisha)	*Cajanus cajanifolius*	Caj-cajanifolius
3	RPRC-C/1	India(Odisha-Kandhamal)	*Cajanus cajan*	Caj-c1
4	RPRC-C/2	India(Odisha-Nayagarh)	*Cajanus cajan*	Ca-c 2/1
5	ICP-7035	India (MP)	*Cajanus cajan*	Ca-c 2/2
6	ICP-7182	India (MP)	*Cajanus cajan*	Ca-c 2/3
7	ICP-7613	India (MP)	*Cajanus cajan*	Ca-c 2/4
8	ICP-9150	Kenya	*Cajanus cajan*	Ca-c 2/5
9	ICP-9880	India (AP)	*Cajanus cajan*	Ca-c 2/6
10	ICP-11975	India (AP)	*Cajanus cajan*	Ca-c 2/7
11	ICP-12746	India (AP)	*Cajanus cajan*	Ca-c 2/8
12	ICP-12825	Tanzania	*Cajanus cajan*	Ca-c 2/9
13	ICP-13434	Malawi	*Cajanus cajan*	Ca-c 2/10
14	ICP-15620	SriLanka	*Cajanus albicans*	Ca-al 1/1
15	ICP-15621	India	*Cajanus albicans*	Ca-al 1/ 2
16	ICP-15622	India	*Cajanus albicans*	Ca-al 1/3
17	ICP-15634	Australia	*Cajanus reticulatus*	Ca-ret 7
18	ICP-15641	India	*Cajanus lineatus*	Ca-lin 4/1
19	ICP-15642	India	*Cajanus lineatus*	Ca-lin 4/2
20	ICP-15643	India	*Cajanus lineatus*	Ca-lin 4/3
21	ICP-15653	India	*Cajanus mollis*	Ca-mo 5/1
22	ICP-15654	India	*Cajanus mollis*	Ca-mo 5/2
23	ICP-15657	India	*Cajanus mollis*	Ca-mo 5/3
24	ICP-15661	India	*Cajanus platycarpus*	Ca-pl 6/1
25	ICP-15664	India	*Cajanus platycarpus*	Ca-pl 6/2
26	ICP-15665	India	*Cajanus platycarpus*	Ca-pl 6/3
27	ICP-15760	India	*Cajanus sericeus*	Ca-se 8/1
28	ICP-15762	Australia	*Cajanus sericeus*	Ca-se 8/2
29	ICP-15767	India	*Cajanus crassus*	Ca-cs 3/1
30	ICP-15770	India	*Cajanus crassus*	Ca-cs 3/2

Note: ICP is an acronym for ICRISAT accession number

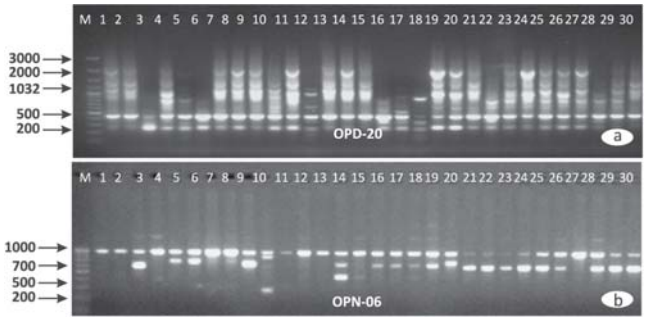

Fig.1. RAPD banding pattern of different species and accessions of *Cajanus* with the use of primers (a) OPD20 (b) OPN-6

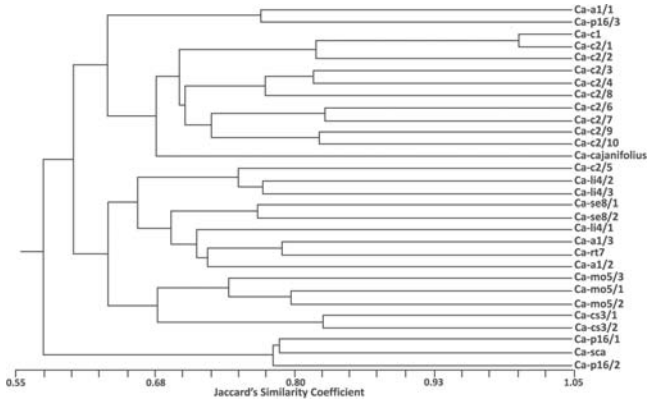

Fig. 2. Dendrogram showing genetic relationship among different species of *Cajanus* and accessions of *C. cajan* as revealed from RAPD

Table 2
Analysis of RAPD primers and bands details

Primer/Primer Combination	Sequences	Range of amplicons	Total bands	Polymorphic bands	Monomorphic bands	Unique bands	% of Polymorphic Band (PPB)	Resolving Power	Primer Index
OPD-08	TGCCGAGCTG	2800-350	12	12	0	0	100	0.67	0.41
OPA-03	AGTCAGCCAC	2900-650	7	3	3	1	42.9	1.14	0.18
OPD-18	AATCGGGCTG	2000-650	5	4	1	0	80	1.75	0.2
OPA-04	GGGTAACGCC	2500-460	4	2	1	1	50	1.16	0.16
OPA-10	GTTGCGATCC	2100-1032	2	1	1	0	50	1.84	0.13
OPD-20	GGACCCAACC	2600-660	7	5	2	0	71.4	1.43	0.26
OPN-06	TTGGCACGGG	2600-100	13	4	2	2	30.8	0.91	0.27
OPAF-14	GTGTGCCCCA	3000-610	10	5	4	1	50	1.15	0.2
OPN-10	CACCGTATCC	2000-220	10	7	3	0	70	1.66	0.24
OPP-02	GAGAGCCAAC	1500-900	2	2	0	0	100	0.58	0.41
OPS-07	GACCGACCCA	1900-250	8	6	1	1	75	1.16	0.26
OPT-04	GAGACGCACA	1850-700	6	4	2	0	66.7	0.98	0.19
OPN-15	AAGCGACCTG	1200-490	4	2	2	0	50	1.16	0.13
OPP-05	GGTGAGGTCA	2900-360	7	6	1	0	85.7	1.14	0.36
OPN-18	TTGCGGCTGA	1900-240	8	5	3	0	62.5	1.61	0.24
OPN-20	GGTGCGCACT	1500-220	6	5	1	0	83.3	1.77	0.19
OPN-14	TGATGCTGTC	2300-250	8	6	1	1	75	1.11	0.26
OPN-16	CCGAACACGG	2850-500	9	8	1	0	88.9	1.19	0.38
Total			128	87	29	7			

Table 3
Jaccard's similarity table for different taxa of *Cajanus* as revealed through RAPD analysis

	Ca-a1/1	Ca-pl6/3	Ca-c1	Ca-pl6/1	Ca-pl6/2	Ca-sca	Ca-c2/1	Ca-c2/2	Ca-c2/3	Ca-c2/4	Ca-cajanifolius	Ca-c2/6	Ca-c2/7	Ca-c2/8	Ca-c2/9	Ca-c2/10	Ca-c2/5	Ca-li4/2	Ca-li4/3	Ca-se8/1	Ca-se8/2	Ca-li4/1	Ca-a1/3	Ca-rt7	Ca-a1/2	Ca-mo5/3	Ca-mo5/1	Ca-mo5/2	Ca-cs3/1	Ca-cs3/2
Ca-a1/1	1.00																												
Ca-pl6/3	0.77	1.00																											
Ca-c1	0.64	0.67	1.00																										
Ca-pl6/1	0.58	0.63	0.61	1.00																									
Ca-pl6/2	0.48	0.54	0.52	0.78	1.00																								
Ca-sca	0.49	0.56	0.56	0.79	0.79	1.00																							
Ca-c2/1	0.64	0.67	**1.00**	0.61	0.52	0.56	1.00																						
Ca-c2/2	0.66	0.69	0.82	0.61	0.54	0.56	0.82	1.00																					
Ca-c2/3	0.65	0.68	0.68	0.64	0.53	0.56	0.68	0.74	1.00																				
Ca-c2/4	0.66	0.64	0.64	0.66	0.57	0.58	0.64	0.71	0.82	1.00																			
Ca-cajanifolius	0.53	0.61	0.67	0.57	0.50	0.50	0.67	0.70	0.64	0.65	1.00																		
Ca-c2/6	0.54	0.59	0.65	0.58	0.52	0.49	0.65	0.67	0.66	0.64	0.68	1.00																	
Ca-c2/7	0.61	0.67	0.76	0.61	0.52	0.52	0.76	0.79	0.77	0.71	0.73	0.83	1.00																
Ca-c2/8	0.62	0.71	0.69	0.63	0.57	0.56	0.69	0.77	0.82	0.73	0.70	0.71	0.79	1.00															
Ca-c2/9	0.65	0.70	0.73	0.64	0.51	0.54	0.73	0.76	0.73	0.70	0.72	0.74	0.79	0.78	1.00														
Ca-c2/10	0.57	0.60	0.64	0.57	0.51	0.52	0.64	0.67	0.66	0.63	0.62	0.68	0.70	0.67	0.82	1.00													
Ca-c2/5	0.63	0.68	0.71	0.60	0.50	0.52	0.71	0.76	0.71	0.70	0.78	0.71	0.77	0.76	0.93	0.76	1.00												
Ca-li4/2	0.53	0.54	0.54	0.65	0.59	0.60	0.54	0.58	0.62	0.61	0.58	0.53	0.58	0.61	0.62	0.57	0.60	1.00											
Ca-li4/3	0.59	0.60	0.60	0.66	0.57	0.55	0.60	0.61	0.68	0.64	0.64	0.57	0.64	0.64	0.65	0.65	0.63	0.77	1.00										
Ca-se8/1	0.55	0.61	0.61	0.65	0.55	0.56	0.61	0.58	0.61	0.58	0.65	0.63	0.65	0.67	0.69	0.64	0.67	0.65	0.72	1.00									
Ca-se8/2	0.59	0.62	0.67	0.66	0.55	0.56	0.67	0.61	0.68	0.67	0.66	0.67	0.69	0.66	0.75	0.65	0.73	0.64	0.72	0.77	1.00								
Ca-li4/1	0.50	0.56	0.61	0.67	0.64	0.60	0.61	0.55	0.57	0.63	0.62	0.58	0.58	0.58	0.60	0.57	0.62	0.67	0.66	0.64	0.73	1.00							
Ca-a1/3	0.58	0.60	0.64	0.65	0.57	0.59	0.64	0.65	0.66	0.63	0.69	0.59	0.63	0.67	0.73	0.64	0.73	0.63	0.66	0.67	0.70	0.73	1.00						
Ca-rt7	0.57	0.60	0.67	0.65	0.55	0.57	0.67	0.64	0.65	0.64	0.73	0.61	0.68	0.66	0.72	0.62	0.70	0.66	0.71	0.73	0.72	0.70	0.79	1.00					
Ca-a1/2	0.54	0.56	0.64	0.68	0.60	0.60	0.64	0.66	0.63	0.66	0.66	0.60	0.63	0.64	0.68	0.60	0.66	0.65	0.66	0.63	0.71	0.72	0.72	0.73	1.00				
Ca-mo5/3	0.51	0.57	0.66	0.69	0.60	0.63	0.66	0.66	0.69	0.66	0.61	0.55	0.63	0.70	0.67	0.63	0.65	0.63	0.66	0.69	0.66	0.69	0.71	0.66	0.72	1.00			
Ca-mo5/1	0.53	0.58	0.61	0.70	0.57	0.62	0.61	0.61	0.62	0.61	0.57	0.53	0.58	0.66	0.62	0.59	0.60	0.70	0.66	0.67	0.66	0.68	0.65	0.66	0.74	0.79	1.00		
Ca-mo5/2	0.53	0.58	0.57	0.64	0.58	0.59	0.57	0.59	0.58	0.60	0.57	0.52	0.54	0.62	0.59	0.58	0.59	0.72	0.65	0.59	0.62	0.68	0.61	0.62	0.66	0.70	0.80	1.00	
Ca-cs3/1	0.53	0.55	0.58	0.61	0.54	0.55	0.58	0.59	0.61	0.60	0.54	0.52	0.52	0.60	0.58	0.58	0.57	0.57	0.60	0.56	0.60	0.62	0.64	0.59	0.64	0.66	0.74	0.69	1.00
Ca-cs3/2	0.49	0.53	0.54	0.60	0.55	0.52	0.54	0.56	0.55	0.56	0.53	0.50	0.52	0.56	0.57	0.54	0.55	0.56	0.59	0.60	0.63	0.60	0.65	0.62	0.59	0.65	0.68	0.66	0.83

