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Unifying features of a high lipid accumulating marine microalga (*Chlorella* sp.) collected from south coast of Odisha and the effects of nitrate on its growth and lipid profile

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ABSTRACT

The study was undertaken to isolate and screen microalgae from South coast of Odisha and further see the effects of nitrate on growth and lipid accumulating ability of a selected green alga. In all ten isolates were pure cultured, which include both cyanobacteria and a green alga. An identified *Chlorella* sp. was found to accumulate high amount of neutral lipids in its stationary phase of growth and subsequently the effects of nitrate on its growth and lipid profile was carried out. Growth was retarded in cultures without nitrate followed by reduced biomass productivity and specific growth rate. Photosynthetic pigments like chlorophylls and carotenoids were less synthesized in nitrate deprived growth medium. Complete lipid profile analysis under normal growth conditions showed that the alga contained maximum PUFA (49.1 %) followed by saturated fatty acids (32 %) and MUFA (18.9 %). Under deprived N condition the occurrence of PUFA (43.6 %) was reduced while saturated fatty acids (36.1 %) and MUFA (20.3 %) increased to 12.8 % and 7.4 %, respectively. It is necessary to grow the organism both in nutrient optimum or deprived condition so that the alga can be used as a source of high nutraceutical compounds or biofuel.

1. Introduction

Microalgae include the prokaryotic or eukaryotic group of photosynthetic organisms which like land plants capture the solar radiation and could fix CO_2 to biomass. They are present over a wide range of temperature, pH and salinity and found to be present in all type of ecosystems (Falkowski and Raven, 1997). They can modify their physiological and biochemical response according to the changes in the environmental parameters which enable them to inhabit in almost all climatic conditions on earth. They are mostly found as a member in fresh water, marine or brackish water environments (Richmond, 2004).

Their growth requirement is very simple which includes a light source e.g. solar energy or artificial illumination system, water and some mineral nutrients. Nutrients, may be in the form of inorganic or organic compounds, are used for growth apart from CO_2 and water required for cellular functions (Neenan *et al*., 1986). These include macro as well as micro elements and the requirement is different from species to species in regard of one or a few of them. Nitrogen is the most vital element after carbon as a constituent of cellular molecules like protein, nucleic acids and amino acids. Algae take it either in the form of NO_3^-, NH_4 or $(NH_2)_2CO$ (urea). The most used N source for the culture of microalgae is nitrate (NO_3^-).

During favourable conditions of growth the microalgae grow rapidly and accumulate large biomass. They contain very high amount of nutrients, especially protein, carbohydrates and lipids. Under optimal growth, the relative content of the various nutrients is fairly similar among species (Hu, 2004). When the nutrient, salinity, temperature and light quality and quantity are not supporting the growth they can modify their metabolism to strive through the harsh conditions. Many algae follow different adaptation strategies

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to survive adverse environmental conditions in different habitats (Seckbach, 2007; Barsanti *et al*., 2008).

Mainly microalgae change the lipid metabolism to unusual pattern under environmental variations and hence able to survive and proliferate under stress (Hu *et al*., 2008). Under unfavourable growth they modify the lipid biosynthetic pathway towards neutral lipids (20-50% of DCW) mostly triacylglycerol (TAG) in nature. In comparision to the algae, cyanobacterial lipids mostly contain diacylglycerol (DAG).

In response to nitrogen limitation, accumulation of lipids, particularly TAG has been observed as a regular strategy in numerous species of various algae (Thompson, 1996; Merzlyak *et al*., 2007). Cyanobacteria are also topic of research and hence subjected to screening for lipid production (Basova, 2005). In contrast to eukaryotic algae, considerable amounts of total lipids have not been found in cyanophycean organisms examined in the laboratory. The accumulation of neutral lipids as triacylglycerols has not been observed in naturally occurring cyanobacteria.

South coast of Odisha harbours many forms of microalgae due to the presence of varied environments such as Chilka Lake, Huma salt pan, Bay of Bengal etc. Chilka is the largest brackish water lagoon of Asia situated at the East coast of India (19°28'2" and 19°54'2" N latitude and 85°06'2" and 85°35'2" E longitude).On one side it is connected with Bay of Bengal and on the other side many rivers like Daya, Bharghavi and Luna fall into it. So it is a habitat for both fresh and brackish water microalgae, due to mixing of sea water with fresh water at many places along the coast. Seasonal fluctuations in the physico-chemical parameters of water along the South coast promote the growth of many species of algae in different seasons.

A random collection of samples from all these environments of South coast of Odisha tempted us to focus on a few of which one was found having high lipid accumulating capacity vis-à-vis variable nitrogen concentrations. The complete lipid profile due to unavailability of nitrate in the culture medium in comparison with the normal growth condition was worked out simultaneously.

2. Materials and methods

2.1 Selection of study sites

South coast of Odisha was chosen as the study site and algal samples were collected during three season viz. winter, summer and rainy for one year from October 2013 to October 2014 taking dry and wet soil, water samples and sediments from Kaluparaghat, Balugaon, Huma salt pan and sea shore of Gopalpur while sampling both planktonic and benthic algal organisms were screened out from the aquatic environments using mess size and macro-forms were rejected at the site. The salinity and temperature of the water was measured using a salinity meter and a thermometer, respectively. The samples were kept in sterile zipper bags and taken to the laboratory. Water (50lit each) from each site was collected for cultivation of the organisms.

2.2 Pre-treatment of the samples

The soiled samples were washed in tap water thoroughly overnight to remove the impurities and again washed along with other samples repeatedly in distilled water. All the samples were serially diluted and used for isolation directly.

2.3 Preparation of culture medium and pure culture

To isolate the organisms BG-11 and nitrogen free BG-11 medium as per composition provided by Rippka *et al.* (1979) along with f/2 medium as per Guillard (1973) was used. The medium was prepared in water collected from the sampling site (autoclaved and kept standing in dark for 24hrs). After sterilisation, the liquid mediums in 250 ml Erlenmeyer flasks were kept for cooling. Solid agar plates were prepared using 1.5% algae culture agar (Hi-Media) in the BG-11, N_2 free BG-11 and f/2 liquid medium.

The water samples were transferred to agar plates using spread plate technique. The soil and sediment samples were transferred to the BG-11and N_2 free BG-11 agar plates. All the plates were then incubated at 28 ± 2^0 C and 250 µmol m^{-2} s^{-1} light intensity using white fluorescent tubes in a culture room. After 7 days all plates and flasks were checked for the growth and appearance of any organisms. The difference in any colour, texture in the coloured patches was marked and again subcultured in fresh plates. After sufficient growth, it was again sub cultured in fresh plates. The pure cultured organisms were observed under an inverted microscope and colonies were subsequently transferred to fresh, sterile liquid medium and incubated under above-mentioned conditions. With every subculture, the organisms were checked under a microscope for purity and transferred to fresh medium.

2.4 Maintenance of pure cultures

All the pure isolates were maintained in the laboratory at 28 ± 2^0 C and 250 µmol $m^{-2}s^{-1}$ light intensity using white fluorescent tubes with continuous illumination. All the flasks were manually shaken twice daily for aeration and to avoid settling down of the organisms at the bottom.

2.5 Selection of the experimental isolate

2.5.1 Morphological identification

The morphology of all the isolates was examined under normal phase contrast microscope using 40X objective. Micrographs were taken using Leica software. The fluorescence property of chlorophyll molecule was used to get a clear picture of the isolates. All the pure isolates were observed under 20X objective in TCS SP5 confocal microscope exciting with a blue light.

2.5.2 Spectral characteristics

Absorption spectra (360-800 nm) of the whole suspension were recorded using carry 300 Bio spectrophotometer at room temperature. The isolates were found to give different wavelength dependent electronic transitions referring to varied pigment types. The absorbance peak at specific wavelengths varied for cyanobacteria and algae, which was used to single out the cyanobacterial or green algal species.

2.5.3 Effects of nitrate on the experimental algae

Out of all only the selected alga proved to be a *Chlorella* sp. JD-2016 as released by NCBI (KU497645) was grown in 250ml Erlenmeyer flask using complete f/2 medium and nitrate deprived f/2 medium under laboratory conditions with 16:8 L: D photoperiod, 100 µmol/m^2S light intensity (white fluorescent bars) and at 24±2 °C. The organism cultured previously by Dash *et al*. (2016) exhibited growth curve in which the exponential growth phase was appeared between 6 to 12 days of culture. Accordingly the dry weight was estimated on 5, 8 and 13 days which fall in the linear region of the growth curve. The photosynthetic pigments concentrations were determined at different phases of the growth curve.

2.5.4 Growth and photosynthetic pigments under altered nitrogen doses

For dry weight determination of the algal biomass, 10 ml algal culture were harvested, washed and filtered through pre-weighed Whatman GF/C filters (0.45 µm) and dried at 80^0C till constant weight was obtained. Specific growth rate and biomass productivity was calculated from the data according to the equation; $K' = Ln (N_2/N_1)/(t_2-t_1)$ where N_1 and N_2 are biomass at time (t_1) and (t_2), respectively (Levasseur, 1993).

The pigment concentration (Chl-a, Chl–b and carotenoids) of the alga in the methanolic extract was determined following Porra *et al*. (1989) and Lichtenthaler (1987). The methanolic extract of the pigments was achieved by percolation method. In order to percolate the pigments, 1ml algal culture was centrifuged at 8000 g for 10 min. The pellet was washed with distilled water and incubated with 2 ml of absolute methanol keeping at - 4° C for 48-h in dark, by which time the extraction of the pigment was completed leaving behind a colourless pellet. The respective absorption peak values for carotenoids (470nm), Chl-b (652 nm) and Chl-a (665nm) in the methanolic extract were corrected for turbidity, if any, by subtracting the values obtained at 750nm.

2.5.5 Extraction of lipids and analysis of Fatty Acid Methyl Esters (FAME) profile

The *Chlorella* sp. grown in culture medium with optimum nitrate (0.082 mM) and without nitrate (0 mM) were further investigated to compare the effects of nitrate on the lipid profiles of the alga. Algal cells were harvested till early stationary phase of growth. The cells on the 14 days of culture were taken, centrifuged and lyophilized. Dry algal biomass (0.2 g) treated with 2% H_2SO_4 in methanol (5 ml) at reflux temperature (65° C) for 4 h was used for the conversion of lipid component present in the biomass to its fatty acid methyl esters. The reaction mixture was extracted with ethyl acetate (3 x 15 ml) and the extract was washed with water. The organic layer was dried over anhydrous sodium sulphate and concentrated to get the fatty acid methyl ester (FAME) mixture.

The FAME mixture was dissolved in minimum amount of chloroform and analysed by GC for fatty acid composition. GC was carried out with Agilent 6890 N-Series gas chromatograph equipped with a FID detector. The GC was performed using DB-225 capillary column (30 m × 0.25 mm × 0.25 µm) and the oven temperature was programmed for 2 min at 160 °C, raised to 230 °C at 5 °C/min and finally maintained at 230° C for 20 min. The carrier gas, N_2 flew at 1.0 ml / min maintaining the split ratio at 50:1.

3. Results

3.1 Isolation and maintenance of pure culture

The water temperature was almost equal everywhere with little variation (22 ± 5° C) in the predetermined sites. However, the salinity differed to a large extent. Gopalpur estuary had recorded maximum salinity of (35 ppt) as compared to water of Kaluparaghat, Balugaon, and Huma, which had salinity range 20-25 ppt.

After repeated culture and subculture, total 10 numbers of pure cultures were isolated. Isolates were named as S-1 to S-10 (S being abbreviated for sample).

Upon microscopic observation it was found that except two microalgal isolates (S-8 and S-9) all others were filamentous algae of varied lengths. All were blue green or green in colour while isolates S-1, S-3, S-4, S-6, S-7 and S-10 were found to belong to different species of *Oscillatoria*,

S-2 and S-5 to be *Phormidium* sp. and S-8 was a unicellular microalga. On the otherhand, S-9 with very small filaments, under the confocal microscope appeared in diad and tetrad (Fig. 1a and Fig. 1b).

3.2 Screening of the isolates

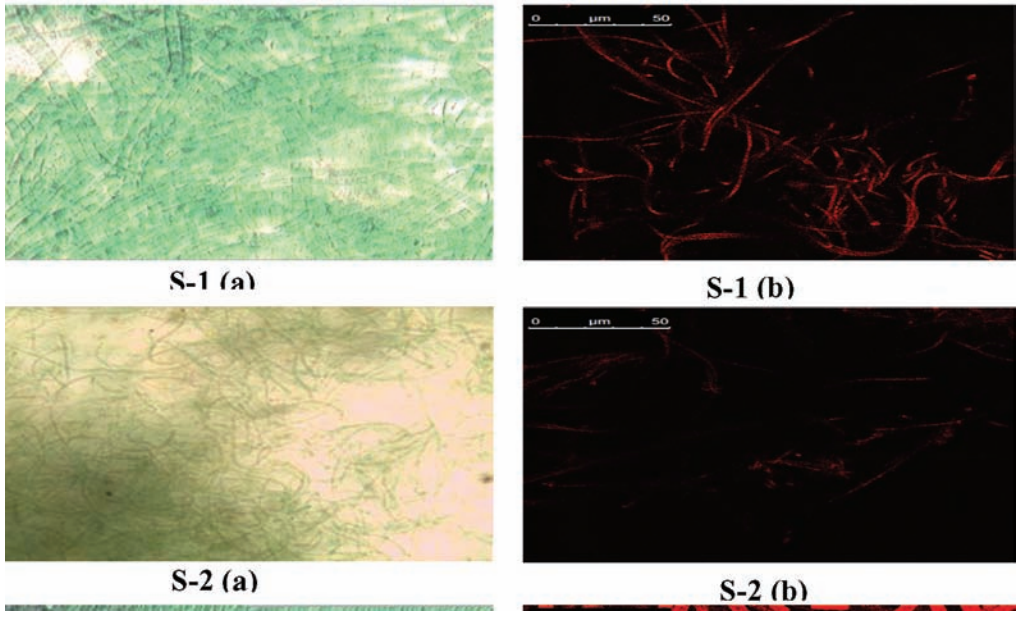
Taking the whole cell absorbance by scanning the cell suspensions of all the isolates were studied in a UV-VIS spectrophotometer from wavelength 360-800 nm. The absorbance peak at specific wavelength varies for cyanobacteria and algae, which was used to single out the cyanobacterial or green algal species. The cyanobacteria and algae, as expected, gave different wavelength dependent electronic transitions referring to the presence of various pigment types. All the isolates, except one, showed characteristic signature of electronic transition for cyanobacteria having a prominent transition peak at 620 nm due to phycocyanin pigments. Isolate S-9 which was apparently viewed as rods in diad and tetrad form under a confocal microscope, was confirmed to be cyanobacteria from its spectral characteristics. Only a single isolate (S-8) exhibited the presence of electronic transition for Chl-b at 652nm (Fig. 2 A) referring to a green alga.

3.3 Effect of nitrate on growth, chlorophyll and lipid profile of the Chlorella sp.

The biomass productivity per day of the alga declined in the cultures with low nitrate. The cell dry weight was lower in the nitrate deprived medium (301 mg/l) as compared to the optimum (610 mg/l) medium. So it is evident that growth in the limited supply of nitrate is reduced as compared to the optimum nitrate. Similarly, the cell division per day has decreased and doubling time has increased suggesting reduced metabolism. The specific growth rate of the alga in optimum nitrate culture medium was higher in comparison to the zero nitrate condition. Similar effects were observed on the synthesis of chlorophyll due to nitrogen deprivation in the culture medium. The synthesis of the chlorophyll per ml of the culture was affected by the absence of nitrate in the medium (Table 2). The chlorophyll synthesis was always more in higher nitrate concentrations. At a particular day of growth the C_a/C_b and total chlorophyll to carotenoids ratio were almost constant. But when the cultures were compared in the stationary phase (24 day), it is noticed that the chl / carotenoids ratio decreased as compared to exponential (12 day) and early stationary phase (18 day). This suggests that the low nitrate in the medium is not only the stressor but the cultures with ageing also exhibit stress which is indicated by the low ratio of total chlorophylls to carotenoids

To further know the normal lipid profile of the alga and if any change upon omitting N from its culture medium is there, FAME were analyzed using gas chromatography with a flame ionizing detector (FID). The chromatogram is also provided that contains peaks for various fatty acids given by their area (Fig. 3 A, B; Fig. 4 A, B) The occurrence of saturated, monounsaturated and polyunsaturated fatty acids (%) upon deprivation and supplementation of nitrate in the culture mediums are tabulated (Table 3).

As it can be seen from the table that under normal growth conditions (optimum nitrate in culture), the algae contain maximum PUFA (49.1 %) followed by saturated fatty acids (32 %) and MUFA (18.9 %). When nitrate is not supplied in the culture medium i.e. under deprived N condition the occurrence of PUFA (43.6 %) has reduced while saturated fatty acids (36.1 %) and MUFA (20.3 %) increased to 12.8 % and 7.4 % respectively.



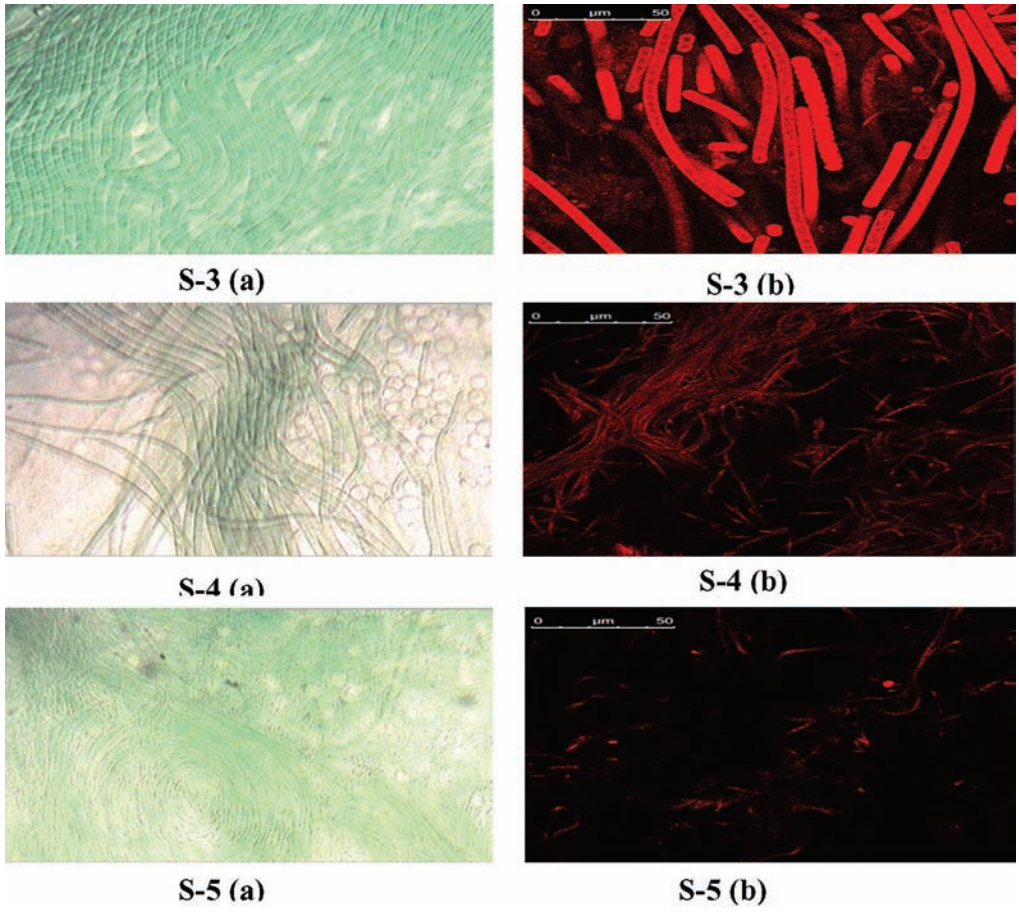
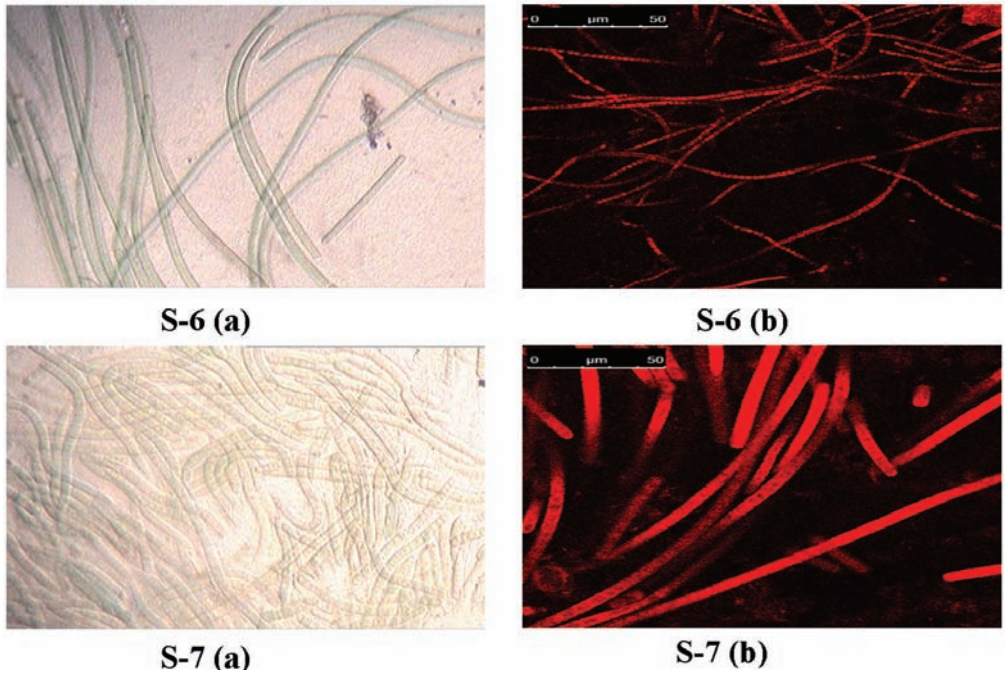


Fig.1a. Phase contrast (a) and confocal (b) micrograph of the isolates



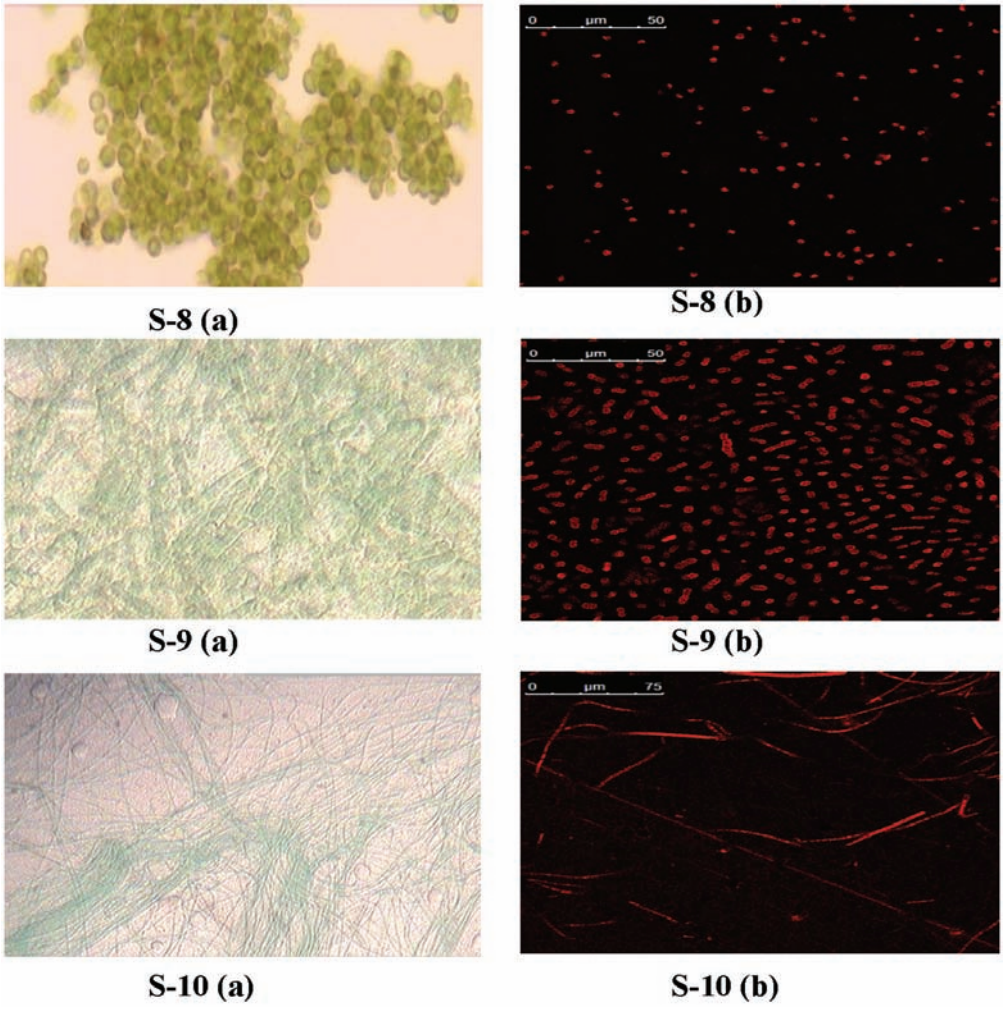
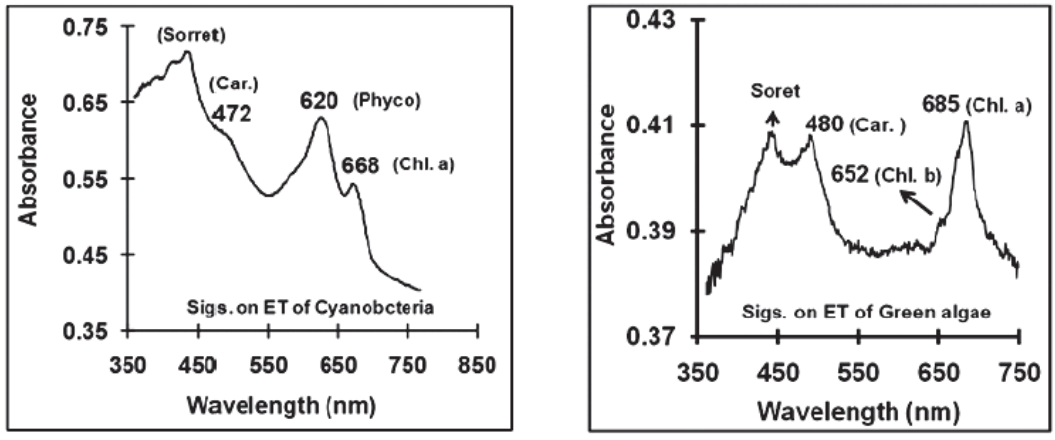


Fig.1b. Phase contrast (a) and confocal (b) micrograph of the isolates



Spectral analysis of the isolates

Fig. 2. Screening of green alga using spectral characteristics of the isolates

Table 1

The effects of nitrate on growth and biomass productivity of the *Chlorella* species

Concentration of Na NO_3	Dry weight (mg/l)	Biomass Productivity mg /l/day	Specific growth rate μ/day	Division/day	Generation/ doubling time
0	301±20	23	0.063	0.09	10.92
0.882	610±66	46.9	0.155	0.22	4.45

Values represent mean ± SE and results were significant at $P< 0.01$

Table 2

Change in pigment concentrations of the *Chlorella* sp. due to absence of nitrate in the medium.

Concentration $NaNO_3$	12 days			18 days			24 days		
	$C_a+ C_b$ (μg/ml)	*Car* (μg/ml)	*C*/*Car*	$C_a+ C_b$ (μg/ml)	*Car* (μg/ml)	*C*/*Car*	$C_a+ C_b$ (μg/ml)	*Car* (μg/ml)	*C*/*Car*
0	0.132	0.088	1.5	0.111	0.109	1.018	0.094	0.116	0.810
0.8	2.224	0.836	2.66	1.799	1.078	1.687	1.415	1.071	1.321

Values represent mean of three replications and results were significant at $P< 0.001$

Table 3

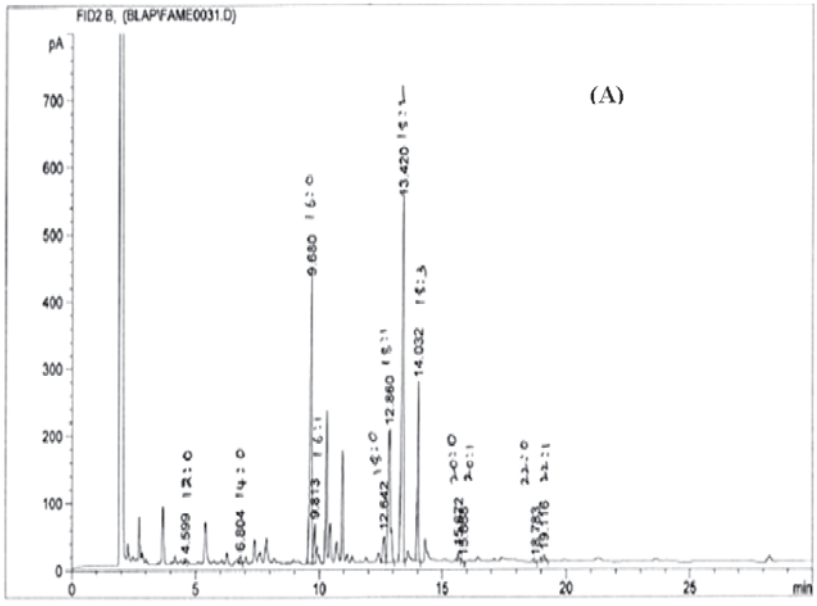
Fatty acids profile of *Chlorella* sp. JD-2016 under normal and nitrates stress conditions.

	TYPES	% FATTYA CID	
		+ N	- N
Saturated	12:0	0.3	0.7
	14:0	0.6	0.9
	16:0	27.7	28.2
	18:0	2.6	4.6
	20:0	0.7	1.5
	22:0	0.1	0.2
		32	36.1 (12.8 % more)
Monounsatureted (MUFA)	16:1	2.5	1.5
	18:1	15.6	18.6
	20:1	0.1	0.1
	22:1	0.7	0.1
		18.9	20.3 (7.4 % more)
Polyunsaturated (PUFA)	18:2	36.8	35.4
	18:3	12.3	8.2
		49.1	43.6 (11.2 % less)

4. Discussion

Out of the selected ten isolates in this study based on morphological features and absorption spectroscopy of the whole organisms, only one eukaryotic alga (isolate S-8) was screened out among all other cyanobacteria. Absorption spectra of algae provide the information of different types of pigments and their concentrations. Such useful information in photosynthesis is also used for phylogenetic and taxonomic purposes (Govindjee and Braun, 1974). The same analogy was used here with absorption spectroscopy to differentiate between prokaryotic cyanobacteria and the singled out eukaryotic alga.

While the cyanobacterial group of algae under stress condition could produce large amount polar lipids like diacylglycerols (DAG), in eukaryotic algae these are stored as triacylglycerols (TAG). Saha *et al.* (2003) reported reduced synthesis of lipids and fatty acids in *Oscillatoria willie* BDU 130511 under nitrogen starvation.

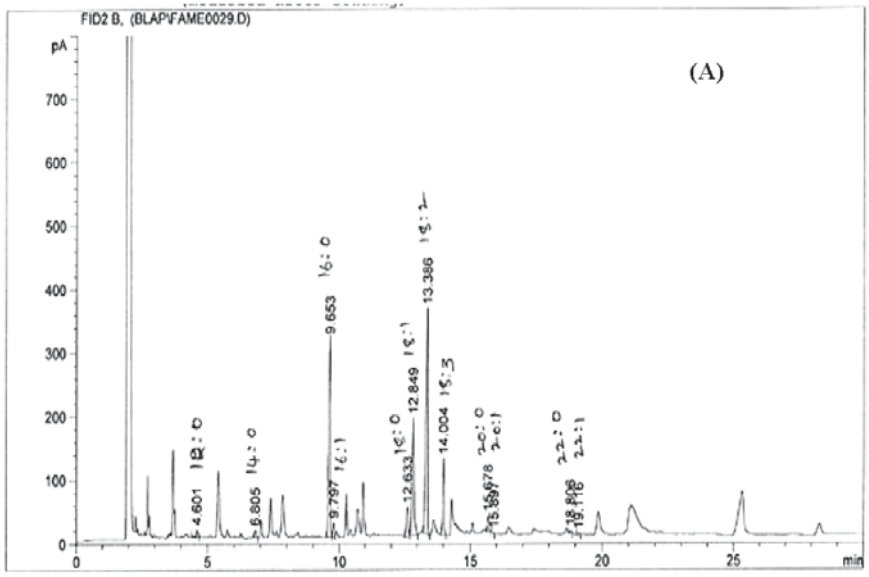


(B)

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	4.599	MM R	0.0502	17.71000	5.88512	0.21190
2	6.804	BB	0.0633	46.95006	11.57809	0.56176
3	9.680	BV	0.0767	2331.54858	420.39017	27.89707
4	9.813	VV	0.0555	218.31042	58.57164	2.61209
5	12.642	VV	0.0840	216.72295	39.49641	2.59310
6	12.860	VB	0.1015	1302.51563	195.99301	15.58465
7	13.420	BP	0.0789	3068.70679	535.19983	36.71719
8	14.032	BP	0.0611	1028.31396	266.21429	12.30382
9	15.672	PB	0.0597	59.71416	15.23206	0.71448
10	15.868	MM R	0.0895	2.29509	7.43828e-1	0.03134
11	18.780	MM R	0.0600	3.97759	1.10419	0.05431
12	19.122	PB	0.0964	59.94469	9.15633	0.81845
Totals :				7324.19105	1460.08929	

Fig. 3. Chromatogram obtained for fatty acids profile of *Chlorella* sp. JD-2016 grown in optimum nitrogen medium (A) and the area percent report (B).