A dendrogram was constructed to derive the relationship among 30 different taxa of the genus *Cajanus* (Fig 2)**,** which separated all of them into two distinct clusters of 27 and 3, sharing a common node at 57% similarity level. The smaller cluster had two accessions of *Cajanus platycarpus* (Ca-pl6/1 and Ca-pl6/2) and *Cajanus scarabaeoides* (Ca-sca) and the similarity among them was about 79% but *Cajanus platycarpus* (Ca-pl6/1) was closer to *Cajanus scarabaeoides* (Ca-sca) than *Cajanus platycarpus* (Ca-pl6/2). The larger sub-group was further divided into 2 sub-clusters (14+13) where cultivated taxa got separated from wild species and accessions. The first sub-cluster again had two divisions of 5 and 9 taxa. Of the five taxa namely *Cajanus crassus* (Ca-cs3/1 and Ca-sc3/2), *Cajanus mollis* (Ca-mo5/1, Ca-mo5/2 and Ca-mo5/3), *Cajanus crassus* (Ca-cs3/1) and *Cajanus crassus* (Ca-cs3/2) formed one group with 81% similarity and got separated from the rest, sharing a common node at 68% level of similarity. *Cajanus mollis* (Ca-mo5/1, Ca-mo5/2 and Ca-mo5/3) came in a sub-cluster with similarity of 72% and *Cajanus mollis* (Ca-mo5/1) and *Cajanus mollis* (Ca-mo5/2) were found to be genetically closely related. The second sub-group included 8 genotypes of wild *Cajanus* species which subsequently segregated into two groups of 6 [*Cajanus albicans* (Ca-a1/2), *Cajanus reticulatus* (Ca-rt7), *Cajanus albicans* (Ca-a1/3), *Cajanus lineatus* (Ca-li4/1), *Cajanus sericeus* (Ca-se8/1) and *Cajanus sericeus* (Ca-se8/2)] having similarity of 68% among them and rest two accessions of *Cajanus lineatus* (Ca-li4/2 and Ca-li4/3) had a similarity of 77%.

The other sub-group included most of the cultivated accession of *Cajanus cajan* which got separated from wild species (e.g. *C. cajanifolius* and *C.sericeus*) sharing a common node at 60% similarity level. This group included the accession of *Cajanus cajan* (Ca-c1, Ca-c2/1, Ca-c2/2, Ca-c2/3, Ca-c2/4, Ca-c2/5, Ca-c2/6, Ca-c2/7, Ca-c2/8, Ca-c2/9, Ca-c2/10), *Cajanus cajanifolius* (Caj-cajanifolius), *Cajanus platycarpus* (Ca-pl6/3) and *Cajanus albicans* (Ca-a1/1). *Cajanus cajan* (Ca-c1) and *Cajanus cajan* (Ca-c2/1) showed maximum similarity of 100%. *Cajanus albicans* (Ca-a1/1) and *Cajanus platycarpus* (Ca-pl6/3) were observed to exhibit a similarity of 78% between them and this group was segregated from *Cajanus cajan* with whom it shared 65% genetic similarity.

3.2 Inter simple sequence repeat (ISSR) analysis

The results obtained from the molecular fingerprinting by ISSR primers in 30 accessions of *Cajanus* representing 10 species are presented in Table 4. Out of the 35 ISSR primers tested, only 18 primers produced good and reproducible amplified product. A total of 147 bands were amplified which include 125 polymorphic, 10 monomorphic and 12 unique bands. The size of amplicons ranged from 200bp to 3000bp. The resolving power of primers ranged from 0.51 [G $(CTGT)_4$] to 1.53 [$(CA)_8$ AG] and the primer index varied in the ranges of 0.16 - 0.44 for the $(CA)_8$ AG and $(CT)_8$G respectively. The ISSR banding pattern is shown in the (Fig. 3). $(AG)_{10}$ produced highest number of amplified loci (14) whereas $(CT)_8$ G and $(GACA)_4$ T produced least number (5) of bands. Nine primers yielded 100% polymorphic bands but the polymorphism observed with $(CA)_8$ AG and $(GGGGT)_3$ primers was only 50%. The average no of amplified bands and polymorphic bands per primer was 8.16 and 6.94 respectively. $(CA)_8$ AG and $(AG)_{10}$ amplified maximum no. of monomorphic loci (3 and 4), and the primer $(GGAGA)_3$ and $(GGGGT)_3$ produced 3 and 4 unique bands respectively.

All the 30 taxa genetically analysed had an average similarity of 56% as per the Jaccard's similarity coefficient analysis Table 5. The highest similarity (0.96) was observed between two accessions of cultivated pigeonpea (Ca-c2/6 and Ca-c2/7) and the lowest (0.22) between *Cajanus platycarpus* (Caj-pl6/2) and *Cajanus mollis* (Ca-mo5/1). The highest numbers of bands (90) were amplified in case of *Cajanus cajan* (Ca-c2/10) and the lowest (33) in *Cajanus mollis* (Ca-mo5/1). The dendrogram (Fig 4) divided the taxa into two distinct clusters of 1 and 29. The first and smallest cluster contained a single accession of *Cajanus mollis* (Ca-mo5/1) and both the groups shared a common node at 25% similarity. The large cluster was further divided into two subclusters (3+26) and the small sub-cluster contained *Cajanus crassus* (Ca-cs3/1 and Ca-cs3/2) and *Cajanus mollis* (Ca-mo5/2). Of these three, *Cajanus crassus* (Ca-cs3/1) and *Cajanus crassus* (Ca-cs3/2) had 78% similarity between them.

The second and larger cluster was divided into two sub-clusters (1+25). The lone accession *Cajanus mollis* (Ca-mo5/3) formed a separate cluster, sharing 45% similarity with the cluster of 25 taxa. The smaller sub-cluster contained

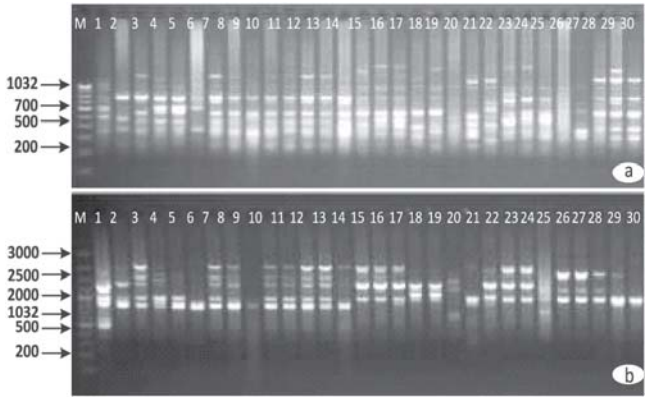

Fig. 3. ISSR banding pattern of different species and accessions of *Cajanus* with the use of primers: a. (AG)10, b. (CT)8A