After growing the *Chlorella* sp. JD-2016 in nitrate plus and minus media it was found that, growth was reduced in the nitrate deprived medium heavily with low specific growth rate, biomass productivity, fewer divisions per day besides a prolonged generation time followed by nitrate starvation. Similar observations under nitrogen starvation experiments were made by Illman *et al.* (2000) and Solovchenko *et al.* (2008) in their respective studies. In response to the lack of nitrogen in microalgae almost complete cell proliferation inhibition was reported (Phadwal and Singh, 2003; Cakmak *et al.,* 2012). Nitrogen is required by photosynthetic organisms to synthesize proteins, nucleic acids, and chlorophylls, among other important cell molecules. The estimated chlorophyll levels were lower in N-starved cells. This is a commonly observed phenomenon in N-starved algal cell (Berges *et al.,* 1996). The limiting nitrogen in N-starved cultures might affect the cells' ability to synthesize amino acids like glycine and glutamate, limiting the synthesis of 5-aminolevulinic acid, a precursor of chlorophyll that in turn leads to lower chlorophyll levels in the algal cells (Ellis *et al.,* 1975). Similar to chlorophyll, carotenoid levels were also found lower in *Chlorella* sp. cultured in lower nitrate, suggesting that the availability of nitrogen affects the level of carotenoids in the cells. This finding corroborates similar other reports of lower carotenoids levels under nitrogen limitation (Li *et al.,* 2012; Kim *et al.,* 2013). However, the chlorophyll / carotenoids ratio was lower in the N-starved cultures and is consistent with most of the nitrogen limitation studies with algae (Berges *et al.,* 1996).The decrease in carotenoid level may be due to the limiting step of dimerization of geranyl-pyrophosphate as suggested by Richmond (1986). Kim *et al.* (2013) clearly showed that higher light intensities accompanied by N limitation usually lead to higher carotenoid levels, whereas lower light intensitithis experiment (100 µmol/m^{2}S) was not high enough to induce higher photo-



Signal 1: FID2 B, (B)

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	4.601	BB	0.0533	39.62854	10.68496	0.75347
2	6.805	BB	0.0627	47.48923	11.38678	0.90293
3	9.653	BV	0.0692	1476.43396	311.82153	28.07197
4	9.797	VV	0.0555	80.84467	22.71822	1.53713
5	12.633	PV	0.0786	240.87051	44.88251	4.57976
6	12.849	VP	0.0843	979.87903	183.35698	18.63079
7	13.386	BP	0.0714	1862.43213	353.94131	35.41109
8	14.004	BB	0.0537	428.41840	119.91370	8.14567
9	15.678	MM R	0.0564	82.20908	24.28224	1.56307
10	15.897	MM R	0.0413	3.48730	1.40784	0.06631
11	18.806	MM R	0.0788	12.89614	3.44990	0.24520
12	19.116	MM R	0.0879	4.87054	1.19403	0.09261
Totals :				5259.45952	1089.04001	

Fig. 4. Chromatogram obtained for fatty acids profile of *Chlorella* sp. JD-2016 grown in nitrogen deprived medium (A) and the area percent report (B).

protective carotenoid production (Couso *et al.,* 2012). The observed decrease in photosynthetic pigments in N- starved cells as compared to normal cells growing in nitrate supply, clearly indicates the importance of nitrogen for chlorophyll and carotenoid synthesis.

There are reports that nitrogen deprivation is the best strategy for neutral lipid induction in microalgae. In case of the tested *Chlorella* sp., it is already reported using NR that large amount of neutral lipids are accumulated during the stationary growth phase (Dash *et al*., 2016). In order to study the changes in the lipid composition of the organism under normal and under stressful conditions, the FAME analysis was performed using Gas chromatography. Although the lipid content of algal species remains congruous if grown under the similar set of conditions, the lipid profile which generally remains constant in a species changes under limiting growth conditions.

Fatty acids are long aliphatic carbon chains that vary in length, degree of unsaturation, and structure. The quantitative analysis of the lipids through determination of FAME profiles revealed that 70 % of the fatty acids belonged to C16–C18 type with USFA/SFA ratio (2:1) being in the range of 1.9–2.3% in the strains. N limitation exhibited a differential effect, it was clearly noted that during the nitrate starvation in the medium the saturated fatty acids (SFA) were many folded. The overall increase in SFA was around 13 %. The abundance of saturated fatty acids is of extreme significance for consideration as a source of biodiesel, because such oils have higher cetane number (CN), decreased NOx emissions, a shorter ignition delay time and oxidative stability (Antolin *et al.,* 2002).

Polyunsaturated fatty acids (PUFA) are of the utmost importance like antibacterial, anti-inflammatory, antioxidant, prevention of cardiac diseases and inhibition of tumor

progression. Such properties are indicative of the potential of PUFA for nutraceutical and pharmaceutical purpose (Pereira *et al.,* 2012).

Under normal growth conditions the tested *Chlorella* sp. was found to contain more of unsaturated fatty acids (68%) as compared to saturated fatty acids (32 %). The relative amounts of SFA (25% -38%) and the PUFA (37%–64%) values were found within reported value in the literature (Li *et al.,* 2002). Among all the fatty acids most abundant was linoleic acid (36.8 %) followed by palmitic acid (27.7 %) and oleic acid (15.6 %). This was in agreement with results obtained by Sahu *et al.,* 2013 in Chlorophyceae who examined 12 different strains of microalgae. Linoleic acid (LA; C18:2*n*-6) was the main PUFA of most chlorophytes. The only exception is *Ulva* sp., in which higher percentages of ALA (16%) were detected, in comparison to LA (5.7%) (Pereira *et al.,* 2012). In Chlorophyceaen members, the prominent fatty acids like C16:0, C18:0, C18:1n9c and C18:2n6 were found similar to the earlier report of Lee *et al.* (2010).

The deficiency in nitrogen did not bring significant difference in the levels of linoleic acid and palmitic acid suggesting that the tested alga can be used as a regular source of these valuable fatty acids. The alga in its optimum growth and metabolism produces very high amount of 49.1 % PUFA in which upon change of nutrient (nitrate) the internal adjustment of lipids brought about uniform decrease in PUFA and same quantity of increase in SFA. This could be a very good strategy to use this alga both the ways i.e. grow normally to use for high value metabolites (MUFA, PUFA, pigments, antioxidants such as carotenoids and phenolics) and impose nitrate stress to synthesize more of SFA to be exploited for biofuel purpose.

5. Conclusion

Microalgae grown in any habitat like south coast of Odisha are potent with so many biotechnological applications some of which are presently unexplored to a large extent. Lipid profile of the tested alga i.e. *Chlorella* sp. is greatly influenced by the change in nitrate concentration. The nutrient manipulation experiment was used as a progressive starvation strategy, instead of first growing it in N- rich medium and then imparting stress, which is generally adopted for lipid induction in case of algae. The lipid profile of the tested alga is very rich in PUFA which is also valuable nutraceutical, as well as can act as antioxidants. Depriving nitrate completely from the medium results in a double fold increase in the quantity of saturated fatty acids, which suggests that this alga could be a promising candidate to be used as biofuel.

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Soil microbial enzyme activities in different age series sponge iron solid waste dumps with respect to reclamation

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ABSTRACT

Soil enzymes are primarily derived from over all microbial population and activities of microorganism. Study of soil enzymatic activities provide an insight in to the microbial biotransformation process and soil enzymes could be used as the potential biochemical indicators of soil quality. In the present investigation soil microbial enzymatic activities of dehydrogrogenase, amylase, invertase, urease, protease and phosphtase were measured in different age series i.e. 0, 1, 3 and 5 years old sponge iron solid waste dumps to determine probable biotransformation of the waste. The study indicated that the soil microbial enzymatic activities were not detected in fresh dump (0 year old). But, there was an increasing trend in soil microbial enzymatic activities from 1 year to 5 year old waste dumps. The comparative analysis of the soil microbial enzymatic activities in different age series sponge iron solid waste dumps reflects the gradual improvement of the functionality in the older dumps.

1. Introduction

Land degradation due to the alteration and destruction of terrestrial habitat is a major environmental concern throughout the tropics and one of the major factors responsible for such degradation is rapid industrialization and spread of mining activity (Ezeaku and Davidson, 2008). Sponge iron or direct reduced iron (DRI) is mostly used for steel making through secondary sector and India continues to be the largest producer of coal based sponge iron in world (Dey *et al.*, 2016). The availability of principal raw materials like high grade iron ore and non-coking coal have created a favorable atmosphere for development of sponge iron industry in the central eastern belt of India including the states of Odisha, West Bengal, Jharkhand and Chhattisgarh (Patra *et al.,* 2008). From the coal based sponge iron industry huge amount of solid waste is generated in form of char, dust and accretion material (CPCB, 2007). Majority of the solid wastes are dumped on land which creates large areas of black calcareous derelict land that apart from reducing the productivity, also reduce aesthetic value of the region (Roy *et al*., 2002). In developing country like India, reclamation of such derelict land is an urgent necessity for restoration of the self sustaining capacity of the ecosystem and its delicate equilibrium.

Soil microorganisms and their activities contribute a wide range of essential services to the sustainable functioning of ecosystem (Langer and Mubarack, 2007). They are the driving force behind nutrient transformations and thus make an essential contribution to soil fertility and ecosystem functioning (Smith and Paul 1990; Shentu *et al.,* 2008). Thus, the soil microbiological parameters are considered as a sensitive indicator of soil quality, ecological stress and restoration process (Pascual *et al.*, 2000; Filip, 2002; Bending *et al.*, 2004).

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Soil enzymes are essential for catalyzing reactions necessary for organic matter decomposition and its mobilization to different soil strata (Pavel *et al.*, 2004; Shi *et al.*, 2006) and are believed to be primarily originated from soil microbes (Ladd, 1978). They are usually associated with viable proliferating cells, but enzymes can be excreted from a living cell or be released into soil solution from dead cells (Tabatabai, 1994). The soil enzyme activities have often been used as indices of microbial activity and soil fertility (Kennedy and Papendick, 1995) because they are strictly related to nutrient transformations, they rapidly respond to the changes in soil properties and it is easy to measure their activities (Gianfreda and Bollag, 1996; Nannipieri *et al.*, 2002). Therefore in present study attempt is being made to study the soil microbial enzymatic activities in different age series sponge iron solid waste dumps with respect to reclamation.

2. Materials and methods

2.1 Study site

The study was carried out in solid waste dumping site of Scans Steels limited, Sundargrah, Odisha. Geographical location of the area is between 20º11' North Latitude and 84º19' East Longitude. Altitude of the area is about 213m above the mean sea level. The area experiences tropical climate with three distinct seasons i.e. summer, rainy and winter. The mean annual rainfall in the area is 1422mm and mean air temperature of the area varies from 10ºC to 45ºC. The relative humidity fluctuates from minimum of 40% to maximum of 83%. In the sponge iron solid waste dumping site, accumulation of solid waste over years resulted in formation of different age series of dumps. Dump age is expressed as time since the establishment of dump in the site. For the present study freshly laid dump (D_0), 1 year (D_1), 3 year (D_3) and 5 year (D_5) old dumps were selected. During dumping of the solid waste, when the dump attains sufficient height, soil of the adjacent area is covered over the dump for stabilization. Thus, D_1, D_3 and D_5 were with soil cover, where as D0 was without soil cover. A natural site adjacent to the waste dumping site was been taken as control site (C) for reference.

2.2 Sample collection

Sampling was done in accordance with the general methods for soil microbiological study (Parkinson *et al.,* 1971). Waste samples from different age series dump (D_0, D_1, D_3 & D_5) and soil from control site were collected by random sampling method from a depth of 0-15cm by digging pits (15 X 15 X 15 cm). For each site, waste sub-samples were collected from five locations. These sub- samples were brought to the laboratory in sterilized polythene bags and mixed thoroughly to form a composite sample. After sorting out larger pieces of materials, each of the samples was divided into three replicates for analysis.

2.3 Enumeration of microbial population

Microbial population of different age series waste dumps and control site was enumerated in terms of total bacterial and fungal colony forming units (CFUs), following the serial dilution plating technique (Parkinson *et al.,* 1971). For bacterial count diluted suspension was spread over Nutrient agar media by spread plate technique and incubated at 30°C for 24 hours. For total fungal count diluted suspension was spread over Rose Bengal agar media by spread plate technique and incubated at 30°C for 7 days. The numbers of colonies isolated were expressed as the CFU (Colony Forming Unit).

2.4 Estimation of soil microbial enzymatic activities

The soil enzymatic activities in different age series waste dumps and control site was analysed with respect to dehydrogrogenase, amylase, invertase, urease, protease and phosphtase by standardized protocols. Dehydrogenase activity was determined spectrophotometrically at 485nm by measuring reduction of 2, 3, 5 - triphenylotetrazolium chloride (TTC) to red-coloured triphenyl formazon (TPF) following Nannipieri *et al.* (1990) and enzyme activity was expressed as mg TPF/g soil/hr. Amylase activity was measured following the protocol of Somogyi (1952) and Roberge (1978) by taking starch as substrate and enzyme activity was expressed as µg glucose/g soil/hr. Invertase activity was determined by spectrophotometric method at 540nm (Ross, 1983) by taking sucrose as substrate and enzyme activity was expressed as µg glucose/g soil/hr. The urease activity was determined by following the method of Tabatabai and Bremner (1972), and enzyme activity was expressed as µg NH_4^+/g soil/hr. Protease activity was measured spectrophotometrically at 700nm (Ladd and Butler, 1972), by taking sodium caseinate as substrate and enzyme activity was expressed as µg tyrosine/g soil/hr. Phosphatase activity was estimated spectrophotometrically following the method of Tabatabai and Bremner (1969) and enzyme activity was expressed in µg p-nitrophenyl phosphate (PNP) /g soil/hr.

3. Results and discussion

Total bacterial and total fungal colony forming units (CFUs) of different age series waste dumps and control site were presented in Fig. 1a and 1b respectively. Among the waste dumps the bacterial and fungal CFUs could not be isolated from D_0. However, in rest of the waste dumps different numbers of bacterial and fungal CFUs were isolated,

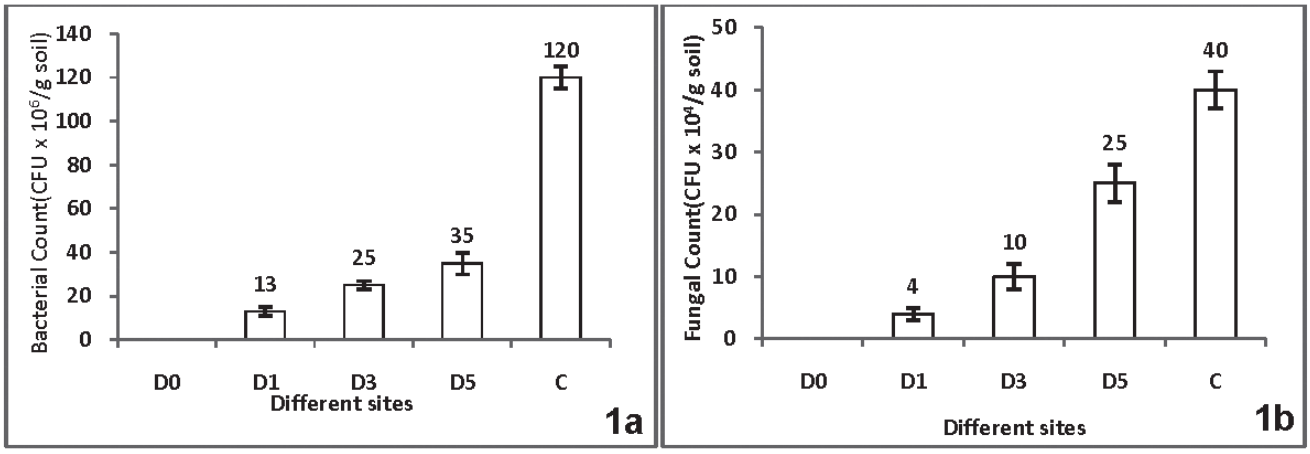

Fig 1a-b: Total bacterial (1a) & Total fungal (1b) Colony Forming Units (CFU) in different age series sponge iron solid waste dumps (D_0, D_1, D_3 and D_5) and control site (C).

which showed gradual increasing trend with increasing age of the waste dumps. Further analysis of variance (ANOVA) indicated that the total bacterial (F = 714.48) and fungal (F = 259.45) CFUs in different sites was observed to be statistically significant at $p< 0.001$.

Such findings can be correlated with fact that solid waste freshly released from the sponge iron industry was dumped on D_0. The microbial inoculums from the surrounding region might not have established them in the freshly laid waste dump, which might explain the present finding. Further, with increase in the age of the waste dumps there was gradual increase in the bacterial and fungal population. Several factors including physicochemical properties of soil (Sexstone *et al.*, 1985), soil organic carbon (He *et al.*, 2012), changes in pH (Lauber *et al.*, 2009), soil water content (Zhou *et al.*, 2002), plant diversity and composition (Wardle *et al.*, 2004; Carney and Matson, 2006) and soil mineral nutrient availability (MacKenzie and Quideau, 2010; Nannipieri *et al.*, 2003) have all been shown to influence soil microbial communities. The improvement in physicochemical properties (Kullu and Behera, 2015) and increase in plant diversity and composition (Kullu and Behera, 2011) of the different age series sponge iron waste dumps has already reported, which resulted in increased microbial population with increase in age of the waste dumps.

The soil microbial enzymatic activities in different age series waste dumps and control site was analysed with respect to dehydrogrogenase, amylase, invertase, urease, protease and phosphtase were presented in Figs. 2 (a-f). It was observed that none of the microbial enzymatic activities could be detected in freshly laid dump D_0. Dehydrogenase is an oxidoreductase group of enzyme is considered to be an index of microbial activity (Tabatabai, 1982; Dick, 1994, 1997). It is an intracellular enzyme and it is not active as extracellular enzymes in soil, hence considered as a good indicator of overall microbial activity (Garcia *et al.,* 1997; Taylor *et al.,* 2002). Absence of microbial population in D_0 resulted in non detection of dehydrogenase activity in the D_0. Amylase, invertase, urease, protease and phosphatase are substrate induced hydrolytic enzymes (Roberge, 1978; Ross, 1983; Nannipieri *et al.,* 1990; Beyer *et al.,* 1992). Therefore, absence or inadequacy of the substrate such as organic carbon, nitrogen and extractable phosphate (Kullu & Behera, 2015) may be the reasons for such non-detection of enzymatic activities in freshly laid waste dump (D_0).

However all the enzyme activity were recorded in D_1, D_3 and D_5, showing an increasing trend with increasing age of the waste dumps. The increase in the microbial population with increasing age of the waste dump has resulted in increased dehydrogenase activity which can be considered as an indication of the recovery of soil functionality (Harris and Birch, 1989; Fiedler *et al.,* 2004). Amylase is complex enzymes that hydrolyze starch to reducing sugar. Change in amylase activity related to increase in the soil organic carbon, microbial biomass fluctuations and diversity of soil microbiota (Pati and Sahu, 2004; Anjaneyulu *et al.,* 2011). The increase in invertase activities might also be related to increase in the soil organic carbon, microbial biomass over time (Tscherko and Kandeler, 2000; Luxhfi *et al.,* 2002; Bol *et al.,* 2003). Soil invertase activity is significantly correlated with the soil organic matter (Shi *et al.,* 2008). Generally, protease activity depends upon distribution of proteolyitc bacteria and the amount of proteinaceous substrate availability in soil organic matter (Subrahmanyan *et al.,* 2011). The increase in the protease activity is closely related to gradual accumulation of soil organic carbon, NH_4-N accumulation and microbial biomass content (Sardans *et*

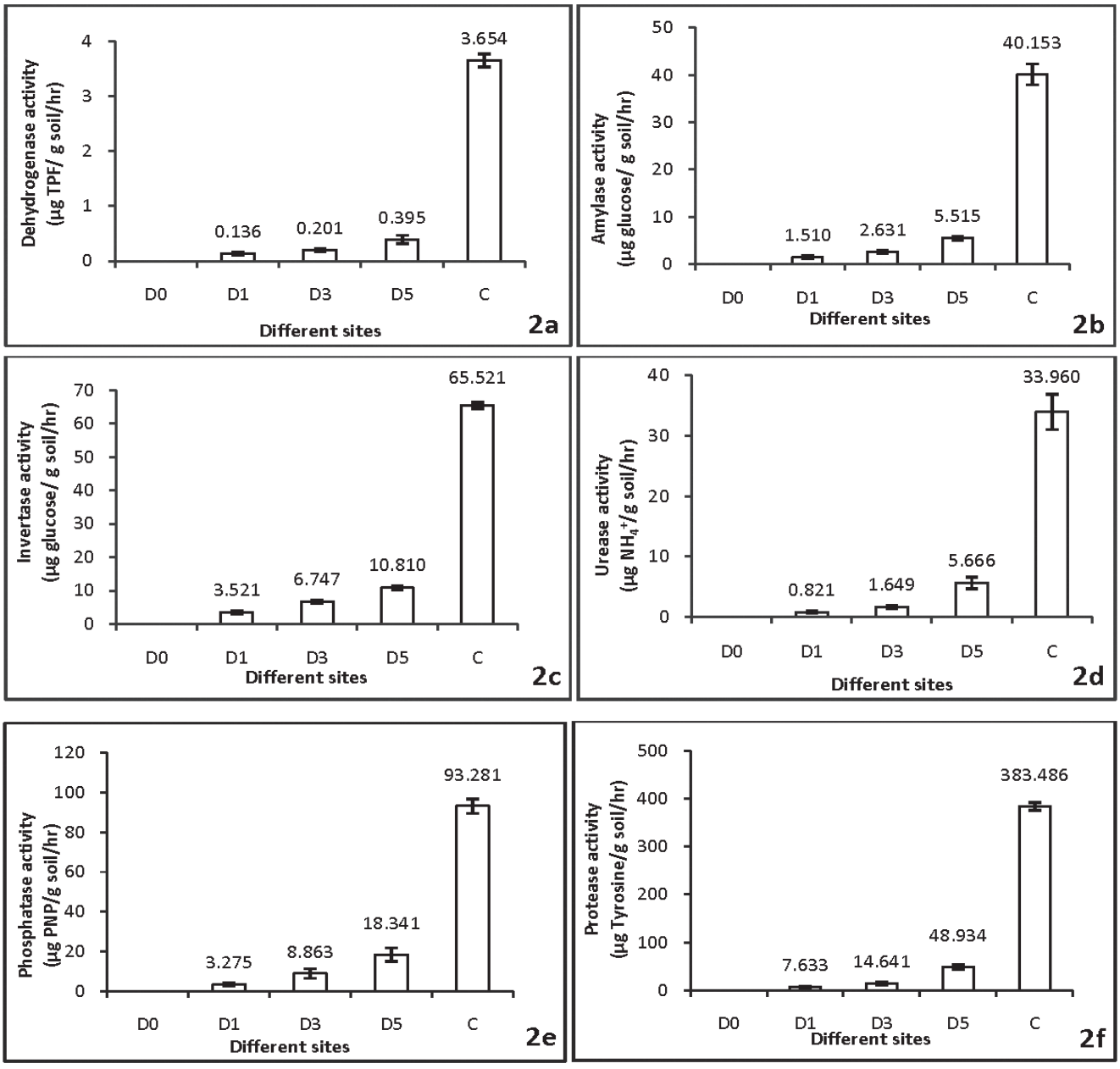

Fig. 2a-f. Soil microbial enzyme Dehydrogenase (2a), Amylase (2b), Invertase (2c), Urease (2d), Phosphatase (2e) & Protease (2f) activities in different age series sponge iron solid waste dumps (D_0, D_1, D_3 and D_5) and control site (C).

al., 2008; Anjaneyulu *et al.,* 2011). Urease activity has been reported to be closely associated with nitrogen mineralization, specifically the conversion of organic nitrogen to inorganic form (Pascual *et al.,* 1998, Giridhara *et al.,* 2003; Yang *et al.,* 2006). Phosphatase acts as an intermediary enzyme for the transformation of organic phosphorous to inorganic form and has a role in the soil phosphorous cycling (Appiah and Thomas, 1982; Kramer and Green, 2000). Thus in general, increase in the respective substrate level with increasing age of the waste dump, the different hydrolytic enzymes showed increasing activity.

The analysis of variance (ANOVA) indicated that the dehydrogrogenase, amylase, invertase, urease, protease and phosphtase enzyme activities in different sites was observed to be statistically significant at $p<0.001$.The relationships between different soil enzyme activities and the age of the waste dumps were expressed in form of linear equations followed by coefficient of determinant (R^2), correlation coefficient (r) and their level of significance (Table -1). There was a positive correlation between the different soil microbial enzyme activities and age of the waste dumps, indicating the process of reclamation.

Table 1

Relationship between different soil enzymatic activity and age of the waste dump (year).

Parameters x	Parameters y	Equation	Coefficient of determinant (R^2)	Correlation coefficient(r)	Level of significance
Age of waste Dump (Year)	Dehydrogenase activity (µg TPF/g soil/hr)	y = 0.072x + 0.020	R^2 = 0.953	0.976	P >0.001
Age of waste Dump (Year)	Amylase activity (µg glucose/g soil/hr)	y = 1.001x + 0.214	R^2 = 0.939	0.980	P >0.001
Age of waste Dump (Year)	Invertase activity (µg glucose/g soil/hr)	y = 1.791x + 2.068	R^2 = 0.784	0.885	P >0.001
Age of waste Dump (Year)	Urease activity (µg NH_4^+/g soil/hr)	y = 1.070x - 0.374	R^2 = 0.892	0.944	P >0.01
Age of waste Dump (Year)	Protease activity (µg Tyrosine/g soil/hr)	y = 9.220x - 2.944	R^2 = 0.896	0.945	P >0.01
Age of waste Dump (Year)	Phosphatase activity (µg PNP/g soil/hr)	y = 3.592x - 0.466	R^2 = 0.984	0.992	P >0.001

4. Conclusion

Sponge iron solid waste dumps represent a distressed habitat and posses scare in the natural landscape which need urgent attention for their reclamation. To understand the natural process of reclamation, among the various features, study of microbiological feature of the sponge iron waste dumps is necessary, so that reclamation process can be favorably manipulated. The findings of the present study clearly revealed that with increasing age of the waste dump there is improvement in the microbiological status of waste dumps with respect to increase in the bacterial and fungal population and microbial enzyme induced operation of nutrient cycling, consequently paving the path way for reclamation.

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Cadmium modulated defense of rice plant against fungal blast (*Pyricularia oryzae* Cav.) as evident by morphological and physiological changes

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ABSTRACT

Cadmium at lower concentration induces resistance to rice plants against *Pyricularia oryzae*. Seeds of *Oryza sativa* var. Khandagiri were tested in hydroponics treated with 50µM of $CdCl_2$, against control and infected with *Pyricularia oryzae* (casual organism of rice leaf blast). Morphological and physiological changes were monitored in 7d, 15d and 21d intervals after treatment with fungus. Significant decrease of plant height, chlorophyll pigment, carotenoids and increase of proline content were noticed in fungal treated plants that became normalized near control in co-stress plants. It was evident from the experiments that lower dose application of Cd provides resistant to rice against fungal pathogen.

1. Introduction

Rice (*Oryza sativa* L.) provides the staple food for more than half of the world's population (Sasaki and Burr 2000). Several biotic and abiotic stresses affect the plant growth and productivity among which are heavy metals and plant pathogens. Cadmium is phytotoxic at high doses which inhibits photosynthetic activity, retards the overall growth and physiological processes (Sheoran *et al*. 1990, Krupa *et al*. 1993, Chugh and Sawhney 1999, Jali *et al*. 2014). However, its yield and quality are also seriously compromised by infectious diseases caused by many pathogens. The rice blast fungus, *Pyricularia oryzae* Cav., is the most serious and destructive pathogen of rice plant. Mittra *et al*. (2004) reported low doses of cadmium provides resistant against fungal disease in wheat. Cadmium salts are also used against fungal infections (Osbourn 1996, Mittra *et al*. 2004). Use of fungicide to control the disease is ban in most of the developed countries due its accumulation as residual deposits in plant parts and various side effects on human health. Hence, an attempt was made to reduce the fungal incidence in rice plants by applying a micro dose of cadmium for developing systemic resistance.

2. Materials and methods

2.1 Plant material collection and seed treatment

Rice seeds (*Oryza sativa* L.) were collected from the Plant Genetics and Breeding Department, Orissa University of Agriculture and Technology (OUAT), Bhubaneswar, Odisha. *Oryza sativa var.* Khandagiri was used for all experiments in this study. Seeds were treated with 0.1% bevistine for 15 min., then washed three times with distilled water and kept in 70% alcohol for 30 sec and washed off with sterilized distilled water followed by treatment with 0.1% mercuric chloride for 5 min (Mittra *et al*. 2004). Seeds were finally washed three times with sterile distilled water and processed for seed germination.

2.2 Collection and storage of Pyricularia oryzae

The freeze-dried fungus (*Pyricularia oryzae* Cav.) culture was obtained from Institute of Microbial Technology (IMTECH), Chandigarh (India), bearing MTCC NO. 1477. Fungal spore suspension were routinely maintained on Oat meal Agar (OMA) slant and was inoculated into flask containing 100 ml Oat meal broth (Fig.1) and kept under

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aerobic condition in the dark at 25±1°C for 7 d in an incubator.

2.3 Hydroponic culture

Healthy germinated seeds were selected to be grown in thermocol cups containing Hoagland's nutrient solution and treated further with Cd^{2+} and *P. oryzae*. Seedlings grown in only nutrient solution without any other treatment served as control. The hydroponics were kept in culture room at 27°C temperature and 90% Relative Humidity and adequate light condition for proper growth.

2.4 Cd treatment and fungal infection

Seven days old seedlings were treated with 50 µM Cd. After 48 h the plant leaves were pricked with sterilized needle and 1ml (1×10^5 spores/ml) of *P. oryzae* broth culture was sprayed over the leaves of rice plant.

2.5 Antifungal activity

OMA media (19 ml) along with 1 ml of $CdCl_2$ was poured into petriplates and allowed to solidify. After complete solidification of the medium, fourteen day old culture of the *P. oryzae* was inoculated over the center of the media. The petri dishes containing OMA media devoid of $CdCl_2$ served as control. The plates were incubated at 25±1°C for seven days. After incubation the colony diameter was measured in mm (Singh and Tripathi 1999). Experiment was carried out in triplicates. The toxicity of $CdCl_2$ in terms of percentage inhibition of mycelial growth was calculated using the formula :

Percent inhibition = C - T / C × 100, where C = Average increase in mycelial growth in control plate and T = Average increase in mycelial growth in treatment plate.

2.6 Extraction and estimation of photosynthetic pigments

Fresh leaves (0.5 gm) were thoroughly homogenized in chilled 80% acetone in a mortar and pestle. The homogenates were centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant were collected and absorbance at 470 nm, 646.8 nm and 663.2 nm were recorded using UV–Visible double beam spectrophotometer for estimation of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids (Arnon 1949).

2.7 Extraction and estimation of proline

Proline content was estimated following the methods of Bates *et al*. (1973). 0.5gm fresh leaves were homogenized with sulfosalicylic acid and filtered through Whatman No. 2 filter paper. 2ml of the filterate was mixed with 2ml ninhydrin and 2ml glacial acetic acid; the mixture was then incubated at 100°C for 1h. The reaction was stopped quickly by keeping the test tubes in ice chamber; 4ml toluene was added and the mixture was shaken vigorously for 15 - 20 sec. The aqueous toluene layer was separated and warmed to room temperature; the red color was measured at absorbance 520 nm. The experiments were repeated three times with three replicates.

3. Results and discussion

3.1 Germination and plant growth

The seeds found about 89% of germination. In control the rice plant, growth was very good which did not show any symptoms of chlorophyll deficiency and decreased plant height but the *P. oryzae* and Cd treated plants showed chlorophyll deficiency symptoms and reduced plant growth. No such chlorophyll deficiency was observed in co-stress (Fig.2).

3.2 Antifungal activity

Cadmium (50µM) treatment showed inhibition of fungal growth with increasing days of incubation whereas in control the growth increased with increasing days of incubation period. (Fig-3), (Table -1).

3.3 Photosynthetic pigments

There was a significant decrease of chlorophyll b except for control plants . Total chlorophyll and carotenoid contents decreased in pathogen treated plant as compared to Cd treatment which is at par with 21d control and Cd+Pathogen treated seedlings. The total chlorophyll and carotenoid contents in 21 d treatment were 428.29 µg/g in Cd treated plant and 325 µg/g in fungus treated plant and

Table 1

Percent mycelia inhibition

Days of treatment	Control (mm)	50 µM Cadmium (mm)	% Inhibition
7	15 ± 0.81	10.33 ± 0.47	31.11
15	18 ± 1.63	11.33 ± 0.94	37.03
21	25.33 ± 0.94	12 ± 1.63	52.63

*Values in the table are mean ± SD of 3 replicates

145.36µg/g and 63.51 µg/g respectively. The carotenoid was 173.67 µg/g for co-culture which is significantly higher than single treatments. The co-culture showed increased chlorophyll as compared to single treatments but was lower than control. Thus, the fungus and cadmium treated plants did not showed enhance chlorophyll and carotenoid content, but in co-stress the photosynthetic pigment content increased significantly (Table 2).

3.4 Proline content

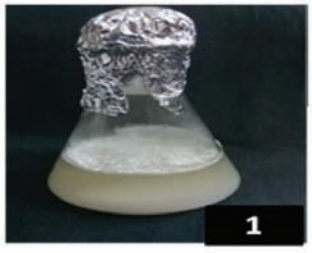
The proline content found 7.19µg/g to 10.71µg/g in control plants (7d to 21d). In fungus treatment, the proline content was increased significantly up to 29.78µg/g which was found less as compared to Cd treated plants (27.03 µg/g f.w.). In co-culture, after 21 d proline content decreased up to 10.095 µg/g (Fig. 4). Enhanced level of proline was reported to reduce oxidative stress damage to cells in rice. Thus, the co-stress infected plants have synergistic effect of PR protein along with Cd treated plant. Further it was observed that phytoallexin like substance due to fungal infection reduces production of proline concentration in accordance with accumulation of proline which is a general phenomenon in all the stressed plants (Lee *et al.,* 2003).

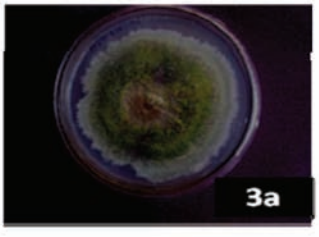
Table 2

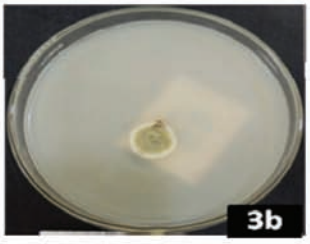
Effect on photosynthetic pigments of rice.