Table 4
Details of ISSR primers used and bands amplified

Primer/Primer Combination	Sequences	Range of amplicons	Total bands	Polymorphic bands	Monomorphic bands	Unique bands	% of Polymorphic Band (PPB)	Resolving Power	Primer Index
(GA)8G	GAGAGAGAGAGAGAGAG	2200-370	8	8	0	0	100	0.87	0.42
(CA)8AG	CACACACACACACACAAG	1700-300	6	3	3	0	50	1.53	0.16
(AG)10	AGAGAGAGAGAGAGAGAGAG	2000-250	14	9	4	1	64.3	1.09	0.25
(CT)8A	CTCTCTCTCTCTCTCTA	2100-480	10	10	0	0	100	0.96	0.4
(AG)8C	AGAGAGAGAGAGAGAGC	1900-350	8	6	1	1	75	0.97	0.29
(GGAGA)3	GGAGAGGAGAGGAGA	1500-250	11	8	0	3	72.7	0.7	0.27
(GGGGT)3	GGGGTGGGGTGGGGT	1500-300	10	5	1	4	50	0.64	0.22
(AG)8	AGAGAGAGAGAGAGAG	1700-460	9	8	0	1	88.9	0.8	0.3
(GAA)6	GAAGAAGAAGAAGAAGAA	2700-550	7	7	0	0	100	1.16	0.4
(AGG)6	AGGAGGAGGAGGAGGAGG	1900-380	6	6	0	0	100	1	0.3
(CT)8G	CTCTCTCTCTCTCTCTG	1500-700	5	5	0	0	100	1.05	0.44
T(GACA)4	TGACAGACAGACAGACA	1900-300	8	7	1	0	87.5	1.11	0.33
(GA)9T	GAGAGAGAGAGAGAGAGAT	1800-400	7	7	0	0	100	1.11	0.36
G(CT)8	GCTCTCTCTCTCTCTCT	2500-450	9	9	0	0	100	0.74	0.33
(GATA)4C	GATAGATAGATAGATAC	2800-300	10	10	0	0	100	0.83	0.43
(GACA)4G	GACAGACAGACAGACAG	1200-500	7	6	0	1	85.7	0.74	0.39
G(CTGT)4	GCTGTCTGTCTGTCTGT	1500-600	7	6	0	1	85.7	0.51	0.3
(GACA)4T	GACAGACAGACAGACAT	2200-400	5	5	0	0	100	0.91	0.29
Total			147	125	10	12	86.7		

one accession of each of *Cajanus platycarpus* (Ca-pl6/3), *Cajanus lineatus* (Ca-li4/1), *Cajanus albicans* (Ca-a1/3), *Cajanus reticulatus* (Ca-ret7) and *Cajanus sericeus* (Ca-se8/2) sharing varying levels of similarity among them. The larger sub-cluster was comprised of all accessions of cultivated *Cajanus cajan*, *Cajanus cajanifolius* and few accessions of *Cajanus albicans, Cajanus scarabaeoides, Cajanus lineatus* and *Cajanus sericeus*.

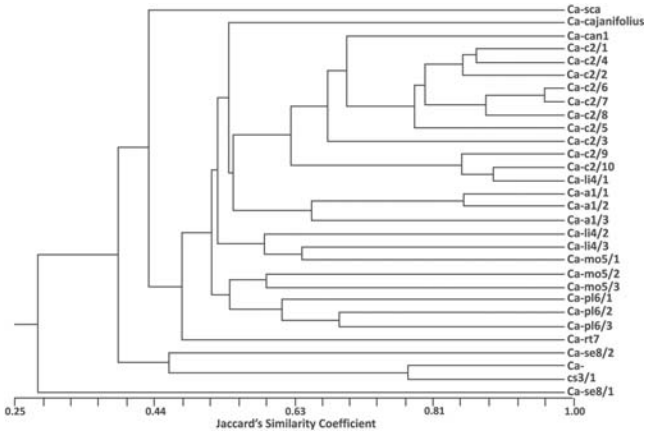

Fig.4. Relationship among different species of *Cajanus* and accessions of *C. cajan* through ISSR analysis

Most of the wild species formed one sub-group having an average similarity of more than 58% among them. The cultivated accessions formed another sub-cluster. The maximum similarity of 96% was obtained between two *Cajanus cajan* accessions (Ca-c2/6 and Ca-c2/7). Similarly, three *Cajanus cajan* accessions namely Ca-c2/1, Ca-c2/4 and Ca-c2/2 got separated in the cluster with more than 81% similarity among them. *Cajanus cajanifolius* (Caj-cajanifolius) showed close affinity with cultivated *Cajanus cajan* (Ca-c2/10 and Ca-c2/9), whereas *Cajanus cajanifolius* (Caj-cajanifolius) was closer to *Cajanus cajan* (Ca-c2/10) than *Cajanus cajan* (Ca-c2/9). *Cajanus platycarpus* (Ca-pl6/2) showed similarity of 50% with cultivated pigeonpea cluster.

3.3 RAPD and ISSR combined markers

The molecular phylogeny of the species of *Cajanus* inferred from data obtained from a combination of RAPD and ISSR markers has been discussed here. A total of 36 RAPD and ISSR primers produced good and reproducible amplification products. The highest (172) number of bands were amplified in case of an accessions of *Cajanus cajan* (Ca-caj2/10) and lowest (113) in *Cajanus mollis* (Ca-mo5/1)**.** Relationships among the 30 taxa containing 10 species of *Cajanus* were determined through analysis of Jaccard's similarities coefficient (Table 6). From the Jaccard's table it was observed that all the species were related to each other with an average similarity of 0.63. Highest similarity (0.89) was observed between two accession of *Cajanus cajan* (Ca-c2/6 and Ca-c2/7) and lowest (39%) between *Cajanus mollis* (Ca-mo5/1) and *Cajanus platycarpus* (Ca-pl6/1) [Table 6].

The cladogram (Fig 5) constructed taking both RAPD and ISSR data in respect of all the 30 taxa of *Cajanus* showed grouping of them into 2 distinct clusters of 26 and 4. Both these clades shared a node at 46% similarity level. The small cluster of 4 taxa was comprised of two accessions from each of *Cajanus crassus* and (Ca-cs3/1 and Ca-cs3/2) and *Cajanus mollis* (Caj-mo5/1 and Caj-mo5/2) and these two species had 55% similarity between them. The large cluster which included 26 taxa of *Cajanus* had two groups of very unequal sizes of 24 and 2. The small group had species like *Cajanus platycarpus* (Ca-pl6/1 and Ca-pl6/2), which got separated in the first place and shared 61% similarity with the other 24 species. The next group was formed of 2 accession of *Cajanus albicans* (Ca-al 1/1, Ca-al1/ 2) and *Cajanus scarabaeoides* (Ca-sca) which had more than 70% similarity among them and 55% with rest of the species in the dendrogram. Five accessions one from each species namely *Cajanus mollis* (Ca-mo5/3), *Cajanus platycarpus* (Ca-pl6/3), *Cajanus lineatus* (Ca-li4/1), *Cajanus albicans* (Ca-al1/3) and *Cajanus reticulatus* (Ca-ret7) got separated in the next level leaving the rest 16 taxa in a bigger cluster. Of these 16 taxa, 2 accessions of *Cajanus lineatus* (Ca-li 4/2 and Ca-li 4/3) and 2 accessions of *Cajanus sericeus* (Ca-se8/1 & Ca-se8/2) got out of the cluster justifying their species status and this clade had 65% similarity with rest others. Further, 11 accessions of cultivated pigeonpea (*Cajanus cajan*) along with one accession of *Cajanus cajanifolius* (Ca-cajanifolius) come together in a bigger clade with varying level of similarities among them. Of the pigeonpea accessions, Ca-c1, Ca-c2/1, Ca-c2/6) and Ca-c2/7 had more than 90% similarity among them.

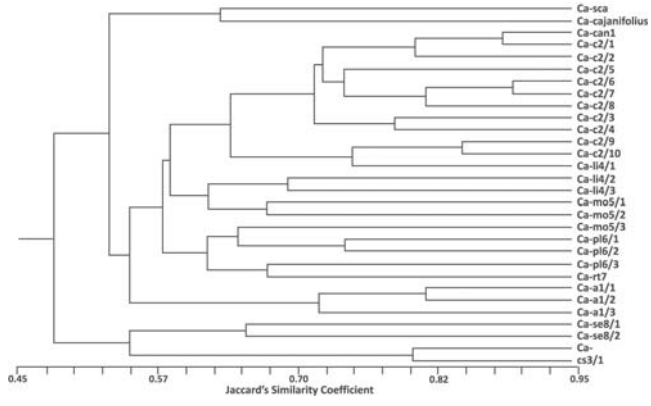

Fig. 5: Phylogeny of different species of *Cajanus* and accessions of *C. cajan* as inferred revealed from combined RAPD and ISSR markers

Table 5
Jaccard's similarity table for different species of *Cajanus* as inferred from ISSR analysis