Treatment	*Chl a* (µg/g f.w.) ± SD			*Chl b* (µg/g f.w.) ± SD			Total*Chl*(µg/g f.w.) ± SD			Carotenoid (µg/g f.w.) ± SD		
	7d	15d	21d	7d	15d	21d	7d	15d	21d	7d	15d	21d
Control	355.9± 8.8	365.9± 9.7	373.5± 13.6	365.3± 38.1	356.8± 80.4	424.8± 28.8	721.2± 30.3	722.7± 10.7	798.4± 6.7	169.8± 10.7	177.6± 1.4	180.0± 1.6
50 µM Cd	336.9± 8.2	279.5± 14.2	260.0± 11.5	257.2± 17.3	177.4± 5.8	168.2± 0.7	594.1± 11.0	456.9± 13.9	428.2± 8.9	166.0± 2.2	154.1± 2.4	145.3± 1.3
P. oryzae	256.5± 2.9	256.1± 10.6	200.8± 58.1	181.8± 1.9	145.8± 24.7	124.1± 21.6	438.39± 3.4	402.0± 11.2	325± 3.7	121.0± 7.03	112.4± 4.8	63.5± 1.8
50 µM Cd + *P. oryzae*	329.0± 5.3	355.3± 7.2	382.0± 9.9	360.2± 4.8	366.9± 20.4	429.81± 1.1	689.3± 1.5	722.2± 13.2	811.8± 8.7	166.2± 1.1	143.5± 2.4	173.6± 3.4

*Values in the table are mean ± SD of 3 replicates.







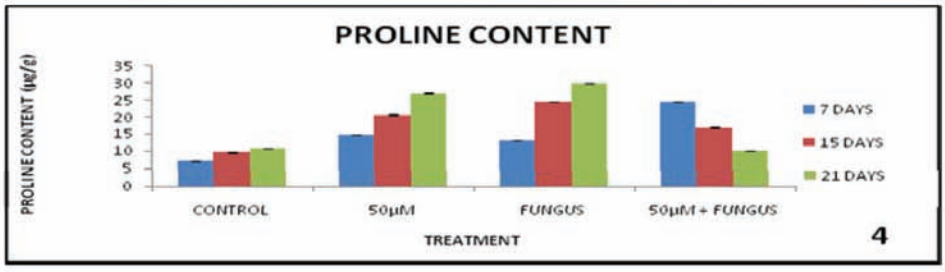


Fig 1. *Pyricularia oryzae* (Broth)
Fig 2. Plant growth : a- control, b- 50µM Cd, c- *P.oryzae*, d- 50µM+ *P.oryzae*
Fig 3. Antifungal activity: 3a- *Pyricularia oryzae* (Control) ,
3b- 50µM Cadmium+ *Pyricularia oryzae*
Fig 4. Proline content of *Oryza sativa* L. with different treatments

4. Conclusion

Metal ions are toxic in nature but in low dose prevent fungal infection in plants. Cadmium application (50µM) to rice produced resistance that prevented *P. oryzae* infection. Chlorophyll and carotenoid, content increased in control and decreased in Cd and *P. oryzae* treated plants. In contrast, proline content increased with the day of treatment. In co-stress, *P. oryzae* and Cd at 7d, 15d and 21d intervals showed general improvement of plant growth accompanied with the increase of pigments as in control.

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Biodegradation of insecticides and herbicides by *Aspergillus* species: A review

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ABSTRACT

Use of synthetic pesticides during past decades has led to serious environmental pollution and residual toxicity to soil organisms. In view of this, efforts have been made all over to study degradation of pesticides and their residues by microorganisms. Bacterial degradation of pesticides has been extensively studied and several strains have been recommended for application and accelerated degradation of residues but fungal degradation has attracted less attention. Several filamentous fungal species belonging to ascomycetes have been found effective in degrading the insecticides and herbicides. Moreover the spore forming ability enable them more persistence in the nature even during harsh climate making them a better choice with less number of applications. It is a review article and mainly focuses on the bioremediation of pesticides using *Aspergillus* species.

1. Introduction

Agricultural use of pesticides dates back to 2500 BC when these were applied for on-field and off-field protection of crops. The first known pesticide was elemental sulphur dusting of which used in ancient Sumer about 4,500 years ago in ancient Mesopotamia. During the 15th century, toxic chemicals such as arsenic, mercury and lead were applied to crops to kill pests. During the 17th century, nicotine sulphate was extracted from tobacco leaves to use as an insecticide (Ashman and Puri, 2002). Subsequently synthetic pesticides entered into agriculture during 1940s resulting in rapid progress and development of different pesticides of widely diverse chemical nature. Extensive use of the synthetic pesticides and their prolonged residual toxicity in the soil environment has affected the soil microflora and fauna leading to loss of soil productivity (Hewitt, 1998). This has become a major concern not only in our country but also throughout the world.

In India 15–20% of all produce is destroyed by insect pests (Bhalerao and Puranik, 2007). This emphasizes the paramount importance of insecticides in India in preventing agricultural loss and enhancing production. The enormous use of pesticides, has added to environmental pollution. However, a number of bacteria, cyanobacteria and fungal species are known to metabolically degrade the insecticides and other organochemicals causing decrease in their environmental toxicity. Members of phycomycetes, ascomycetes and white-rot fungi (*Pleurotus ostreatus, Trametes versicolor, Trametes hirsutus, Bjekandera adusta, Stereum hirsutum, Hypholoma fasciculare, Flammulina velutipes, Lentinus ododes, Penicillium steckii, Phlebia acanthocystis, Phlebia aurea, Phlebia lindtneri, Phlebia vrevispora, P. chrysosporium* and *P. sordid)* have been noted to be very useful for biodegradation of pesticides (Bending *et al.,* 2002; Jauregui *et al.,* 2003). Many a different species of *Aspergillus* can degrade the pesticides under ambient environmental set up, even when exposed to high concentrations (Anderegg and Madisen, 1983; Hasan, 1999; Bhalerao and Puranik, 2009; Jain and Garg, 2013; Jain *et al.,* 2014; Yadav *et al.,* 2015; Oliveira *et al.,* 2015). However, little attempt has been made to comprehensively evaluate the potential of *Aspergillus* species in degrading the pesticides in the field. This review presents the researches

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made on the evaluation of various species and strains of *Aspergillus* for biodegradation of pesticides and use of these fungi in bioremediation.

2. Degradation of insecticides

2.1 Organophosphates

Marine-derived fungus *Aspergillus sydowii* performs the degradation of organophosphorus insecticide methyl parathion (commercially named Folisuper 600 BR) even at high concentrations. Different concentrations (50-300 mg/l) of the insecticide could be effectively degraded by the fungus in solid medium but better degradation could be achieved in liquid medium *A. sydowii* shows (98% degradation in 10 days and 100% degradation in 20 days) with a pesticide concentration of 50 mg/l (Alvarenga *et al*., 2014). Two other isolates of *A. sydowii* (CBMAI 935 and CBMAI 1241) also degraded profenofos applied at 100 mg/l (da Silva *et al*., 2013). These strains degraded up to 98% and 92% in 30 days in a concentration of 50 mg/l and 100 mg/l, respectively (da Silva *et al.,* 2013). The fungus could also utilize 1.65 g/l malathion and lancer as phosphorus sources (Hasan, 1999). *Aspergillus glaucus* degraded malathion to non-toxic levels when the insecticide was applied in stored grains (Anderegg and Madisen, 1983). Similarly soil derived fungus *Aspergillus oryzae,* isolated from the soil using a 100 mg/l of monocrotophos, grew well up to 500mg/l and showed tolerance upto 900 mg/l of monocrotophos in liquid medium (Bhalerao and Puranik, 2009)(Table 1).

Yadav *et al.* (2015) observed that chlorpyrifos is degraded very efficiently by *Aspergillus sp*. in continuous flow bioreactor as compared to degradation by bacteria. The operating range for continuous bioreactor is found to be in the range of 180 to 250 mg/l. d. This shows that fungus is a better agent for biodegradation of chlorpyrifos as compared to other microbes. Taking different OP insecticides (Pirimiphos-methyl, Pyrazophos, Dimethoate, Malathion, Lancer, Profenfos), Hasan (1999) reported that two isolated phosphatase-producing fungi *A. flavus* and *A. sydowii* caused a general degradation of the insecticides up to a concentration of 1000 mg/l. Soluble phosphorus increased distinctly under the action of these species by enhanced phosphatase activity. The mineralization of insecticide residues was higher in soil amended with wheat straw than in unamended soil.

A. fumigatus utilized pirimiphos-methyl and lancer, as sole phosphorus sources. More than 60 % of *A. flavus* and *A. sydowii* isolates utilized malathion and lancer (1.65 g/l) as phosphorus sources. In enrichment culture *A. sydowii* followed *A. niger* and *A. flavus* were best degrading species by producing more than 50% of biomass. *A. fumigatus* and *A. terreus* also utilized the pesticides but produced less than 50 % biomass (Hasan, 1999). Liu *et al.* (2001) observed that *A. niger* degraded dimethoate by showing enhanced expression of OP acid anhydrase (Phosphotriesterase). The OP degrading activity of the fungus was significantly enhanced by Cu^{2+} as observed in bacteria (Mulbry and Karns, 1989; Liu *et al.,* 2001). The tolerant strain of *A. niger* degraded formothion and malathion with almost equal efficiency whereas it was unable to degrade parathion and dichlorovos indicating the chemical specificity of the enzyme (Liu *et al.,* 2001).

Fungi are also known to degrade OP insecticides by enhanced phosphatase activity (Hasan, 1999). *A. sydowii* phosphatase was highly active against pyrazophos followed by lancer and malathion. All added pesticides except profenfos could be degraded by the fungus during three weeks of exposure (Hasan, 1999). *A. flavus* and *A. sydowii* were the first fungi isolated from wheat straw capable of degrading organophosphate pesticides and utilizing these compounds as sole phosphorus and carbon sources by releasing phosphorus from these pesticides through the action of their phosphatases. These strains could be beneficial as fungal inoculums for efficient hydrolysis of pesticides (Hasan, 1999).

By the application of 150 mg/l concentration of monocrotophos at pH 8 and temperature 30°C Jain *et al.* (2014) reported that *A. falvus* caused significant (up to 1.16 fold) increase in degradation. The fungus degraded 91.59 ± 4.31% of pesticide in a concentration of 150 mg/l within 15 days. The released inorganic phosphate content remained almost same showing the metabolic utilization of the nutrient by the fungus (Jain *et al.,* 2014). *A. niger* and *A. flavus* degraded monocrotophos in phosphorus-free liquid medium with the degrading ability of *A. niger* being more than *A. flavus*. The implementation of degrading enzyme (extracellular hydrolase) from the fungus was more effective than application of whole cell (Jain and Garg, 2013).

Silambarasan and Abraham (2013a,b) observed that *A. terreus* showed 100% degradation of 300 mg/kg of chlorpyrifos and its major product within 24 hours and 48 hours respectively, with media supplemented with nutrient and media with no addition of nutrient but only supplemented with insecticide. Chlorpyrifos could also be efficiently degraded by *A. niger* both in nutrient enriched and nutrient deficient media. The degraded increased exponentially with prolongation of incubation indicating the enhanced synthesis of the degrading enzyme by the fungus and metabolic utilization of the insecticide (Mukherjee and Gopal, 1996).

Aspergillus niger utilised glyphosate mainly through the cleavage of carbon-phosphorus (C-P) bond, resulting in

the release of sarcosine and a phosphate group. The phosphate group was utilized as a source of phosphorus for fungi growth. The released sarcosine was possibly further degraded to other products. A small fraction of glyphosate was degraded through the cleavage of carbon-nitrogen(C-N) bond, with the release of aminomethyl phosphonic acid and also the growth if the fungus was enhanced in the presence of pesticide, which shows the degrading power of the fungus (Adelowo *et al.,* 2014).

Chlorfenvinphos, however on the other hand, was degraded to very low level by the use of the fungal species. Combination of both the species was much effective rather than using each fungus at a time indicating cooperativity between the species (Oliveira *et al.,* 2015). *A. niger* and *A. smithii* metabolically degraded dichlorvos efficiently in nutrient enriched medium. There was increased growth performance of the fungi in the culture with the insecticide on prolonged exposure indicating the utilization of the insecticide as a substrate by the fungus (Mohapatra, 2006). Effective degradation of diazinon has been achieved with *A. oryzae* and *A. niger* (Mostafa *et al*., 1972).

2.2 Organochlorines

Among the various OC insecticides, extensive work has been done on the fungal degradation of endosulfan. Strains *A. sydoni* have been found to degrade endosulfan very efficiently and use it as a source of carbon in broth medium as well as in soil microcosm (Goswami *et al.,* 2009). It has been reported that the fungus degraded both á endosulfan and â endosulfan with almost equal efficiency (95% and 97%, respectively) through oxidative and hydrolytic pathways (Goswami *et al.,* 2009). *A. niger* degraded technical grade endosulfan to almost complete removal within 12 days at 400mg/ml concentration under laboratory condition (Bhalerao and Puranik, 2007). However, the fungal degradation of the insecticide caused formation of various less toxic products like endosulfan diol, endosulfan sulfate, and an unidentified metabolite. Nevertheless metabolic utilization of the insecticide and its degradation products was performed by the fungus as evidenced by the increased CO_2 evolution (Bhalerao and Puranik, 2007). Hussaini *et al.* (2013) observed that *A. niger* degraded endosulfan (59%) more efficiently than lindane (29%). DDT was, however, not degraded by the fungus. There is no report on the efficient fungal degradation of DDT showing its recalcitrant behaviour in the environment. However, microbial consortia can be tried for removal of the residual DDT from the environment. Indigenous *A. niger* (ARIFCC 1053) isolated by Bhalerao (2013) could tolerate and utilize higher concentration (1,000 mg/l) of endosulfan. In vitro degradation was marked with increase in the amount of released chlorides, dehalogenase activity, and released proteins. The organism was able to degrade half of the initial endosulfan within 96 h of inoculation and complete degradation was achieved after 168 h of incubation. The study also identified sulfurous acid, glyoxal, and protonated formic acid, which, in the environment, are generally converted to CO_2, SO_2, and H_2O (Bhalerao, 2013).

Mukherjee and Mittal (2005) recorded degradation of endosulfan by *A. terreus*. The degradation was not very remarkable in the first three days but it increased quite drastically with prolongation. The strain *A. tamarii* JAS9 isolated from endosulfan spiked soil was able to tolerate higher doses of endosulfan up to 1300 mg/l and grew well up to 1000mg/l (Silambarasan and Abraham, 2013b). Another important feature was that these particular strains were capable of degrading endosulfan sulphate which is more persistent and hence need to be degraded (Silambarasan and Abraham, 2013a).

Microbial degradation of DDT has been recorded from 1960 but fungal degradation is limited. Many strains of *Trichoderma viride* metabolize DDT by producing DDE, DDA and DDNS (Singh and Dwivedi, 2004). Mehrotra *et al.* (2004) have reported that *A. flavus* and *A. parasiticus* converted DDT to DDE in nutrient enriched medium. The species were, however, not efficient degrader of the insecticide when compared to that performed by *T. viride*.

A. flavus and *Penicilium notatum* metabolically degraded aldrin and dieldrin on prolonged incubation and on increase of the inoculums density (Mehrotra *et al*., 2004). Syntrophic activity of *Aspergillus* and bacteria had shown more efficient degradation of these chemicals than by individual strains. Similarly chlordane and heptachlor were found degraded to their epoxides by syntrophy of *A. niger* and *Pseudomonas urticae* (Singh and Dwivedi, 2004). *Aspergillus* species could also effectively degrade ã-HCH in nutrient enriched media but metabolic use of the chemical has not been reported (Rani and Dhania, 2014; Javaid *et al*., 2016).

2.3 Pyrethroids

No significant work has been done on the fungal degradation of pyrethroids though algal and cyanobacterial degradation has been reported (Samantarai, 2006; Chandrakala, 2016). Some literature are, however available, to show the efficiency of *Aspergillus* to degrade pyrethroids. *A. niger* could degrade 54.83 % of β-cypermethrin (50 mg/l) in 7 days and could completely degrade 100 mg/l of 3-phenoxybenzoic acid within 22 h, which is considerably higher than the reported degradation rates of some bacteria, such as *Ochrobactrum lupini* DG-S-01 (Chen *et al.,* 2011a)

and *Stenotrophomonas sp*. ZS-S-01 (Chen *et al.,* 2011b). Enzymatic activities of the fungal strain showed that it can effectively degrade α-cypermethrin and its metabolites except permethric acid, which makes it an important biodegradable organism (Deng *et al.,* 2015)

3. Degradation of herbicides

3.1 Chloroacetanilides

The microbial degradation of metolachlor was solely due to the mixed fungal culture of *Aspergillus flavus* and *Aspergillus terricola* in soil which was estimated by difference in degradation in uninoculated and inoculated sterile soils and the net effect was found to be 49.21% in a concentration of 20µg/g (Sanyal and Kulshrestha, 2003). Similarly, the overall degradation due to the combined effect of biotic and abiotic processes was up to 84.24% after 25 days of incubation in soil treated at the 50 mg/g level, out of which, as high as 50.80% degradation was due to the mixed fungal community and only 33.45% was due to other abiotic processes. The mixed culture of fungus was almost able to degrade 100% of pesticide but less efficiency was observed when applied separately (Sanyal and Kulshrestha, 2003).

Crude extract of *Aspergillus flavus* was very much effective at higher concentration of metolachlor causing about 48.2% degradation of the parent chemical within 6 h. The rate of degradation was found proportional to the volume of the extract added and the duration of incubation (Sanyal and Kulshrestha 2004). The evidence from the metabolites formed during the degradation of metolachlor showed that the parent chemical was hydrolyzed by dechlorination, hydroxylation, and dealkylation and that aniline was in minor fractions of the metabolites formed (Sanyal and Kulshrestha 2004).