	Ca-pl6/1	Ca-pl6/2	Ca-c1	Ca-a1/1	Ca-a1/2	Ca-sca	Ca-c2/1	Ca-c2/2	Ca-c2/3	Ca-c2/4	Ca-c2/5	Ca-c2/6	Ca-c2/7	Ca-c2/8	Ca-c2/9	Ca-c2/10	Ca-cajanifolius	Ca-li4/2	Ca-li4/3	Ca-se8/1	Ca-se8/2	Ca-rt7	Ca-a1/3	Ca-li4/1	Ca-pl6/3	Ca-mo5/3	Ca-mo5/1	Ca-mo5/2	Ca-cs3/1	Ca-cs3/2
Ca-pl6/1	1.00																												
Ca-pl6/2	0.51	1.00																											
Ca-c1	0.46	0.61	1.00																										
Ca-a1/1	0.46	0.57	0.64	1.00																									
Ca-a1/2	0.47	0.55	0.66	0.85	1.00																								
Ca-sca	0.52	0.49	0.56	0.63	0.67	1.00																							
Ca-c2/1	0.44	0.59	0.77	0.63	0.63	0.55	1.00																						
Ca-c2/2	0.45	0.57	0.72	0.59	0.59	0.56	0.86	1.00																					
Ca-c2/3	0.36	0.44	0.59	0.48	0.49	0.51	0.68	0.72	1.00																				
Ca-c2/4	0.47	0.58	0.72	0.61	0.59	0.56	0.87	0.85	0.75	1.00																			
Ca-c2/5	0.42	0.53	0.66	0.53	0.51	0.51	0.75	0.77	0.71	0.84	1.00																		
Ca-c2/6	0.42	0.57	0.67	0.60	0.55	0.51	0.85	0.79	0.67	0.87	0.80	1.00																	
Ca-c2/7	0.40	0.55	0.68	0.58	0.54	0.49	0.84	0.78	0.65	0.84	0.81	0.96	1.00																
Ca-c2/8	0.39	0.52	0.65	0.56	0.53	0.47	0.76	0.71	0.60	0.77	0.75	0.89	0.88	1.00															
Ca-c2/9	0.41	0.46	0.50	0.53	0.47	0.47	0.61	0.60	0.51	0.65	0.59	0.66	0.65	0.69	1.00														
Ca-c2/10	0.43	0.50	0.55	0.56	0.50	0.46	0.63	0.61	0.51	0.66	0.62	0.69	0.68	0.71	0.87	1.00													
Ca-cajanifolius	0.45	0.51	0.56	0.57	0.52	0.49	0.65	0.62	0.53	0.69	0.65	0.67	0.66	0.68	0.83	0.89	1.00												
Ca-li4/2	0.47	0.49	0.49	0.49	0.47	0.48	0.52	0.52	0.53	0.57	0.58	0.52	0.49	0.52	0.55	0.58	0.60	1.00											
Ca-li4/3	0.42	0.46	0.47	0.49	0.47	0.48	0.54	0.51	0.50	0.58	0.57	0.53	0.52	0.54	0.58	0.64	0.67	0.61	1.00										
Ca-se8/1	0.41	0.48	0.46	0.44	0.46	0.49	0.49	0.52	0.51	0.56	0.53	0.51	0.50	0.49	0.55	0.53	0.58	0.56	0.64	1.00									
Ca-se8/2	0.44	0.49	0.47	0.51	0.48	0.52	0.52	0.55	0.47	0.56	0.53	0.52	0.51	0.53	0.55	0.50	0.54	0.48	0.53	0.56	1.00								
Ca-rt7	0.40	0.43	0.49	0.54	0.48	0.50	0.55	0.50	0.49	0.55	0.53	0.54	0.53	0.52	0.54	0.56	0.58	0.44	0.59	0.49	0.59	1.00							
Ca-a1/3	0.40	0.49	0.51	0.48	0.50	0.52	0.57	0.57	0.54	0.59	0.55	0.57	0.58	0.53	0.56	0.57	0.60	0.50	0.61	0.54	0.57	0.58	1.00						
Ca-li4/1	0.39	0.46	0.47	0.51	0.51	0.47	0.55	0.54	0.48	0.56	0.52	0.54	0.55	0.51	0.52	0.55	0.56	0.43	0.53	0.46	0.51	0.57	0.69	1.00					
Ca-pl6/3	0.41	0.49	0.46	0.51	0.52	0.48	0.53	0.52	0.47	0.54	0.48	0.49	0.49	0.47	0.50	0.49	0.52	0.41	0.45	0.46	0.50	0.52	0.53	0.69	1.00				
Ca-mo5/3	0.36	0.47	0.42	0.48	0.45	0.44	0.46	0.44	0.44	0.48	0.49	0.47	0.46	0.46	0.49	0.48	0.50	0.44	0.51	0.47	0.41	0.48	0.48	0.52	0.62	1.00			
Ca-mo5/1	0.24	0.22	0.26	0.30	0.31	0.33	0.24	0.25	0.32	0.27	0.27	0.25	0.24	0.28	0.27	0.27	0.29	0.30	0.29	0.34	0.25	0.30	0.26	0.22	0.24	0.29	1.00		
Ca-mo5/2	0.29	0.29	0.33	0.32	0.29	0.30	0.31	0.32	0.38	0.35	0.33	0.34	0.32	0.32	0.34	0.34	0.33	0.35	0.33	0.37	0.32	0.33	0.30	0.30	0.31	0.33	0.44	1.00	
Ca-cs3/1	0.33	0.44	0.43	0.43	0.41	0.43	0.48	0.47	0.42	0.47	0.43	0.47	0.45	0.41	0.42	0.39	0.41	0.43	0.41	0.47	0.43	0.44	0.42	0.43	0.50	0.49	0.30	0.46	1.00
Ca-cs3/2	0.31	0.43	0.38	0.41	0.38	0.39	0.44	0.42	0.39	0.43	0.40	0.45	0.42	0.41	0.40	0.39	0.40	0.41	0.40	0.41	0.40	0.41	0.39	0.40	0.46	0.45	0.32	0.46	0.78	1.00

Table 6
Coefficient of similarity among species of *Cajanus* revealed from combined markers (RAPD + ISSR) analysis.

	Ca-pl6/1	Ca-pl6/2	Ca-c1	Ca-a1/1	Ca-a1/2	Ca-sca	Ca-c2/1	Ca-c2/2	Ca-c2/3	Ca-c2/4	Ca-c2/5	Ca-c2/6	Ca-c2/7	Ca-c2/8	Ca-c2/9	Ca-c2/10	Ca-cajanifolius	Ca-li4/2	Ca-li4/3	Ca-se8/1	Ca-se8/2	Ca-rt7	Ca-a1/3	Ca-li4/1	Ca-pl6/3	Ca-mo5/3	Ca-mo5/1	Ca-mo5/2	Ca-cs3/1	Ca-cs3/2
Ca-pl6/1	1.00																												
Ca-pl6/2	0.63	1.00																											
Ca-c1	0.55	0.64	1.00																										
Ca-a1/1	0.52	0.60	0.62	1.00																									
Ca-a1/2	0.48	0.54	0.58	0.81	1.00																								
Ca-sca	0.51	0.53	0.56	0.71	0.73	1.00																							
Ca-c2/1	0.54	0.63	0.88	0.62	0.57	0.56	1.00																						
Ca-c2/2	0.55	0.63	0.77	0.60	0.56	0.56	0.84	1.00																					
Ca-c2/3	0.51	0.57	0.64	0.56	0.51	0.53	0.68	0.73	1.00																				
Ca-c2/4	0.56	0.61	0.68	0.63	0.58	0.57	0.74	0.78	0.79	1.00																			
Ca-c2/5	0.47	0.57	0.66	0.55	0.50	0.51	0.71	0.73	0.67	0.73	1.00																		
Ca-c2/6	0.48	0.58	0.66	0.59	0.53	0.50	0.74	0.73	0.66	0.75	0.73	1.00																	
Ca-c2/7	0.50	0.61	0.72	0.60	0.53	0.51	0.80	0.78	0.72	0.77	0.77	0.89	1.00																
Ca-c2/8	0.50	0.61	0.67	0.60	0.55	0.52	0.72	0.74	0.71	0.75	0.72	0.80	0.83	1.00															
Ca-c2/9	0.52	0.57	0.61	0.58	0.49	0.51	0.67	0.68	0.62	0.68	0.66	0.70	0.72	0.73	1.00														
Ca-c2/10	0.50	0.55	0.59	0.57	0.51	0.49	0.64	0.64	0.58	0.65	0.62	0.68	0.69	0.69	0.85	1.00													
Ca-cajanifolius	0.50	0.53	0.57	0.58	0.51	0.49	0.62	0.59	0.55	0.64	0.62	0.62	0.60	0.61	0.75	0.75	1.00												
Ca-li4/2	0.50	0.52	0.52	0.57	0.53	0.54	0.53	0.55	0.58	0.59	0.58	0.52	0.54	0.56	0.58	0.57	0.68	1.00											
Ca-li4/3	0.50	0.53	0.54	0.57	0.52	0.51	0.57	0.56	0.59	0.61	0.61	0.55	0.58	0.59	0.61	0.64	0.70	0.69	1.00										
Ca-se8/1	0.48	0.55	0.54	0.54	0.50	0.52	0.55	0.56	0.57	0.57	0.60	0.57	0.58	0.58	0.62	0.59	0.63	0.61	0.68	1.00									
Ca-se8/2	0.52	0.56	0.57	0.58	0.52	0.54	0.60	0.58	0.59	0.62	0.60	0.60	0.60	0.60	0.65	0.57	0.62	0.57	0.63	0.67	1.00								
Ca-rt7	0.45	0.50	0.55	0.60	0.56	0.55	0.58	0.53	0.53	0.59	0.57	0.56	0.56	0.55	0.57	0.57	0.59	0.55	0.63	0.57	0.66	1.00							
Ca-a1/3	0.48	0.54	0.57	0.56	0.54	0.55	0.61	0.61	0.61	0.61	0.62	0.58	0.61	0.60	0.64	0.61	0.61	0.57	0.64	0.61	0.64	0.66	1.00						
Ca-li4/1	0.48	0.53	0.57	0.58	0.53	0.53	0.62	0.60	0.57	0.61	0.63	0.58	0.62	0.58	0.62	0.58	0.60	0.55	0.62	0.60	0.62	0.64	0.74	1.00					
Ca-pl6/3	0.47	0.53	0.55	0.59	0.56	0.54	0.59	0.59	0.56	0.60	0.57	0.55	0.56	0.55	0.59	0.54	0.58	0.52	0.55	0.55	0.61	0.62	0.63	0.71	1.00				
Ca-mo5/3	0.43	0.52	0.54	0.58	0.52	0.54	0.56	0.55	0.57	0.57	0.55	0.51	0.54	0.58	0.58	0.55	0.54	0.54	0.58	0.58	0.54	0.58	0.59	0.59	0.67	1.00			
Ca-mo5/1	0.39	0.41	0.45	0.51	0.45	0.49	0.44	0.45	0.50	0.46	0.44	0.40	0.42	0.48	0.45	0.43	0.46	0.52	0.48	0.53	0.48	0.51	0.47	0.47	0.50	0.55	1.00		
Ca-mo5/2	0.41	0.44	0.45	0.47	0.43	0.45	0.44	0.46	0.49	0.48	0.46	0.43	0.43	0.47	0.47	0.45	0.45	0.54	0.49	0.49	0.48	0.50	0.45	0.47	0.48	0.51	0.65	1.00	
Ca-cs3/1	0.42	0.50	0.51	0.52	0.47	0.49	0.53	0.53	0.52	0.54	0.49	0.49	0.49	0.50	0.49	0.48	0.48	0.50	0.50	0.52	0.52	0.53	0.53	0.51	0.57	0.58	0.53	0.58	1.00
Ca-cs3/2	0.40	0.48	0.46	0.50	0.47	0.45	0.49	0.49	0.48	0.50	0.47	0.47	0.47	0.49	0.49	0.46	0.48	0.49	0.49	0.51	0.52	0.51	0.52	0.51	0.53	0.55	0.52	0.57	0.80

4. Discussion

The revised genus *Cajanus* currently comprises of 18 species from Asia, 15 species from Australia, and one species from West Africa. Of these, 13 are found only in Australia, 8 in the Indian subcontinent, and 1 in West Africa, with the remaining 14 species occurring in more than one country. Based on growth habit, leaf shape, hairiness, structure of corolla, pod size, and presence of strophiole, van der Maesen (1980) grouped the genus *Cajan* into six sections. Eighteen erect species were placed under three sections: seven species in Atylia, nine species in section Fruticosa, and two species in section Cajanus that consists of the cultivated species along with its progenitor, *C. cajanifolius*. Eleven climbing and creeping species were arranged in two sections, Cantharospermum (5) and Volubilis (6) and the remaining three trailing species were classified under Rhynchosoides.