3.2 Sulfonylureas

Aspergillus niger showed total biodegradation of two sulfonylureas- chlorsulfuron and metsulfuron methyl in nutrient rich medium and the degradation pathways were the sulfonylurea bridge cleavage and the hydroxylation of benzene ring (Boschin *et al.,* 2003).

Atrazine, diuron and isoproturon could not be degraded only by fungus like *A. fumigates* and *A. terreus* but a fungi-bacteria consortium was effective to remove these herbicides from the medium (Oliveira *et al.,* 2015). The above study indicated that the initial degradation was performed by bacteria and the subsequent metabolism by the fungi. *A. niger*, isolated from soil samples showed survival in liquid media even in high concentration of about 2mg/ml of chlorimuron-ethyl. The degradation of the chemical by the fungus was by the extracellular enzymes, which converted it into simpler forms that enabled the microorganism to utilise it for growth and maintenance. Fungal consortium with *Aspergillus* as the major partner was more effective in removing chlorimuron-ethyl from soil and water (Sharma *et al.,* 2012).

3.3 OP herbicides

Higher tolerance levels of the local fungal strains (*A. niger* FGP1, *A. terreus* PDP1, *A. terreus* BGCZ3, *A. tamarii* PDCZ1 and *A. flavus* WDCZ2) against glyphosate was reported by Eman *et al.* (2013). The mycoremediation by these fungal strains from liquid media after16 days showed that glyphosate was degraded rapidly in liquid media by *A. flavus* WDCZ2 (99.6%) followed by *A. tamarii* PDCZ1 (96.7%) and *A. flavus* WDCZ2 (90.6%) (Eman *et al.,* 2013). The maximum degradation of triclosan was achieved by *A. versicolor* which was up to 71.91% at 7.5 mg/l triclosan concentration (Tastan and Dönmez, 2015).

4. Conclusion

The literature showed that there are many different species of *Aspergillus*, which can degrade toxic agro-chemicals very efficiently both in vitro and in vivo conditions. Such species should be identified and isolated to be utilized as biodegrading agents. The tolerant strains can be upgraded in the laboratory condition and can be exclusively used in agricultural field for degradation of these chemicals. Alternatively, a consortium of these organisms can also be utilized to achieve more effective degradation in less time and quantity.

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Table 1

Degradation of various pesticides by different strains of *Aspergillus*.

Pesticides	*Aspergillus* spp.	Important Intermediate Product	Reference
Methyl parathion	*A. sydowii CBMAI 935*	p-nitrophenol	Alvarenga *et al.,* 2014
Monocrotophos	*A. oryzae, A. niger* and *A. flavus*	Methylamine and Dimethyl phosphate	Bhalerao and Puranik, 2009; Jain and Garg, 2013; Jain *et al.,* 2014
Profenofos	*A. sydowii CBMAI 935*	Chlorophenol-glicose conjugate and MHPM	da Silva *et al.,* 2013
Chlorpyrifos	*Aspergillus* sp., *A. terreus* JAS1, *A. niger*	3,5,6-Trichloro-2-pyridinol and Diethyl thiophosphate	Mukherjee and Gopal, 1996; Silambarasan and Abraham, 2013a; Yadav *et al.,* 2015
pirimiphos-methyl	*A. fumigates, A. flavus, A. niger* and *A. sydowii*		Hasan, 1999
Pyrazophos	*A. flavus, A. niger* and *A. sydowii*	Phenyl hydrazine and diethyl thiophosphoric acid	Hasan, 1999
Chlorfenvinphos	*A. fumigates* and *A. terreus*	2,2,4-trichloroacetophenone	Oliveira *et al.,* 2015
Dimethoate	*A. flavus, A. niger* and *A. sydowii*	Dimethyl phosphate	Hasan, 1999
Malathion, lancer, profenfos	*A. glaucus, A. flavus, A. niger* and *A. sydowii*	Desmethyl malathion and dimethyl thiosulphate	Anderegg and Madisen, 1983; Hasan, 1999
Glyphosate	*A. flavus, A. tamarii, A. terreus* and *A. niger*	Aminomethylphosphonic Acid	Eman *et al.,* 2013; Adelowo *et al.,* 2014
β-cypermethrin	*A. niger*	3-phenoxybenzoic acid and chrysanthemic acid	Deng *et al.,* 2015
Metolachlor	*A. flavus* and *A. terricola*	2-hydroxy acetamide	Sanyal and Kulshrestha, 2003; Sanyal and Kulshrestha 2004
Chlorsulfuron, metsulfuron methyl	*A. niger*	triazine derivatives	Boschin *et al.,* 2003
α and β endosulfan, endosulfan, endosulfan diol, endosulfan sulphate and lindane	*A. sydoni, A. niger, A. niger* (ARIFCC 1053), *A. terreus, A. tamarii* JAS9	Endosulfan sulphate and endosulfan diol	Bhalerao and Puranik, Mukherjee and Mittal, 2005; 2007; Goswami *et al.,* 2009; Hussaini *et al.,* 2013; Bhalerao, 2013; Silambarasan and Abraham, 2013b
Atrazine, diuron, and isoproturon	*A. fumigates* and *A. terreus*	triazine derivatives	Oliveira *et al.,* 2015
Chlorimuron-ethyl	*A. niger*	ethyl-2-aminosulfonylbenzoate and 4-methoxy-6-chloro-2-amino-pyrimidine	Sharma *et al.,* 2012
Triclosan	*A. versicolor*	2,4-dichlorophenol	Tastan and Dönmez, 2015
DDT	*A. flavus and A. parasitacus*	DDE	Mehrotra *et al.,* 2004

with *Aspergillus glaucus*. J. Econ. Entomol. 76(4): 733-736.

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Rapid *in vitro* shoot multiplication and analysis of plumbagin in *Agrobacterium rhizogenes* mediated hairy root culture of *Plumbago zeylanica* L.

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ABSTRACT

Plumbago zeylanica L. is an important medicinal plant and roots are used for dyspepsia, piles, diarrhoea, skin diseases, leprosy and rheumatism. A protocol for rapid *in vitro*-multiplication through axillary bud proliferation was developed. Approximately 7 shoots were produced from a single nodal segment of a four year old field grown plant after 4 weeks of culture on Murashige and Skoog's (MS) basal medium supplemented with 2 mg/l BAP + 100mg/l adenine sulphate. Optimum number of roots was induced (~15/shoot) upon transferring the individual regenerant to half strength MS medium supplemented with 0.5mg/l IBA. Hairy roots were initiated with the A4 strain of *Agrobacterium rhizogenes* which exhibited optimum growth in half strength MS medium containing 4% sucrose. Growth kinetic studies demonstrated a maximum 11 fold increase in root biomass yield after 6 weeks of culture. The fresh hairy roots produced 0.61% higher amounts of plumbagin over the untransformed control roots. The present research findings revealed for the first time the potentialities of the hairy root cultures of *P. zeylanica* for the production of the important secondary metabolite, plumbagin.

1. Introduction

Plants have been an important source of medicine for thousands of years. Even today, the World Health Organization estimates that up to 80% of people still rely mainly on traditional medicines for remedies. *Plumbago* species L. (Plumbaginaceae) is a medicinal shrub distributed throughout India. The plant is commonly known as Ceylon leadwort (English), Chita or Chitra (Hindi) and Chitramoolam (Tamil). The tuberous roots of *Plumbago* species are used as an important indigenous ayurvedic drug. The root contains plumbagin, 3-chloroplumbagin, 2,3-biplumbagin, 6,6-biplumbagin, zeylinone, isozeylinone, chitranone, droserone, plumbagic acid and plumbazeylanone (Chinnamadasamy *et al.,* 2010). The leaves and stem contains little or no plumbagin. Roots of *Plumbago zeylanica* L. are used for the treatment of various ailments, such as dyspepsia, piles, diarrhoea, skin diseases, leprosy and rheumatism (Rout *et al.,* 1999). Roots are also reported as antibacterial, antifungal, abortifacient (Uma Devi *et al.,* 1999) and reported to be substitute for cantharides (Chetia and Handique, 2000). The pharmacological importance of this perennial shrub lies in its ability to produce a napthoquinone, called plumbagin (Modi, 1961), mainly found in its roots. Discoveries of the tumor inhibitory substance (Krishnaswami and Puroshothaman, 1980) and radiomodifying effects (Uma Devi *et al.,* 1999) of plumbagin have enhanced the demand of this medicinal plant for its roots. Conventional propagation of the plant is rather difficult and insufficient to meet the growing demand owing to the poor germination of seeds and death of young seedlings under natural conditions (Anonymous, 1989). Moreover, indiscriminate collection of the roots from the natural habitat to meet the growing demands of the pharmaceutical companies and various adverse biotic factors affecting the wild population coupled with inadequate attempts for its replenishment have led to

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acute depletion of the natural population. Hence, rapid *in vitro* micropropagation approaches are highly important for steady supply of plants for commercial use. Reports on plant regeneration from callus cultures of *P. zeylanica* (Debata and Susmita, 1998, Rout *et al*., 1999, Verma *et al*., 2002, Mallikadevi *et al*., 2008) and clonal multiplication of *P. indica* (Chetia and Handique 2000) and *P. rosea* (Kumar and Bhavanandan, 1988, Jose *et al*., 2007) through axillary bud proliferation was reported earlier. Establishment of an efficient and reproducible organogenic system in such important medicinal plant species is a pre-requisite not only for basic research but also for commercial exploitation of plumbagin. *In vitro* plant also have greater significance in genetic transformation studies through *Agrobacterium rhizogenes* for hairy root culture. Selvakumar *et al.* (2001) reported shoot multiplication in *P. zeylanica* earlier with very less number of shoot regeneration which need further refinement in rate of *in vitro* shoot multiplication.

Furthermore, as the medicinally active constituents of *P. zeylanica* are mainly obtained from the root tissues, standardization of *Agrobacterium rhizogenes* mediated genetic transformation for hairy root cultures and evaluation of plumbagin production by transformed culture have greater importance. In a wide range of medicinal plants, hairy root cultures have already proven to be an efficient alternative production system for root derived phytochemicals of consistent quality within much shorter time than is commonly expected from *in vivo* grown plants (Tepfer, 1990, Wysokinska and Chimel, 1997, Canto-Canche and Loyola-Vargas, 1999, Banerjee *et al.,* 2002). The present study dealt with *in vitro* multiplication of *P. zeylanica* for large number of micropropagated plant in one hand and standardization of hairy root culture in regenerated elite clones through *A. rhizogenes* and evaluation of plumbagin in hair roots on the other hand.

2. Materials and methods

2.1 Nodal multiplication, rooting and hardening

Plants of *Plumbago zeylanica* are collected from medicinal garden of College of Agriculture, Orissa University of Agriculture and Technology, Bhubaneswar. Young, nodes from newly sprouted young stems (2-3 weeks old) of *P. zeylanica* were cut and washed in distilled water. They were washed with 2% Tween-20 solution for 8-10 min and washed with 70% ethanol followed by 0.1% (w/v) mercuric chloride for a 5 min and thoroughly rinsed three-four times in sterilized distilled water. About 1.0 cm long sprout segments cultured on agar solidified MS (Murashige and Skoog 1962) medium (pH 5.8) supplemented with various concentration of 0.5-5.0 mg/l BAP along with 25-100 mg /l adenine sulphate for induction and proliferation of multiple shoots. Cultures were maintained under cool-white fluorescent light (3,000 lux) at 25±2°C with 16 h photoperiod. Sub-culture was done after 3-4 weeks interval in MS medium with same concentration of hormones. After induction and proliferation of multiple shoots, these are transferred into rooting medium i.e. MS supplemented with 0.1 – 1.0 mg/l IBA. The regenerated rooted plantlets were first pre-hardened with distilled water in culture room for 48 h so as to acclimatize them. Then these plants were transferred to polybags containing sand, soil and vermiculite (1:1:1) under green house condition for 7 d for hardening.

2.2 Hairy root culture through Agrobacterium rhizogenes

The aseptically grown nodal explants and leaves were infected with 48h old suspension of the *A. rhizogenes* both A4 and MTCC532 strain (OD value 0.8 at absorbance 620 nm) grown in liquid YMB medium (Hooykaas *et al.,* 1977) having 200 mM acetosyringone. After co-cultivation with bacteria for 24h to 96 h on semi-solid, hormone-free MS medium, the explants were transferred onto the same medium containing 500mg/l of cephalaxin (Ranbaxy, India) under dark conditions in order to get bacteria free culture. Hairy roots were induced after 7-10 days after transferring of bacteria free culture on hormone free MS medium. The emerging hairy roots were subsequently transferred to half or full strength of liquid MS medium with 3% or 4% sucrose under dark condition for further growth.

2.3 Plumbagin content in transformed and non-transformed roots

Dry hairy roots (1g weight) were consequently chemically extracted (in triplicate) according to the earlier reported protocol (Gupta *et al.,* 1999, Basu and Yogananth 2009) and the plumbagin content was quantified through a HPTLC. Chemical extraction and quantification of plumbagin were also carried out in the fresh, non-transformed control roots as well as in the dry hairy roots at the optimum growth phase for comparative analysis. The soxhlet extraction was done from transformed and non-transformed dried roots using methanol. Plumbagin was identified by TLC method using silica-gelG-60 powder pre-coated TLC plates (E-mark, Germany) for standard and extracted samples. Solvent system used for TLC plates was toluene:glacial acetic acid (99:1) and the light red spot of authentic sample was identified as plumbagin in UV light and after derivatization in anisaldehyde sulphuric acid reagent followed by heating at 110^{0}C for 10 min. Quantification of plumbagin from transformed and non-transformed dried roots was carried out through HPTLC method using Camag TLC applicator. The HPTLC finger prints profile was snapped

by Cammag Reprostar III, before derivatization under UV light 254 nm, 366 nm and after derivatization. The isolated plumbagin peak was confirmed by comparing the spectrum obtained by TLC scanner, which was completely in agreement with the reference standard.

3. Results and discussion

3.1 Nodal shoot multiplication, rooting and hardening

The best multiple shoot initiation response was noted in MS medium supplemented with 2mg/l BAP along with 100mg/l adenine sulphate. Approximately 7 numbers of shoots per node were recorded after three to four weeks of culture (Table 1; Figs. 1 and 2). Root induction was observed with all media combinations tried with IBA (Table 1). The average number of roots (~15) with root length (~2-3 cm) were induced on half strength MS medium containing 0.5 mg/l IBA and well developed plants with good root systems were produced within two weeks (Table 1, Fig. 3). Well developed plants with good root systems were produced within two weeks of transfer to this medium. Sub-culturing was done after 3-4 weeks intervals in MS medium in same concentrations of growth hormones to provide proper nutrients for growth of the *in vitro* regenerated plants. The reported direct shoot regeneration protocol can be exploited commercially to multiply elite clones more rapidly and within a shorter time period, and also can be used for developing *in vitro* strategies for the conservation of this useful medicinal plant. Adenine sulphate with BAP in the present experiment showed very exciting result in shoot multiplication in *P. zeylanica* with average of 7 shoots as compared to only one shoot per explants with IBA and adenine sulphate combination as reported by earlier (Selvakumar *et al.,* 2001). Lowering the concentrations of both the plant growth regulators to half of their original strengths improved the growth, and elongated shoots with 8–10 inter-nodes could be obtained within the next 3 weeks of culture. Lowering the concentration of BAP improved differentiation of shoot buds has also been reported in case of *P. indica* (Anonymous, 1989). Hence, the multiplication rate as revealed in the present study significantly exceeds that of the report of Selvakumar *et al.* (2001) within a relatively shorter time period. More than 90 % of plantlets survival was observed on hardening for one week. However, the rate of survival decreased to 55% after two three weeks of acclimatization. It was observed that gradual acclimatization of *in vitro* grown plants to external environment is most essential for *P. zeylanica.* More than 85 % of the plants transferred to pots survived under field conditions (Fig. 4).

Table 1

Effect of various concentrations of plant growth regulators on shoot and root formation in *P. zeylanica* culture.