With a view to conserving germplasm of the diverse array of species of *Cajanus* and allied taxa and to incorporate desirable genes from these plants into cultivated *C. cajan,* emphasis has been laid on the need to understand the phylogenetic relationships of these species more completely (Reddy 1981a, b & c; Reddy and De, 1983; Pundir and Singh 1985a, b & c; Saxena & Sharma, 1990). Although related wild species are a rich reservoir of not only resistance genes against various biotic and abiotic stresses but also of genes responsible for yield components, use of closely related species in pigeonpea improvement have been limited. Ongoing efforts using molecular tools to examine taxonomic relationships within the subtribe Cajaninae would throw light on the phylogenetic relationships within the group, and may suggest parsimonious routes for trait introgression.

4.1 Analysis of genetic diversity and phylogeny among species of Cajanus

The members of the genus *Atylosia* closely resemble the genus *Cajanus* in vegetative and reproductive characters and were relegated to two separate genera mainly on the basis of the presence or absence of a seed strophiole. Although some earlier taxonomists pointed out the unsatisfactory placement of *Atylosia* and *Cajanus* under two different genera, the irrefutable experimental evidence from the studies on inter-specific hybridization, cytotaxonomy and chemotaxonomy led to merger of the two under *Cajanus* (van der Maesen, 1986).

Baker (1876), besides considering *Cajanus* and *Atylosia* as two separate genera, divided the genus Atylosia into two sub-genera i. e. Atylia (containing species like *A. lineate*, A. *trinervia, A. sericea, A. mollis, A. heynei*) and Cantharospermum (with species like *A. scarabaeoides, A. albicans, A. platycarpa. A. goensis*). Taking into account a few key characters like growth habit, leaf shape, hairiness, nature of corolla, pod size, and strophiole characteristics, van der Maesen (1985) divided the genus into six sections namely, Cajanus (2 spp.), Atylia (7 spp.), Fruticosa (9 spp.), Cantharospermum (5 spp), Volubilis (6 spp.) and Rhynchosoides (3 spp). While suggesting sectional arrangement within the genus, van der Maesen (1985) himself admitted that this classification into sections would not always exhibit natural relationships and members of one section share a number of characters with the species of another section.

In the present investigation, the genetic relationships among 10 species of *Cajanus* were assessed using RAPD and ISSR markers. The dendrogram constructed using UPGMA method based on molecular data revealed the grouping of species under different sections of the genus (Fig 5) as proposed by van der Maesen (1986). All the accessions of cultivated pigeonpea (*Cajanus cajan*) and its wild progenitor *Cajanus cajanifolius* formed a single cluster conforming their placement under the sect. Cajanus. While the two species (*C. crassus* and *C. mollis*) of the sect. Volubilis came together in the phylognetic tree, two members of sect. Cantharospermum namely, *C. scarabaeoides and C. albicans* formed another sub-cluster. Species like *C. lineatus, C. sericeus, C. reticulatus*, classified under the sect. Atylia by van der Maesen (1986) also formed a cluster with few accessions of other species from different sections. The findings of the present study, to a large extent, are in agreement with the sectional classification of the genus *Cajanus*.

The closer affinity between pigeonpea and its wild relative *C. cajanifolius* has been established through the study of morphological traits (Mallikarjuna *et al*., 2011), analysis of esterase isozymes (Krishna and Reddy, 1982) and SDS-PAGE (Panigrahi *et al*., 2007). In the case of SDS-PAGE, the banding patterns revealed *C. cajanifolius* to be the closest to *C. cajan*, with *C. platycarpus* as an outgroup species justifying its status as a tertiary gene pool species (van der Maesen 1986). The close affinity between *C. cajan* and *C. cajanifolius* has also been observed through RFLP (Nadimpalli *et al*., 1994) and SSR analyses (Mallikarjuna *et al*., 2011). There is further evidence from cytology that *C. cajanifolius* is the progenitor species of *C. cajan* as the two have a similar karyotype, and the hybrids between the two species show normal meiosis with high pollen fertility and high seed set (Pundir and Singh, 1985a).

In the present study, *C. platycarpus*, belonging to the sect. Rhynchosioides formed an isolated cluster with its two accessions. Sivaramakrishnan *et al*. (2002), while assessing the genetic diversity in 12 species of *Cajanus*

including other accessions of pigeonpea and species of *Rhynchosia* using RFLP made similar inference on status of *C. platycarpus.* The distinctness of *C. platycarpus* in this study also corroborates well with the earlier reports on the interrelationships of *C. platycarpus* and other wild relatives of *Cajanus* (Krishna & Reddy, 1982 and Pundir & Singh, 1985a).

The dendrogram constructed using UPGMA method based on molecular data in the present analysis revealed the grouping of the two species namely, *Cajanus scarabaeoides* and *Cajanus albicans*, which correspond to the sect. Cantharospermum of the genus *Cajanus* proposed by van der Maesen (1986). Similar conclusion was drawn by Krishna & Reddy (1982) based on their study of esterase isozymes among *Cajanus cajan* and 6 species of *Atylosia* (now *Cajanus*), who detected 3 common bands justifying the close relationship between the two species. Karyotypes of *C. albicans* and *C. scarabaeoides* were very similar and none of the two species had a chromosome pair with *r*-index >2.0, which is reflected in very similar symmetry indices (Ohri & Singh, 2002). The findings of the present work are in accordance with the relationship established by the above studies.

The results of the present study led to placement of 3 accessions of *C. lineatus* and 2 accessions of *C. sericeus* in a tight sub-cluster and 1 accession of each of *C. reticulatus, C. albicans, C. platycarpus* and *C. mollis* in another sub-cluster; both the sub-cluster share a common node at 57% level of similarity. Three of the above species namely, *C. lineatus*, C. sericeus and *C. reticulatus* belong to the sect. Atylia. According to Ohri & Singh (2002), the karyotypes of *C. lineatus* and *C. sericeus* belonging to sect. Atylia were similar in respect of maximum *r*-index and the ratio of longest and shortest chromosomes in their respective complements. Using RAPD marker, Ratnaparkhe *et al*. (1995) found a similar affinity between *C. lineatus* and *C. sericeus* but contrary to the sectional arrangement of the genus, *C. albicans* belonging to the sect. Cantharospermum formed a cluster with the above two taxa. The grouping of an accession of *C. albicans* with members of the sect. Atylia in the current piece of work corroborates the findings of Ratnaparkhe *et al*. (1995) and Nadimpalli *et al*. (1992). Though closeness between Australian *C. reticulatus* and Indian *C. platycarpus* has been reported in one of the studies (Parani *et al*., 2000) based on ribosomal DNA variation, inclusion of an accession of *C. mollis* in the cluster is difficult to explain.

The genetic relatedness between the two species of the sect. Volubilis namely, *C. crassus* and *C. mollis* was very close as could be established from the present study. In a number of molecular phylogenetic studies, the relationship between *C. volubilis* and *C. mollis* have been derived (Jha & Ohri, 1996; Sivaramakrishnan *et al*., 2002) but no reference was found with regard to the genetic similarity between *C. mollis* and *C. crassus*. Upadhyaya *et al*. (2012) assessed 18 species of the genus *Cajanus* including *C. mollis and C. crassus* for 27 morpho-agronomic traits and found them in two different clusters. However, there were similarities in respect of climbing habit, larger sized leaves, higher seed protein contents etc. among these two species.

4.2 Genetic diversity analysis in pigeon pea (Cajanus cajan) accessions

In the present study, 2 local accessions of *Cajanus cajan* (Pigeonpea) collected from Kandhamal and Nayagarh districts of Odisa (India) and 9 accessions procured from ICRISAT were analysed to derive the genetic relationship among them using RAPD and ISSR markers. Intra-specific genetic diversity analysis of pigeon p*e*a with the use of a total of 36 RAPD and ISSR primers revealed distinct segregation of genotypes of Indian and African origin. Among the accessions from Indian states, those from Madhya Pradesh, Andhra Pradesh and Odisha formed separate clusters justifying the proximity of the geographical area of their occurrence and cultivation. Two local landraces collected from Kandhamal and Nayagarh of Odisha (locally known as "Kandula") were genetically very close to each other and to the accession from adjoining state of Madhya Pradesh. Songok *et al*. (2010) found similar distinction of pigeon pea genotypes from India and East Africa. While analysing phylogenetic relationships of *Cajanus* and allied genera using AFLP markers, Nadimpalli *et al*. (1992) also observed grouping of accessions of individual species from different countries.

The dendrogram constructed based on UPGMA method using data obtained from RAPD and ISSR markers, revealed the clear segregation of pigeonpea genotypes of African origin. The accession Tanzania (ICP No. 12825) and Malawi (ICP No. 13434) got separated from rest of the Indian genotypes (except 1 from Kenya) sharing a similarity of 63%. Songok *et al.* (2010) also found clear distinction of pigeonpea genotypes from India and East Africa. They apprehended that after domestication, pigeonpea is believed to have been taken from India, the country of its origin, to Malaysia, then to East Africa and then to Egypt through the Nile valley around 2000 BC (Songok *et al*., 2010; van der Maesen, 1990; Smartt, 1990). Though a self-pollinated crop, out-crossing (40-70%) does occur through insect pollination and over the years, substantial genetic variability among these geographically isolated populations of India and Africa might have been taken place.

However, Wasike *et al*. (2005) using AFLP studied the genetic variability and relatedness between Asian and African pigeonpea cultivars found no major clustering patterns according to country of origin. Though there was a close genetic relationships between them, East African pigeonpeas are less genetically diverse than Indian cultivars. It was opined that the Indian cultivars could be used as a source of germplasm for future improvement of East African pigeonpea. The non-clustering of the accession from Kenya (ICP No. 9150) with African genotypes may be due to the reason explained above or it might have been a recent introduction from an Asiatic country. In a similar type of work, Boehringer *et al*. (1991) used allozymes to detect polymorphism between Indian and Zambian genotypes of pigeonpea.

Among the collections from Indian states, which came in a separate cluster, the genotypes from Madhya Pradesh (ICP No.7182 & 7613), Andhra Pradesh (ICP No.9880, 11975 and 12746) and Odisha (Ca-c1 and Ca-c2/1) formed separate clusters justifying their geographical area of occurrence and cultivation. Two local cultivars collected from Kandhamal and Nayagarh Districts of Odisha and locally known as "Kandula" were very close with about 88% similarity between them and along with an accession from adjoining state of Madhya Pradesh (ICP No. 7035), they occupied a distinct sub-cluster in the dendrogram. Kandula, the local variety of pigeonpea has a different taste and the 'dal' prepared out of it is somewhat pasty in nature. The relatively close relationship of pigeonpea accessions from Odisha and Andhra Pradesh indicates gene flow among populations of these adjoining coastal states and perhaps adaptation to prevailing environmental conditions and cultural practices. Thus, a clear distinction of genotypes from different states/ geographical locations could be observed in the present investigation as has been established by Songok *et al*. (2010), who analysed cultivars/ accessions from Indian states of Odisha, Madhya Pradesh, Andhra Pradesh, Tamilnadu, Maharasthra and Gujrat along with those from Africa.

In the face of decreasing production of pulses in several parts of the world including India, there is an increasing need to broaden the genetic base and introduce traits for various biotic stresses and desirable traits. There is a renewed interest to exploit more wild relatives and such efforts would have a considerable impact on broadening the genetic base of variation of different leguminous crops and introduction of useful biotic, abiotic and agronomic traits. The possibility of exploiting wild relatives from the various gene pools of cultivated crops especially of grain legumes, has opened up new vistas for enhancing the genetic variability and the findings of the present work will help in the process of evaluation of genetic diversity and selection of species, accessions and landraces of legumes for utilization in breeding and crop improvement programmes in India or elsewhere.

References

Baker, J. G. (1878). Leguminosae. In: Hooker, J. D. (ed.) Flora of British India, Part-V. L. Reeve & Co, London, pp. 56-306.

Boehringer, A., Lebot. V. and Aradhya, M. (1991) Isozyme variation in twenty-one perennial pigeonpea genotypes. Int. Pigeonpea Newslett. 14: 6-7.

De, D. N. (1974). Pigeonpea. In: Hutchinson, J. (ed.) Evolutionary Studies on World Crops. Cambridge Press, London, UK, pp. 79-87.

Deodikar, G. B. and Thakar, C. V. (1956). Cytotaxonomic evidence for the affinity between *Cajanus indicus* Spreng. and certain erect species of *Atylosia* W. and A. Proc. Indian Acad. Sci. 43: 37–45.

Doyle, J. J. and Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Dutta, S., Kumawat, G., Singh, B. P., Gupta, D. K., Singh, S., Dogra, V., Gaikwad, K., Sharma, T. R., Raje, R. S., Bandhopadhya, T. K., Datta, S., Singh, M. N., Fakrudin, B., Kulwal, P., Wanjari, K. B., Varshney, R. K., Cook, D. R., Singh, N. K. (2011). Development of genic-SSR markers by deep transcriptome sequencing in pigeonpea [*Cajanus cajan* (L.) Millspaugh]. BMC Plant Biol. 11: (doi: 10.1186/1471-2229-11-17).

Ganapathy, K. N., Gnanesh, B. N., Byre Gowda, M., Venkatesha, S. C., Gomashe, Sunil S. and Channamallikarjuna, V. (2011). AFLP analysis in pigeonpea [*Cajanus cajan* (L.) Millsp.] revealed close relationship of cultivated genotypes with some of its wild relatives Genet. Resour. Crop Evol. 58: 837–847.

Grear, J. W. (1978). A revision of the New World species of *Rhynchosia* (Leguminosae-Faboideae). Mem. New York Bot. Gard. 31 (1): 1-168.

Haines, H. H. (1919). Some new species of plants from Behar and Orissa. J. Asiatic Soc. Beng. (NS) 15: 309-317.

Jha, S. S. and Ohri, D. (1996). Phylogenetic relationships of pigeonpea [*Cajanus cajan* (L.) Millsp.] and its wild relatives based on seed protein profiles. Genet. Resour. Crop Evol. 43: 275–281.

Krishna, T. G. and Reddy, L. J. (1982). Species affinities between *Cajanus cajan* and some *Atylosia* species based on esterase isoenzymes. Euphytica 31: 709-713.

Kumar, L. S. S., Thombre, M. V. and D'Cruz, R. (1958). Cytological studies of an intergeneric hybrid of [*Cajanus cajan* (L.) Millsp.] and *Atylosia lineata* Wt. & Arn. Proc. Indian Acad.Sci. Section B. 47 (4): 252-262.

Lackey, J. A. (1978). Leaflet anatomy of Phaseoleae (Leguminosae: Papilionoideae) and its relation to taxonomy. Bot. Gaz. 139 (4): 436-444.

Mallikarjuna, N., Saxena, K. B. and Jadhav, D. R. (2011). *Cajanus*. In: Cole, C. (ed.) Wild Crop Relatives: Genomic and Breeding Resources of Legume Crops and Forages. Springer, pp. 21-33.

Nadimpalli, R. J., Jarret, R. L., Pathak, S.C. and Kochert, G. (1992). Phylogenetic relationships of the pigeonpea [*Cajanus cajan* (L.) Millsp.] based on nuclear restriction fragment length polymorphisms. Genome 36: 216-223.

Odeny, D. A., Jayashree, B., Gebhardt, C., and Crouch, J. (2009). New microsatellite markers for pigeonpea [*Cajanus cajan* (L.) Millsp.]. BMC Res. Notes (doi: 10.1186/ 1756-0500-2-35).

Ohri, D. and Singh, S. P. (2002). Karyotypic and genome size variation in pigeonpea [*Cajanus cajan* (L.) Millsp.] and some of its relatives. Genet. Resour. Crop Evol. 49: 1–10.

Panguluri, S. K., Janaiah, K., Govil, J. N., Kumar, P. A. and Sharma, P. C. (2006). AFLP fingerprinting in pigeonpea [*Cajanus cajan* (L.) Millsp.] and its wild relatives. Genet. Resour. Crop Evol. 53 (3): 523-531.

Panigrahi, J., Kumar, D. R., Mishra, M., Mishra, R. P. and Jena, P. (2007). Genomic relationships among 11 species in the genus *Cajanus* as revealed by seed protein (albumin and globulin) polymorphisms. Plant Biotechnol. Rep. 1: 109–116.

Parani, M., Lakshmi, M., Senthil Kumar, P. and Parida, A. (2000). Ribosome DNA variation and phylogenetic relationships among [*Cajanus cajan* (L.) Millsp.] and its wild relatives. Curr. Sci. 78 (10): 1235–1238.

Pundir, R. P. S. and Singh, R. B. (1985a). Cytogenetics of F1 hybrids between *Cajanus* and *Atylosia* species and its phylogenetic implications. Theor. Appl. Genet. 71: 216–220.

Pundir, R. P. S. and Singh, R. B. (1985b). Biosystematic relationships among *Cajanus, Atylosia and Rhynchosia* species. Theor. Appl. Genet. 69: 531-534.

Pundir, R. P. S. and Singh, R. B. (1985 c). Crossability relationships among *Cajanus*, *Atylosia* and *Rhynchosia* species and detection of crossing barriers. Euphytica 34: 303-308.

Ratnaparkhe, I. B., Gupta, V. S., Venmurthy, M. R. and Ranjekar, P. K. (1995). Genetic fingerprinting of pigeonpea [*Cajanus cajan* (L.) Millsp.] and its wild relatives using RAPD markers. Theor. Appl. Genet. 91: 893-898.

Reddy, L. J., and De, D. N. (1983). Cytomorphological study in *Cajanus cajan* X *Atylosia lineata.* Indian J. Genet. 43: 96-103.

Reddy, L. J. and Faris, D. G. (1981). A cytoplasmic genetic male-sterile line in pigeonpea. Int. Pigeonpea Newslett. 1: 16–17.

Reddy, L. J. (1981a). Pachytene analyses in *Cajanus cajan* x *Atylosia lineata* and their hybrid. Cytologia 46: 397-412.

Reddy, L. J. (1981b). Pachytene analyses in *Atylosia scarabaeoides* and *Cajanus cajan* X *A. sericea* hybrid. Cytologia 46: 576-577.

Rohlf, F. J. (2000). NTSYS-pc: numerical taxonomy and multivariate analysis system, version 2.1. Exeter Software: Setauket, New York.

Saxena, K. B. and Sharma, D. (1990). Pigeonpea. In: Nene, Y. L., Hall, S. D. & Sheila, V. K. (ed.) The Pigeonpea. CAB International, UK, pp. 137-157.

Sivaramakrishnan, S., Kannan, S. and Reddy, L. J. (2002). Diversity in selected wild and cultivated species of pigeonpea using RFLP of mtDNA. Euphytica 125: 21–28.

Smartt, J. (1990). Grain Legumes: Evolution and Genetic Resources. Cambridge University Press, Cambridge, pp. 278-293.

Songok, S., Ferguson, M., Muigai, A. W. and Silim, S. (2010). Genetic diversity in pigeonpea [*Cajanus cajan* (L.) Millsp.] landraces as revealed by simple sequence repeat markers. African J. Biotechnol. 9 (22): 3231-3241.

Stirton, C. H. (1981). Psoraleceae. In: Polhill, R. M. and Raven, P. H. (ed.) Advances in Legume Systematics, Part 2. Royal Botanic Gardens, Kew, UK, pp. 337–343.

Upadhyaya, H. D., Kashiwagi, J., Varshney, R. K., Gaur, P. M., Saxena, K. B., Krishnamurthy, L., Gowda, C. L. L., Pundir, R. P. S. *et al*. (2012). Phenotyping chickpeas and pigeonpeas for adaptation to drought. Frontiers Plant Physiol. 3 (179): 1-10.

Van der Maesen, L. J. G. (1980). India is the native home of the pigeonpea. Miscellaneous papers 19, Landbouwhoge school, Wageningen, pp. 257-262.

Van der Maesen, L. J. G. (1986). *Cajanus* DC. and *Atylosia* Wt. & Arn. (Leguminosae). Agricultural University Wageningen Papers 85 (4): 1–225, Wageningen, Netherlands.

Van der Maesen, L. J. G. (1985). Revision of the genus *Pueraria* DC with some notes on *Teyleria* Backer (Leguminosae), Agricultural University Wageningen Papers 85 (1): 1–132, Wageningen, Netherlands.

Van der Maesen, L. J. G. (1990). Pigeonpea: Origin, history, evolution and taxonomy. In: Nene, Y. L., Halls, D. and Sheila, V. K. (ed.) The Pigeonpea. CAB International, UK, pp. 15–46.