	Shoot multiplication			Rooting of micropropagated shoot	
BAP (mg/l)	ADS (mg/l)	No. of shoots/node	IBA(mg/l)	No. of roots/shoot	Rooting response
0.5	0	1.0±0.2	0.1	0	No rooting
1.0	25	2.2±0.6	0.2	1.0±0.25	No rooting
1.5	50	4.6±0.5	0.3	2.3±0.55	Slow rooting
2.0	100	7.5±0.4	0.4	7.2±0.75	Profuse rooting
2.5	100	4.2±0.5	0.5	15.1±0.45	Profuse rooting
3.0	100	3.7±0.9	0.6	9.5±0.33	Profuse rooting
3.5	100	2.5±0.24	0.7	5.2±0.65	Slow rooting
4.0	100	2.2±0.54	0.8	4.8±0.44	Slow rooting
4.5	100	1.7±0.46	0.9	3.2±0.05	Slow rooting
5.0	100	1.5±0.87	1.0	3.0±0.15	Slow rooting

3.2 Hairy root culture and kanamycin based selection

Nodal and leave explants from such *in vitro* raised plantlets served as explants for genetic transformation studies. The relative transformation frequency in A4 strain of *A. rhizogenes* was recorded to be 78.29% and 50.35% in shoot and leaf explants respectively in 150 min of co-culture after 3 weeks of bacterial infection (Table 2). No transformation was recorded in and MTCC532 strain. Excision and sub-culturing of the emerging hairy root clones (20-30 in number) on full and half strength MS medium with 3% sucrose exhibited a very thin and fragile appearance with limited growth characteristic. However, half strength liquid MS medium containing 4% sucrose supported active proliferation of one healthy looking root line (Fig. 5) as has also been reported earlier by Banerjee *et al.* (1998) in case of *Valeriana wallichii*. A gradual increase in the hairy root induction was

noticed with an increase in co-cultivation from 1 to 3 days. The developed roots exhibited fast growth and high lateral branching on growth regulator free MS medium and plagiotropism. The biomass in hairy root culture was higher than in non transformed root culture. The survival percent of regenerated transformed plants from callus (20%) and multiple shoots (30%) by *A. rhizogenes* (A4) strain in 120 min infection time and 2d co-cultivation periods. No regeneration was found from leaf and multiple shoots in *A. rhizogenes* (MTCC532) strain. The growth inhibiting dose of kanamycin was determined by transfering the transformed and non transformed lines on MS medium supplemented with various concentrations of kanamycin (0-60 mg l^{-1}). It was observed that lethal dose for control plant 60 mg l^{-1} kanamycin used for primary screening of putative transformants. The optimized selection method eliminates the regeneration of non-tranformed plants.

3.3 *Plumbagin content transformed and non-transformed roots*

Growth index analysis of this hairy root clone revealed a gradual increase in fresh weight up-to 6 weeks of culture at which a maximum 11 fold increase in biomass could be recorded (Fig. 5). The plumbagin production potentials of the fresh hairy root cultures paralleled with the different growth phases and reached its maximum during the optimum growth period, i.e. 6 weeks of culture. Plumbagin content of the fresh hairy roots with that of the fresh, non-transformed control roots and the dry hairy roots at the optimum production phase (i.e. 6 weeks of culture), the fresh hairy roots were found to possess 8.13% plumbagin than non-transformant roots (7.52%) (Figs. 6 & 7). Besides this, the fresh weight : dry weight ratio of the hairy roots being 20:1, the fresh hairy roots were found to possess 49.4 times higher amount of plumbagin than the dry *in vitro* roots in *Plumbago zeylanica.* The present study demonstrates for the first time, successful induction and establishment of *A. rhizogenes* mediated hairy root cultures in *P. zeylanica* with higher potential for the production of the active compound – plumbagin. The production of this compound from hairy root cultures can further be optimized for commercial production.

Table 2

Effect of co-culture period on mean percentage of root induction in shoot tips and leaves (A4 strain)

Co-culture period	Response of shoot tips			Response of leaves		
	7 d	15 d	21d	7 d	15 d	21d
30 min	22.27±0.25	38.89±0.35	44.44±0.36	0	0	0
60 min	33.33±0.79	45.23±0.44	50.30±0.87	0	0	0
90 min	38.78±1.01	51.25±0.26	55.29±0.96	11.10±0.03	16.66±0.34	27.89±0.12
120 min	52.23±0.96	56.26±1.04	62.15±0.16	22.21±0.33	28.24±0.24	35.25±0.15
150 min	55.15±0.39	63.67±0.65	78.29±0.97	23.26±0.43	38.89±0.45	50.35±0.67
180 min	35.25±1.12	42.30±0.26	50.29±0.88	5.52±0.24	12.14±0.33	16.34±0.45

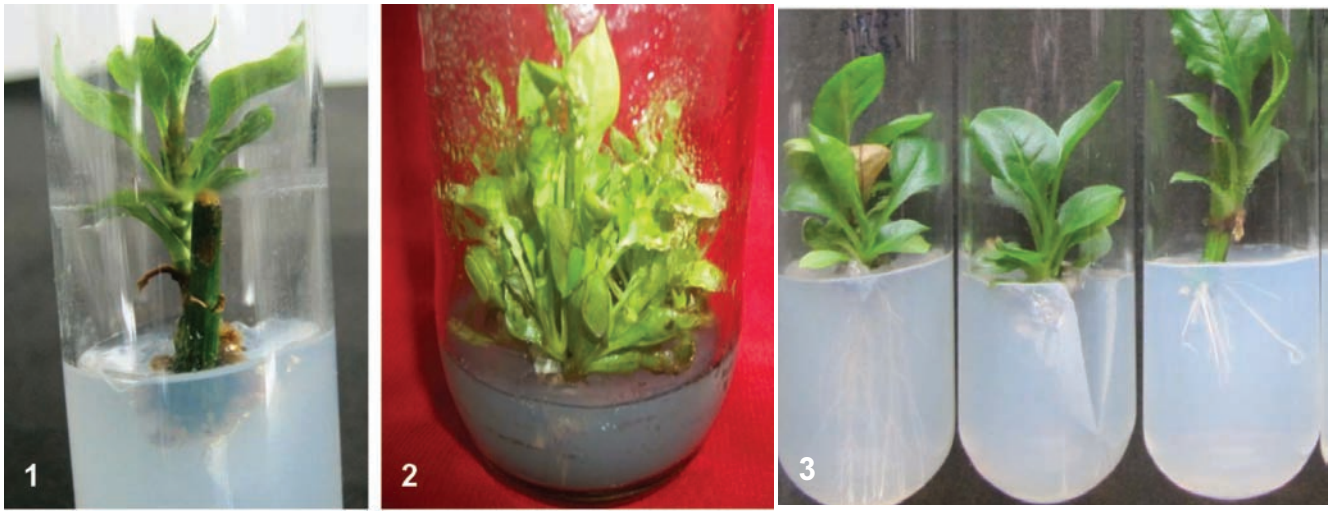

Fig. 1. Aseptic nodal culture establishment of *P. zelinica* in MS medium supplement with 1.5 mg/l BAP. Fig. 2. Nodal shoot multiplication of *P. zelinica* in MS medium supplement with 2mg/l BAP + 100 mg/l adenosine sulphate. Fig. 3. *In vitro* rooting of multiple shoots of *P. zelinica* in MS medium supplemented with 0.5 mg/l IBA.

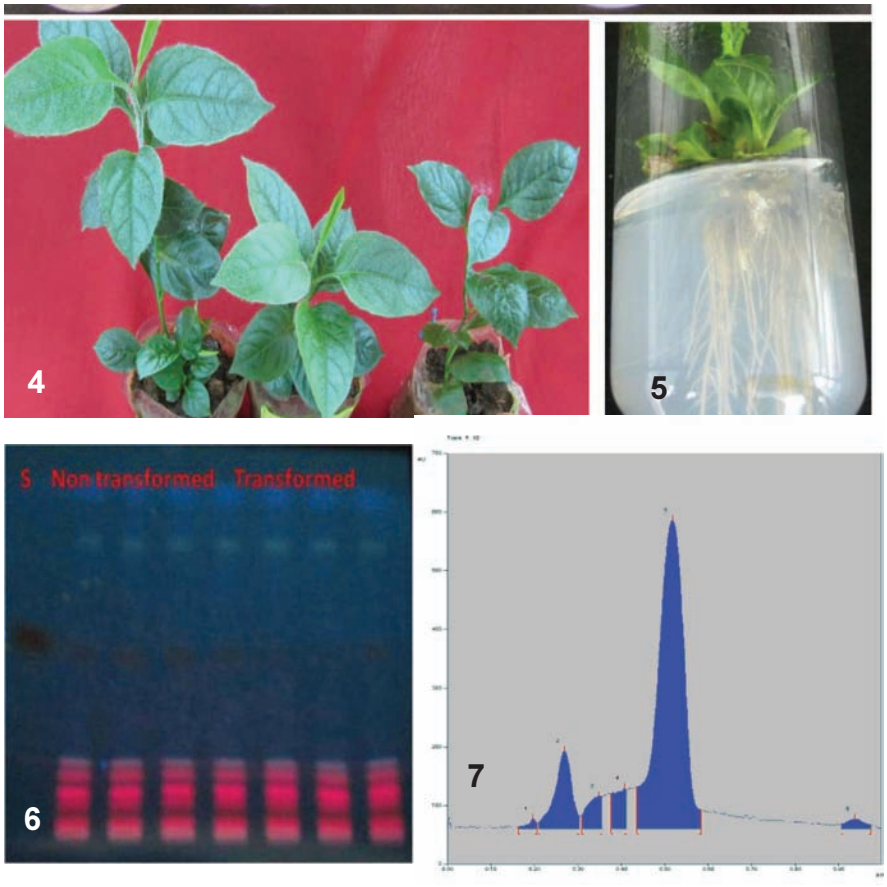

Fig. 4. Hardening of rooted plants of *P. zelinica* in net house. Fig. 5. Hairy root initiation of *P. zelinica* aseptically grown nodal shoot tip transformed with *Agrobacterium rhizogenes.* Fig. 6. HPTLC plats showing plumbagin in transformed and non transformed roots. Fig. 7. HPTLC picks showing plumbagin content in transformed and non-transformed root samples.

4. Conclusion

In vitro nodal multiplication, rooting and hardening of *Plumbago zeylanica,* a medicinal plant having potential active principle napthoquinone - plumbagin, is standerized. Hairy root culture through *Agrobacterium rhizogenes* mediated genetic transformation is also successful with A4-strain. Pricking method in aseptically grown *in vitro* generated multiple shoots and leaves were found more effective in shoots as compared to leaf explants. The establishment of hairy root cultures through *Agrobacterium rhizogenes* mediated genetic transformation could produce more roots as compared to normal root produced with a higher production of a bioactive compound, plumbagin, in transformed root. Plumbagin content was found to be more in transformed roots (8.13%) than non- transformed roots (7.52%) as detected by HPTLC method.

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Effect of plant growth regulators and explant types on micropropagation of an endangered medicinal plant *Blepharispermum subsessile* DC.

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ABSTRACT

A reproducible protocol for *in vitro* plant regeneration of an endangered medicinal plant *Blepharispermum subsessile* was developed. The protocols were developed for establishment of secondary culture from shoot tips and nodal segments. They were cultured on MS medium supplemented with different concentration of BAP and Kn either alone or in combination with GA_3, IAA, IBA and NAA. Of the different cytokinin evaluated, bud break with multiple shoot proliferation in nodal segment and shoot tip explants was best achieved in MS medium supplemented with 1.5 and 2.5 mg/l BAP respectively. Percentage shoot development and the number of shoots per shoot tip was maximum in MS augmented with 1.5 mg/l BAP + 0.2 mg/l IAA, where *ca.* 91% cultures produced 6 shoots/explant with an average length of 2.43 cm over a period of 4 weeks. The maximum rooting regeneration was observed in ½ MS + 2% sucrose + 1.0 mg/l IAA, with a average 5.83 roots per regenerated shoots. Well rooted plants showed 39% survival during hardening under green house condition.

1. Introduction

Blepharispermum is a genus of the aster family Asteraceae, distributed in Africa, Madagascar, the Arabian Peninsula, India, and Sri Lanka. Out of the reported 16 species, *B. subsessile* DC. is found in moist deciduous forests of Odisha, Andhra Pradesh, Madhya Pradesh, Tamil Nadu, Karnataka and Maharashtra of India. It is known as "Rasnajhadi" in Odisha, "Naama banta" in Karnataka and "Adavi banti" in Andhra Pradesh and Telengana. This plant has been used in Indian system of medicine for formulation of a number of Ayurvedic drugs and also by tribals for ailment from various kind of diseases (Nayak and Kalidass, 2016). Under natural condition, the plant propagates only through seeds. Seed setting in this species is low and germinability of seeds is too poor. As this plant is an important medicinal species, local people harvest its aerial parts and rhizomes indiscriminately for their own use and trade. Human interference coupled with habitat degradation has been the causal factors for endangerment of the species in the wild. Further, no attempts have been made so far for *in situ* conservation of this rare medicinal plant species. However, a recent study reported multiple shoot regeneration from cotyledons of axenically grown seedlings of *Blepharispermum subsessile* (Nayak and Kalidass, 2016). In view of its threat status and problems associated with seed availability and germination, the present study aims at developing secondary cultures using nodal segments and shoot tips from already established primary cultures of this plant species.

2. Materials and methods

2.1 Collection of plant materials

Nodal segments and shoot tips of *B. subsessile* DC. were obtained from aseptically grown cultures of this plant species. The primary cultures were developed from cotyledonary nodes obtained by *in vitro* seed germination.

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The primary cultures were maintained in MS medium (Murashige and Skoog, 1962) supplemented with different concentration of BAP (6-benzyl aminopurine) and GA_3 (gibberellic acid).

2.2 Establishment of in vitro culture

The formulation of Murashige and Skoog's (1962) basal medium (MS) has been used in the present investigation. Shoot tips and nodal segments were aseptically excised from primary shoots and were inoculated in previously sterilized (autoclaved at 121ÚC and 15 psi for 15 – 20 min) MS medium supplemented with BAP (0.5 – 5.0 mg/l)or Kn (kinetin; 0.5 – 5.0 mg/l) either alone or in combination with Kn (0.5 - 2.0 mg/l), GA_3 (0.2 - 2.0 mg/l), IAA (indole-3-acetic acid), NAA (Naphthalene acetic acid) or IBA(Indole butyric acid) at 0.2 – 1.0 mg/l. Three explants were inoculated onto each culture vial containing 50 ml media. All the cultures were incubated in a growth room with a 16h photoperiod provided by cool, white fluorescent tubes (30 µmol m/s) and the temperature maintained at 25 ± 2°C, with 50-80% relative humidity.

2.3 Subculture of micro shoots and rooting

Proliferated micro shoots were separated and those measuring 2-3 cm and above were individually planted onto the full, half and quarter strength MS basal medium, with different sucrose concentrations and with or without the supplementation of auxins (0.2 - 2 mg/l) like IAA, NAA or IBA for rooting.

2.4 Hardening

Well rooted *in vitro* raised plants were carefully removed from the cultural vials and test tubes. These were washed thoroughly under running tap water to remove agar adhering to their roots. They were treated with (0.5%) bavistin (Bayar, India) solution for 10 minutes and plantlets were transferred to small plastic pots and thermo cool cups containing autoclaved vermiculite, soil and farmyard manure in 1:1:1 ratio. The plantlets were maintained in greenhouse condition and were watered every alternate day with tap water. Well-established and hardened plants were transferred to poly-pots containing growing media.

2.5 Data analysis

Experiments were set up in completely randomized design and repeated thrice with ten explants per replicate. Observations on the number of days taken for bud breaking, percentage response, the number of multiple shoots, and mean shoot length per culture were recorded at 30 days after inoculation in the shoot induction medium. Data on the number of days taken for root initiation, percentage response, the number of roots per shoot, root length and nature of roots were collected from the *in vitro* shootlets after 30 days of culturing in the rooting media. Data were statistically analyzed using analysis of variance (ANOVA) and Duncan Multiple Range Test (DMRT) using SPSS 11.5; SPSS Inc., Chicago IL, USA) software. Significance differences were determined at the $p \leq 0.05$ level. The photographic illustrations were also provided wherever necessary.

3. Results and discussion

The most common approach in tissue culture is to isolate organized meristems like shoot tips or axillary buds and induce them to grow into complete plants. Out of the two explants taken for this study, shoot tips showed better response in culture. In the present study, shoot tips and nodal segments inoculated in MS basal medium devoid of growth regulators failed to show any regenerative response (Table 1 & 2). The addition of plant growth regulators especially cytokinins (BAP and Kin) to the basal culture medium resulted in bud break and shoot proliferation. About 91% shoot tips cultured in MS media augmented with BAP (1.5 mg/l) and IAA (0.2 mg/l) responded well producing an average of 6.33 shoots per explants with an average length of 2.43 cm (Table 1; Fig. 1A & B). On the other hand, about 88% nodal segments cultured in MS augmented with BAP (1.5 mg/l) alone responded producing an average of 4.67 shoots per explant with an average length of 3.17 cm per shoot (Table 2; Fig. 1C & D). This result is in conformity with the findings of Gnanaraj *et al.* (2011) in *Alternanthera sessilis* where shoot tips were more responsive than nodal segments. Sudharsan *et al.* (2015) observed that the type of explant markedly influenced the shoot organogenesis and growth of the regenerated shoots in *Hybanthus enneaspermus*. They found that the number of shoots formed per regenerative explant was greater with shoot tips in BAP (2.5mg/l) than nodal segments in BAP (2.0 mg/l). However, in *Sphaeranthus amaranthoides* nodal segments were found to be more responsive than shoot tips (Ravipaul *et al.*, 2008; Devika *et al.*, 2012). Out of the two cytokinins tested, BAP was most effective in inducing multiple shoots (Fig. 1E) in both the explants. The highest response was scored at the optimal concentration of BAP (2.5 mg/l) for shoot tips. Increasing concentrations of BAP beyond 2.0 mg/l reduced the number of shoot buds formed per explant. Highest elongation of regenerated shoots (average length 2.9 cm/shoot) was observed in BAP (1.5 mg/l). Shoot length decreased with increasing concentration of BAP beyond 1.5 mg/l. These shoots had narrow leaves and they showed stunted growth with minimum internodal elongation (Fig. 1F). Supplementations of Kn in the culture medium failed to show multiple shoot proliferation. The existing shoot bud

elongated producing a single shoot in both the explants (Table 1 & 2). Jawahar *et al.* (2008), Komalavalli and Rao (2000) and Reddy *et al.* (1998) reported that the MS medium containing BAP was more effective than Kn for induction of multiple shoots. Contrary to the findings of this study, Sudharsan *et al.* (2015) found that in *Hybanthus enneaspermus*, multiple shoots can be induced from explants treated with Kn. No significant variation was seen in multiple shoot induction and shoot elongation in all the combined concentrations of BAP & Kn (Table 1 & 2). In *Hybanthus enneaspermus,* shoot tips produced more shoot buds than nodal segments when BAP was used in combination with Kn (Sudharsan *et al.*, 2015).