Wasike. S., Okori. P. and Rubaihayo. P. R. (2005) Genetic variability and relatedness of the Asian and African pigeonpea as revealed by AFLP. African J. Biotechnol. 4 (11): 1228-1233.

Plant Science Research

ISSN 0972-8546

Ethnobotany and bioactive compounds in leaf of *Bixa orellana* L. and its toxicity to *Artemia salina* L.

S. Kumar, P. K. Tripathy and P. K. Jena[Ψ]

Department of Botany, Ravenshaw University, Cuttack- 753003, India

ARTICLE INFO

Article history:
Received : 14 November 2012
Received in revised form : 11 December 2012
Accepted : 13 December 2012

Keywords:
Bixa orellana
ethnobotany
bioactive compounds
toxicity

ABSTRACT

Bixa orellana L. (Kum Kum) belonging to the family Bixaceae is a indegineous plant in Odisha. It is also cultivated at some places. The leaves and fruits of the plant are used as dye and as natural lipstick. They are also used as dye for colouring the vegetables and dishes. The plant and its parts are used in treatment of different diseases by the rural and some peripheral tribal communities of Odisha, such as those residing in Simlipal Biosphere Reserve Forest. Experiments were designed to study the ethnobotanical use of the plant among tribal groups of SBR, Odisha and the bioactive compounds presents in the plant and or its leaves and also whether the leaf extracts posses any toxic effect or not. The results revealed the potent bioactive compounds in the leaves of *Bixa orellana* L. The study further exhibited that the leaf extracts posses no toxic action against *Artemia salina* L., an experimental arthropod. Thus the studies emphasize upon the potent bioactive compounds present in the leaves of *Bixa orellana* L. without any toxic effect.

Medicinal plants are of great importance to the health of individuals and communities as well. The medicinal value of plants lies in some biochemical substances present in them that produce a definite physiological action on the human body (Okwu, 2001).The use of herbal medicine for the treatment of diseases and infections is an age old practice. The World Health Organization supports the use of traditional medicines provided they are proven to be efficacious and safe. In developing countries, a large number of people live in extreme poverty and many of them are suffering and dying for want of safe water and medicine, they have no alternative for primary health care (WHO, 1985).Therefore, the need to use medicinal plants as alternatives to medicines in the absence of primary healthcare cannot be avoided. More so herbal medicines have received much attention as sources of bioactive compounds since they are considered as time tested and relatively safe for human use and are also environment friendly (Fazly *et al*., 2005) Therefore, there is the need to search for more and more herbal medicinal plants with an aim to validate the ethno-medicinal use and subsequently the isolation and characterization of compounds in them which can be added to the potential list of drugs. *Bixa orellana* L. is a well studied plant of the family Bixaceae which is either naturally grown or cultivated in Odisha. *Bixa orellana* L. is a small tree with a round head, generally grown as an ornamental plant in rural areas because of its lovely flowers of various colors, such as dark red, light red and shiny red. The height ranges from 3-10 meters, leaves glossy ovate and ever green with reddish veins; heart shaped base and a pointed tip. The plant is with a thin, long stem and the leaves are between 8-12 cm long and 5-14 cm wide. Fruits round, with dense soft bristle or a smooth surface. Seed numerous, brown or black. Its seeds produce a natural dye used for colouring by the tribal people of Odisha in Simlipal Biosphere Reserve Forest and other forest block. It is used for colouring foods and cosmetics. It is a native of tropical America but is now distributed in most tropical countries in both wild as well as cultivated forms. In India, it is found mainly in Odisha, Andhra Pradesh and Maharashtra and to

[Ψ] *Corresponding author; Email:* padan_jena@rediffmail.com

some extent in Kerala, Karanataka and Tamilnadu (Venugopalan *et al.,* 2011) .It is native to Tropical America because it requires full sunlight and protection from wind. It grows equally well in lowlands as well as mountainous regions and areas of higher elevation. It is believed to come from Brazil and was carried through Central and South America to India. Indians used its color as body paint and also as lipstick. In India, the plant is also used by Ayurveda practitioners as an astringent and mild purgative and is considered by them as a good remedy for treating dysentery and kidney diseases. The root bark is antipyretic too. In Philippines, the leaf decoction is used to cure skin diseases and burns. The leaves are a popular febrifuge in Cambodia. The infusion of leaves is prescribed as a purgative and is used in the treatment of dysentery. In Central America, the oil derived from seeds is used to cure leprosy and decoction is utilized to treat jaundice (Metta *et al.,* 2009).

Bixa orellana L. leaves were collected from Medicinal Germplasm Garden of Regional Plant Resource Centre, Bhubaneswar. The collected plant materials were washed thoroughly by tap water followed by distilled water twice, and were oven dried. The dried materials were crushed to powder with mechanical devise and were kept in an air tight container for qualitative analysis of bioactive compounds. Qualitative phytochemical analysis were done using percolation method for crude extract (Harborne, 1973; Trease and Evans, 1989). The Brine shrimp assay was carried out according to the principle described by Meyer *et al.* (1982) and Krishnaraju *et al.* (2005).

Solvent extract was obtained from 5g of leaf powder which was macerated in solvent for 12 h in refrigerator. After 12 h sample was filtered and residue was again macerated in same solvent and for each solvent the process was repeated thrice. Solvent extraction was started with methanol followed by acetone and chloroform. Aqueous extract was prepared separately by taking the powder in distiled water followed by filtration. Filtrates were dried and concentrated to get semisolid mass. All the phytochemical assays were conducted using the same extracts. The brine shrimp lethality assay (BSLA) has been used routinely in the primary screening of the crude extracts as well as isolated compounds to assess the toxicity towards brine shrimp, which might provide an indication of possible cytotoxic properties of the test materials. Brine shrimp nauplii have been previously utilized in various bioassay systems (McLaughlin *et al.,* 1991). It is a typical primitive arthropod with a segmented body to which is attached broad leaf-like appendages. The body usually consists of 19 segments, the first 11 of which have pairs of appendages, the next two which are often fused together carry the reproductive organs, and the last segments lead to the tail. The total length about 4 mm (Criel and Macrae, 2002). It is found suitable as a "standard" organism in toxicological assays, despite the recognition that it is too robust an organism to be a sensitive indicator species (Criel and Macrae, 2002). Shrimp eggs were kept for hatching in 6 % normal saline for 18 h. Plant extract obtained following the above protocol were subjected to motility assay. Readings were taken every hour up to 4 h and later at 24 h. Motility parameters (Table 1) such as, +4 indicate highly motile, +3 indicate motile, +2 indicate sluggish and +1 indicates slow.

Field survey has indicated sound ethnobotanical uses of Kumkum, particularly leaves among the aboriginals of SBR forest. Leaf juice is used against snakebites among the tribal communities of Kukurvuka village of Ghatkumar range of SBR forest. Leaf juice is used to treat gonorrhoea among the **Lohar** community of Sanuski village of Gurguria range of SBR forest. Leaves paste is applied to cure skin infections among the **Kolhoo** and **Khadia** tribe of SBR forest. This plant was classified by the Food and drug Administration of the U.S.A. as a "Color additive exempt of certification" (Paula *et al.,* 2009). The qualitative analysis of bioactive compounds of the leaves of *Bixa orellana* L. in different organic solvents and in aqueous extract were excellent, In aqueous extract tannis, anthraquinone, flavonoids, saponin, terpenoids and steroids were presents (Table 2). Flavonoids are known to have anti-fungal activities (Havsteen, 1983) which justifies the tribal claim of use of leaf paste is used against skin infections. Tannins were present in aqueous and methanol extracts which is also reported to have anti-fungal activities (Doss *et al.,* 2009). Terpenoids were present in aqueous and acetone extract which is known to be good against cancer (Tsuyoshi *et al.,* 2010). Glycosides were present in methanol, acetone, hexane and ether extracts, which is effective for heart problems (Schoner and Scheines, 2007). Steroids were found only in aqueous extract (Table 2). The study revealed that the leaf of *Bixa orellana* L. is rich in bioactive compounds. In order to asses the toxicity of the extracts BSLA was carried out. The Brine shrimp assay indicated no death of *Artemia salina* L. by organic and aqueous extract of leaves of *Bixa orellana* L. (Table 1).

The results of present study revealed the phytochemical value of *Bixa orellana* L. Qualitative investigation of bioactive compounds of leaves indicates the presence of tannins, glycosides, anthraqunine, flavonoids and saponins. The above observations support the presence of bioactive compounds in *Bixa orellana* L. and justify the usefulness of this plant in the treatment of various diseases. In addition to prepare as medicine, it can also be safely used as a dye or coloring materials due to its

Table 1
Toxicity of different leaf extracts of *Bixa orellana* L. against *Artemia salina* L.

Sample 5µl/ ml	10:30 AM	11:30 AM	12:30 PM	1:30 PM	2:30 PM	After 24 h
Control	4+	4+	4+	4+	4+	4+
Hexane ext.	4+	4+	4+	4+	4+	4+
Ether ext.	4+	4+	4+	4+	4+	3+
Chloroform ext.	4+	4+	4+	4+	4+	4+
Acetone ext.	4+	4+	4+	4+	4+	4+
Meth. ext.	4+	4+	4+	4+	3+	3+
Aqueous ext.	4+	4+	4+	4+	4+	4+

(+4 indicates highly motile, +3 indicates motile, +2 indicates sluggish and +1 indicates slow, ext-extract)

Table 2
Variations of bioactive compounds in the different leaf extracts of *Bixa orellana* L.

Bioactive compounds	Aqueous extract	Methanol Extract	Acetone extract	Chloroform extract	Hexane extract	Ether extract
Tannins	+ve	+ve	- ve	- ve	- ve	- ve
Anthraquinone	+ ve	- ve	- ve	- ve	- ve	- ve
Flavonoids	+ ve	- ve	- ve	- ve	- ve	- ve
Saponin	+ ve	- ve	- ve	- ve	- ve	- ve
Phlobatanin	- ve	- ve	- ve	- ve	- ve	- ve
Terpenoids	+ ve	- ve	+ ve	- ve	- ve	- ve
Glycosides	- ve	+ ve	+ ve	- ve	+ ve	+ ve
Steroids	+ ve	- ve	- ve	- ve	- ve	- ve

(+ve – Present, -ve – Absent)

non-toxic effect. Further work can be carried out to evaluate the specific bioactive compounds against specific pathogens and successful use of these compounds to preparation of medicines.

Acknowledgement

Authors gratefully acknowledge to Field Director, Simlipal Biosphere Reserve forest; Dr. S. Bhatnagar and Ms. Susmita, Regional Plant Resource Centre with employees of Biotechnotrics Pvt. Ltd., Bhubneshwar for their valuable suggestion and provide lab facilities to complete this work.

References

Criel, R. J. and Macrae, H. T. (2002). *Artemia* morphology and structure. Kluwer Academic Publishers. The Netherlands.

Doss, A., Mubarack, H. M. and Dhanabalan, R. (2009). Pharmacological importance of *Solanum trilobatum*. Ind. J. Sci. Tech. 2(2): 41-43.

Fazly, B. S., Khajehkaramadin, M. and Shokooheizadeh, H. R. (2005). *In vitro* antibacterial activity of *Rheum ribes* extract obtained from various plant parts against clinical isolates of Gram-negative pathogens. Iranian J. Pharm. Res. 2: 87-91.

Harborne, J. B. (1973). Phytochemical Methods. Chapman and Hall, Ltd. London.

Havsteen, B. (1983). Flavonoids, a class of natural products of high pharmacological potency. Biochem. Pharmacol. 32: 1141–1148.

Krishnaraju, A. V., Rao, T. V. N., Sundararaju, D., Vanisree, M., Tsay, H. S. and Subbaraju, G.V. (2005). Assessment of bioactivity of Indian medicinal plants using brine

shrimp lethality assay. Int. J. Appl. Sci. Eng. 3(2): 125-134.

McLaughlin, J., Chang, C. and Smith, D. (1991). Proceedings of the 18th National Seminar and UNESCO Workshop on Natural Products. Institute of Advance Studies, University of Malaysia, Malaysia.

Metta, O., Arunsri, J. and Chanapong, R. (2009). Antibacterial effect of crude alcoholic and aqueous extracts of six medicinal plants against *Staphylococcus aureus* and *Escherichia coli.* J. Health Res. 23(3): 153-156.

Meyer, B. N., Ferrigni, N. R., Putnam, J. E., Jacobsen, L. B., Nichols, D. E. and Mclaughlin, J.L. (1982). Brine shrimp: A convenient general bioassay for active plant constituents. Planta. Med. 45: 31-34.

Okwu, D. E. (2001). Evaluation of the chemical composition of indigenous spices and flavouring Agents. Global J. Pure Appl. Sci. 7(3): 455- 459.

Paula, H., Pedrosa, M. L., Rossoni, J. V., Haraguchi, F. K., Santos, R. C. and Silva, M. E. (2009). Effect of an aqueous extract of annatto seeds on lipid profile and biochemical markers of renal and hepatic function in hipercholesterolemic rats. Brazilian Arch. Biol. Tech. 52(6): 1373-1378.

Schoner, W. and Scheiner, G. (2007). Endogenous and exogenous cardiac glycosides and their mechanisms of action. Amer. J. Cardiovasc. Drugs. 7(3): 173-89.

Trease, G. E. and Evans, W. C. (1989). Pharmacognsy. 11th ed. Brailliar Tiridel Can. Macmillian publishers. London.

Tsuyoshi, G., Nobuyuki, T., Shizuka, H. and Teruo, K. (2010).Various terpenoids derived from herbal and dietary plants function as PPAR modulators and regulate carbohydrate and lipid metabolism. PPAR Res. 1: 1-9.

Venugopalan, A., Giridhar, P. and Ravishankar, G. A. (2011). Food, ethnobotanical and diversified applications of *Bixa orellana* L.: a scope for its improvement through biotechnological mediation. Indian J. Fund. Appl. Life Sci. 1(4): 9-31.

World Health Organization (WHO). (1985). Chronicle. 39: 51-59.

Plant Science Research

ISSN 0972-8546

Distribution and composition of halophytes at Vainateyam estuary Andhra Pradesh

G.M. Narasimha Rao[Ψ], V. Lakshmi Narayana, and B. Niranjana Reddi

Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh - 530 003, India

ARTICLE INFO

Article history:
Received : 12 July 2011
Received in revised form : 25 August 2012
Accepted : 27 August 2012

Keywords:
Halophytes
Distribution
Vainateyam estuary
Andhra Pradesh

ABSTRACT

Halophytes are succulents which occur near estuarine and mangrove habitats only. These plants are commercially useful for food, fodder, extraction of antibiotics and bio-fuels. Halophytes of Vainateyam estuary was studied by using 1 X1 m quadrat; 6 study sites were selected in the distributaries of the estuary to collect the data on distribution and abundance of different plant species. A total 60 quadrat samples were collect in various parts of the estuary to get the information on frequency and abundance of halophytic populations. Species such as *Suaeda maritima, Suaeda monoica, Salicornia bracliiata* and *Sesuvium portulacastrum* were reported as dominant forms in this estuary while species such as *Suaeda nudiflora, Heliotropium curassavicum* and *Prosopis chilensis* were reported as rare species. Frequency and abundance were also minimum for these populations in this estuary. Halophytes were distributed along the creeks and drainage canals near the estuary.

Halophytes are transitional plants which occur in between true mangroves and terrestrial plants. They are capable of withstanding high salt concentrations and distributed along the estuaries, back water creeks. Now a days these halophytic populations are highly valuable and their use in bio-saline, agriculture as vegetables and extraction of bio-fuels. Ecology and distribution of halophytic populations along the mangroves and its associates was studied by Umamaheswara Rao and Narasimha Rao (1998), Baskara Rao *et al.* (1992), Venkanna and Narasimha Rao (1993), Naarsimha Rao and Dora (2009), Narasimha Rao and Subba Rangaiah (2010) and Narasimha Rao and Murthy (2010a,b). In the present study observations were collected on distribution and abundance of halophytic plant species in the estuarine region of Vainateyam river, one of the branches of river Godavari.

River Godavari bifurcated into two branches, Gauthami and Vashista. This Vashista branch of Godavari flows south east and divided into two branches namely Vashista and Vainateyam. Vainateyam branch of Godavari opens into Bay of Bengal at Karawaka near Gogannamatam. Mangroves and halophytic species are spreading either side of the estuary. Total six study sites were selected for collection of data. At each station 10 quadrat samples were collected and total 60 quadrat samples were collected to analyze the data on frequency and abundance of halophytes.

Information collected on density of the halophytes occurs in the six different stations of Vainetayam estuary was present in Table l. This branch of Godavari estuary was dominated by the halophytic vegetation. In station 1, *Prosopis chilensis* was having highest density (682 plants/ha) while *Suaeda monoica* showed minimum density (426 plants/ha) with height of 20-25 cm. In station 2, maximum density was reported for the species *Sesuvium portulacastrum* (572 plants/ha) and minimum density for *Arthrocnemum indicum.* Whereas in station 3, highest density (814 plants/ha) was reported for *Suaeda maritima* and minimum density (564 plants/ha) for *Suaeda nudiflora.* In station 4, *Salicornia bracliiata* was the dominating plant

[Ψ] *Corresponding author; E-mail:* gmnrao_algae@hotmail.com

Table: 1
Density of halophytes at various stations of Vainateyam estuary. Values presented are the mean dedensity/ha for each species

Nam of the Plant species	Station 1	Station 2	Station 3	Station 4	Station 5	Station 6
Arthrocnemum indicum	572	466	676	564	782	862
Sesuvium portnlacastrum	614	572	748	492	674	794
Suaeda maritime.	512	492	814	514	628	812
Snaeda monoica	426	556	696	412	814	874
Prosopis chilensis	682	476	582	516	424	762
Salicornia brachiata	636	542	620	584	592	864
Sueda nudiflora	580	496	564	424	618	824

Table: 2
Distribution of halophytes in the Vainateyam estuary

Name	Family
1. *Arthrocnemum indicum*	Chenopodiaceae
2. *Sesuvium portulacastrum*	Aizoaceae
3. Suaeda maritima	Chenopodiaceae
4. Suaed monoica	Chenopodiaceae
5. Prosopis chilens	Fabaceae
6. Salicornia brachiata	Chenopodiaceae
7. Suaeda nudiflora	Chenopodiaceae

than of other species. Species such as *Suaeda monoica* and *Arthrocnemum indicum* was the dominant forms in station 5 and 6 (Table 1). In the present study, seven halophytic species were reported with different densities. These seven species belongs to Chenopodiaceae, Aizoaceae and Fabaceae (Table 2). Plants are distributed very near to water edges where there are no true and associated mangroves. In station 3 and 4 halophytes are present away from the water edge after 30 to 40 meters. Estuarine regions are daily inundated by the high tides of sea. Accumulation of salt content in estuarine soils may be responsible for the distribution of halophytes in estuarine habitat. Composition of soil i.e silt, clay and sand contents also influence the growth, development and distribution of mangrove species and halophytes. Present study agrees with the earlier investigations of Bhaskara Rao *et al.* (1992), Narsimha Rao and Dora (2009) and Narsimha Rao and Murthy (2010 b).

References

Bhaskara Rao, V., Narasimha Rao, G.M., Sarma, G.V.S. and Krishna Rao, B. (1992). Mangrove environment and its sediment characters in Godavari estuary east coast of India. Indian. J. Mar. Sci. 21: 64-66.

Narasimha Rao, G. M. and Murthy, P. P. (2010a). Mangrove populations of Vamsadhara estuary. Int. J. Plant Sci. 5: 698-699.

Narasimha Rao, G. M. and Murthy, P. P. (2010b). Mangroves and associated flora of Vashista and Vainateyam esturies, Andhra Pradesh, India. J. Not. Sci. Biol. 2(4): 40-43.

Narasimha Rao, G. M. and Subba Rangaiah, G. (2010). Distribution of Mangroves and associated flora of the pandi Back waters of guatami Godavari estuary. ANU. J. Nat. Sci. 1.

Narasimha Rao, G. M. and Dora, S. V. V. S. N. (2009). An experimental approach to Mangroves of Godavari estuary. Indian J. of Forestry. 32(3): 263-265.

Umamaheswara Rao, M. and Narasimha Rao, G. M. (1988). Mangrove populations of the Godavari delta complex. Indian J. Mar. Sci. 17: 326-329.

Venkanna, P. and Narasimha Rao, G. M. (1993). Distribution pattern of the mangroves in Krishna estuary. Indian J. Forestry, 16: 48-53.