A combined effect of a cytokinin BAP with GA_3 was also evaluated for multiple shoot induction. Addition of GA_3 (0.2 – 2.0 mg/l) along with BAP (1.5 mg/l) to the basal medium failed to show any significant morphogenic response (Table 1 & 2). Effect of the combination of cytokinin (BAP) with different auxins (IAA, NAA, and IBA) was also evaluated for multiple shoot induction. The addition of auxin (0.2 – 1.0 mg/l) along with a cytokinin BAP (1.5 mg/l) to the basal medium enhanced the morphogenic response significantly in the shoot tip explants and no significant variation was observed in case of nodal segments (Table 1 & 2). Percentage shoot development and the number of shoots produced per explant was maximum in MS basal medium supplemented with 1.5 mg/l BAP + 0.2 mg/l IAA, where *ca.* 91% cultures responded producing 6.33 shoots/ explant with an average length of 2.43 cm over a period of 4 weeks (Table 1 & 2). Shoots produced from shoot tip explants in MS basal medium supplemented with 1.5 mg/l BAP + 0.8 mg/l IAA attained highest shoot length of 3.93 cm/shoot. Among the three auxins tested, IAA at lower concentration was found most effective followed by IBA. NAA-treated explants showed callusing at the base of the regenerated shoots. In IBA treated cultures, the leaves were dark-green in color. Some successful plant growth regulator combinations used for multiple shoot induction have been reported earlier *viz.* BAP + IAA for *Desmodium gangeticum* (Behera and Thirunavoukkarasu, 2006); BAP + NAA for *Rauvolfia serpentina* (Mathur *et al.*, 1987), *Gomphrena officinalis* (Mercier *et al.*, 1992), *Gloriosa superba* (Hassan and Roy, 2005), *Costus speciosus* (Punyarani and Sharma, 2010), *Vernonia cinerea* (Seetharam *et al.*, 2007); BA + IBA for *Rheum emodi* (Lal and Ahuja, 1989); BAP + GA3 for *Saussurea lappa* (Arora and Bhojwani, 1989) and Kn + NAA in *Echinops kebericho* (Manahlie and Feyissa, 2014).

Rooting

Healthy and elongated shoots (2 -3 cm) were excised and cultured in different media for root induction. The effect of strengths of different media (full, half and quarter), sucrose concentration (3% and 2%) and types of growth regulators and concentration were assessed. Shoots cultured in full strength MS basal medium supplemented with 3% sucrose but without any growth regulator failed to produce any root (Table 3). In full strength MS basal medium supplemented with 2% sucrose but without any growth regulator, 42% shoots produced an average of 3.33 roots/shoot with an average length of 2.26 cm/root in 2 weeks time. In half strength MS basal medium supplemented with 2% sucrose but without any growth regulator, 100% of shoots produced an average of 4.14 roots/shoot with an average length of 4.28 cm/root in 2 weeks time. In quarter strength MS basal medium supplemented with 2% sucrose but without any growth regulator, 42% shoots produced an average of 2.67 roots/shoot with an average length of 2.96 cm/root in 2 weeks time. Augmentation of full strength MS basal medium containing 3% sucrose with auxins like IAA, NAA and IBA (0.2 – 2.0 mg/l) resulted in root formation (Table 3). About 100% of the shoots cultured in MS basal medium + 3% sucrose + IAA (1.0 mg/l) produced an average of 1.75 roots/ shoot with an average length of 2.2 cm/root in 2 weeks time. MS basal media supplemented with lower concentration of NAA (0.2 – 1.2 mg/l) failed to induce roots in shoots. Higher concentration (1.4 – 1.8 mg/l) of NAA induced rooting and was accompanied with callus formation at the base of the shoot. Shoots treated with IBA produced comparatively more number of roots, which were thick but failed to elongate further.

Augmentation of half strength MS basal medium containing 2% sucrose with auxins like IAA, NAA and IBA (0.5 – 2.0 mg/l) resulted in root formation (Table 3). But here more days were taken for root initiation compared to control (1/2 MS + 2% sucrose). Though more roots were produced here than control, the roots failed to elongate much in 2 weeks time. About 100% of the shoots cultured in 1/ 2 MS basal medium + 2% sucrose + IAA (1.0 mg/l) produced an average of 5.83 roots/shoot with an average length of 0.51 cm/root in 2 weeks time (Fig. 1G). Different growth regulators used for *in vitro* root induction have been reported *viz.* IBA for *Alternanthera sessilis* (Gnanaraj *et al.*, 2011), *Hybanthus anneaspermus* (Sudharsan *et al.*, 2015), *Phyllanthus urinaria* (Kalidass and Mohan, 2009); IAA for *Psoralea corylifolia* (Anis and Faisal, 2005); *Vernonia cinenea* (Seetharam *et al.*, 2007), *Vernonia amygdalina* (Khalafalla *et al.*, 2007), NAA for *Elephantopus scaber* (Rout and Sahoo, 2013).

Hardening

The plantlets transferred to root trainers/ poly pots containing vermiculite, soil and farmyard manure in 1:1:1

Table 1

Effect of different cytokinins BAP/Kn either alone or in combination with GA_3 and different auxins IAA/IBA/NAA on *in vitro* shoot regeneration from shoot tips of *Blepharispermum subsessile*

MS+3% sucrose+0.6% agar+ growth regulators (mg/l)						Days taken to shoot initiation	% response	No. of shoots/ explant	Shoot length /explant (in cm)
BAP	Kn	GA_3	IAA	NAA	IBA				
-	-	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	5 – 7	71	2.00 fghij	2.03 cdefghi
1.0	-	-	-	-	-	4 – 9	66	4.33 bcd	2.10 cdefghi
1.5	-	-	-	-	-	4 – 6	70	4.00 cde	2.90 abcdef
2.0	-	-	-	-	-	6 – 7	87	5.00 abc	2.47 cdefghi
2.5	-	-	-	-	-	3 – 9	88	5.67 ab	1.53 ghi
3.0	-	-	-	-	-	4 – 9	76	2.67 defghij	1.63 fghi
3.5	-	-	-	-	-	3 – 9	66	3.00 defgh	1.70 efghi
4.0	-	-	-	-	-	5 – 6	67	2.67 defgh	1.53 efghi
4.5	-	-	-	-	-	3 – 7	70	2.33 efghij	1.47 hi
5.0	-	-	-	-	-	3 – 6	81	2.33 efghij	1.50 ghi
-	0.5	-	-	-	-	5 – 7	90	1.00 j	1.90 defghi
-	1.0	-	-	-	-	4 – 6	42	1.00 j	1.90 defghi
-	1.5	-	-	-	-	3 – 9	80	1.00 j	2.00 cdefghi
-	2.0	-	-	-	-	4 – 6	50	1.00 j	1.37 hi
-	2.5	-	-	-	-	3 – 7	86	1.00 j	2.46 cdefghi
-	3.0	-	-	-	-	2 – 8	90	1.00 j	2.88 abcdef
-	3.5	-	-	-	-	4 – 7	66	1.00 j	1.95 defghi
-	4.0	-	-	-	-	3 – 5	57	1.00 j	1.82 defghi
-	4.5	-	-	-	-	5 – 8	43	1.00 j	1.88 defghi
-	5.0	-	-	-	-	4 – 7	40	1.33 hij	2.43 cdefghi
1.0	0.5	-	-	-	-	5 – 8	80	2.67 defghij	2.67 cdefgh
1.0	1.0	-	-	-	-	3 – 5	88	2.67 defghij	3.20 abc
1.0	1.5	-	-	-	-	3 – 7	76	2.67defghij	3.00 abcde
1.0	2.0	-	-	-	-	2 – 6	66	2.33 efghij	3.00 abcde
1.5	-	0.2	-	-	-	4 – 6	67	3.33 defg	1.50 ghi
1.5	-	0.4	-	-	-	3 – 7	57	4.33 bcd	2.60 cdefghi
1.5	-	0.6	-	-	-	4 – 9	42	2.67 defghij	3.10 abcd
1.5	-	0.8	-	-	-	5 – 7	57	2.33 efghij	2.93 abcde
1.5	-	1.0	-	-	-	4 – 9	61	2.67defghij	2.43cdefghi
1.5	-	1.2	-	-	-	3 – 8	70	3.00 defgh	3.73 ab
1.5	-	1.4	-	-	-	4 – 7	76	2.00 fghij	1.30 i
1.5	-	1.6	-	-	-	5 – 6	81	2.00 fghij	1.43 hi
1.5	-	1.8	-	-	-	3 – 9	88	2.00 fghij	2.13 cdefghi
1.5	-	2.0	-	-	-	4 – 6	78	2.33 efghij	1.53 ghi
1.5	-	-	0.2	-	-	3 – 9	91	6.33 a	2.43 cdefghi
1.5	-	-	0.4	-	-	4 – 7	57	2.33 efghij	2.80 abcdef

1.5	-	-	0.6	-	-	2 – 8	78	5.00 abc	3.93 a
1.5	-	-	0.8	-	-	4 – 9	87	3.33 defg	3.93 a
1.5	-	-	1.0	-	-	4 – 8	76	2.67 defghij	1.37 hi
1.5	-	-	-	0.2	-	3 – 6	66	1.67 ghij	2.07 cdefghi
1.5	-	-	-	0.4	-	3 – 5	76	2.75 defghi	2.32 cdefghi
1.5	-	-	-	0.6	-	4 – 9	57	2.33 efghi	2.33 cdefghi
1.5	-	-	-	0.8	-	4 – 6	59	2.50 efghij	1.80 defghi
1.5	-	-	-	1.0	-	4 – 8	43	1.25 ij	1.32 i
1.5	-	-	-	-	0.2	5 – 7	55	2.67 defghi	1.47 hi
1.5	-	-	-	-	0.4	4 – 7	73	3.67 cdef	1.83 defghi
1.5	-	-	-	-	0.6	3 – 8	63	2.50 efghij	1.95 cdefghi
1.5	-	-	-	-	0.8	3 – 9	66	3.00 defgh	2.37 cdefghi
1.5					1.0	4 – 7	67	2.33 efghij	2.10 cdefghi

Data pooled from three independent experiments each with 10 replicates per treatment. Data presented of 4 weeks old cultures. Mean values within column followed by the same letter are not significantly different ($p<0.05$; Duncan's Multiple Range Test)

Table 2

Effect of different cytokinins BAP/Kn either alone or in combination with GA_3 and different auxins IAA/IBA/NAA on *in vitro* shoot regeneration from nodal segments of *Blepharispermum subsessile*

MS + growth regulators (mg/l)						Days taken to shoot initiation	% response	No. of shoots/ explant	Shoot length /explant (in cm)
BAP	Kn	GA_3	IAA	NAA	IBA				
-	-	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	4 – 10	70	3.33^{bcde}	2.27 bcdefghij
1.0	-	-	-	-	-	3 – 6	78	4.33^{b}	2.47 abcdef
1.5	-	-	-	-	-	2 – 6	88	4.67 a	3.17 a
2.0	-	-	-	-	-	3 – 8	86	4.00 abc	2.53 abcde
2.5	-	-	-	-	-	4 – 6	87	3.67 abcd	1.93 defghijkl
3.0	-	-	-	-	-	6 – 9	63	2.00 fghi	1.8 defghijkl
3.5	-	-	-	-	-	4 – 6	57	3.00 cdef	1.57 ghijkl
4.0	-	-	-	-	-	3 – 12	81	3.67 abcd	1.3 l
4.5	-	-	-	-	-	5 – 9	87	3.67 abcd	1.73 efghijkl
5.0	-	-	-	-	-	3 – 7	69	3.67 abcd	1.5 hijkl
-	0.5	-	-	-	-	5 – 9	87	1.00 i	1.94defghijkl
-	1.0	-	-	-	-	4 – 7	81	1.00 i	2.32 bcdefgh
-	1.5	-	-	-	-	3 - 9	76	1.00 i	2.42 abcdefg
-	2.0	-	-	-	-	4 – 9	63	1.00 i	1.84 defghijkl
-	2.5	-	-	-	-	3 - 6	86	1.00 i	2.32 bcdefgh
-	3.0	-	-	-	-	4 – 6	64	1.00 i	2.41 abcdefg
-	3.5	-	-	-	-	5 - 7	62	1.00 i	1.98 defghijkl
-	4.0	-	-	-	-	4 – 7	70	1.00 i	2.84 abc
-	4.5	-	-	-	-	3 – 8	62	1.00 i	2.86 abc

-	5.0	-	-	-	-	3 – 6	76	1.00 i	1.86 defghijkl
1.0	0.5	-	-	-	-	4 – 8	66	2.67 defg	2.63 abcd
1.0	1.0	-	-	-	-	2 – 4	88	3.33 bcde	3.23 a
1.0	1.5	-	-	-	-	2 – 6	76	2.67 defg	3.00 ab
1.0	2.0	-	-	-	-	3 – 4	63	1.67 ghi	2.63 abcd
1.5	-	0.2	-	-	-	6 – 8	66	2.33 efgh	2.03 bcdefghijkl
1.5	-	0.4	-	-	-	4 – 6	76	1.67 ghi	1.5 hijkl
1.5	-	0.6	-	-	-	3 – 8	70	2.33 efgh	1.5 hijkl
1.5	-	0.8	-	-	-	6 – 9	76	2.33 efgh	1.4 jkl
1.5	-	1.0	-	-	-	4 – 5	84	2.33 efgh	2.3 bcdefghi
1.5	-	1.2	-	-	-	3 – 5	81	3.33 bcde	1.33 kl
1.5	-	1.4	-	-	-	3 – 7	57	2.00 fghi	1.77 efghijkl
1.5	-	1.6	-	-	-	4 – 8	86	2.33 efgh	1.83 defghijkl
1.5	-	1.8	-	-	-	3 – 9	76	2.67 defg	1.97 defghijkl
1.5	-	2.0	-	-	-	5 – 6	66	2.00 fghi	1.67 fghijkl
1.5	-	-	0.2	-	-	3 – 5	77	2.33 efgh	1.57 ghijkl
1.5	-	-	0.4	-	-	2 – 4	87	3.33 bcde	1.53 hijkl
1.5	-	-	0.6	-	-	4 – 7	67	1.67 ghi	2.2 bcdefghijk
1.5	-	-	0.8	-	-	3 – 6	63	2.00 fghi	2.23 bcdefghij
1.5	-	-	1.0	-	-	3 – 5	77	3.33 bcde	1.43 kl
1.5	-	-	-	0.2	-	4 – 8	66	2.33 efgh	1.9 defghijkl
1.5	-	-	-	0.4	-	4 – 6	70	2.33 efgh	1.63 fghijkl
1.5	-	-	-	0.6	-	3 – 7	76	2.25 efghi	1.45 hijkl
1.5	-	-	-	0.8	-	2 – 7	71	1.33 hi	1.33 kl
1.5	-	-	-	1.0	-	4 – 7	66	1.00 i	1.2 l
1.5	-	-	-	-	0.2	4 – 8	70	2.67 defg	1.53 hijkl
1.5	-	-	-	-	0.4	5 – 7	82	3.00 cdef	1.6fghijkl
1.5	-	-	-	-	0.6	5 – 8	63	1.67 ghi	2.3 bcdefghi
1.5	-	-	-	-	0.8	3 – 7	66	2.00 fghi	2.3 bcdefghi
1.5	-	-	-	-	1.0	6 – 8	87	3.33 bcde	1.57 ghijkl

Data pooled from three independent experiments each with 10 replicates per treatment. Data presented of 4 weeks old cultures
Mean values within column followed by the same letter are not significantly different ($p<0.05$; Duncan's Multiple Range Test)

Table 3

Effect of different media strength, sucrose concentration and concentrations of auxins IBA/NAA/IAA on induction of roots in *in vitro* regenerated shoots of *Blepharispermum subsessile*

MS+ 3% sucrose+ growth regulators			Days taken to root initiation	% response	No. of roots per shoot	Root length per shoot (in cm)
IAA	NAA	IBA				
-	-	-	-	-	-	-
0.2	-	-	-	-	-	-
0.4	-	-	-	-	-	-
0.6	-	-	10 – 12	60	1.00d	0.46ij

0.8	-	-	-	-	-	-
1.0	-	-	8 – 10	100	1.75bcd	2.22bcdefg
1.2	-	-	8 – 9	76	1.67bcd	1.30cdefghij
1.4	-	-	7 – 10	76	1.67bcd	2.30bcde
1.6	-	-	10 – 12	75	1.33cd	2.03bcdefg
1.8	-	-	9 – 10	75	2.67bcd	1.60cdefghi
2.0	-	-	8 – 10	60	1.67bcd	1.80bcdefgh
-	0.2	-	-	-	-	-
-	0.4	-	-	-	-	-
-	0.6	-	-	-	-	-
-	0.8	-	-	-	-	-
-	1.0	-	-	-	-	-
-	1.2	-	-	-	-	-
-	1.4	-	8 – 12	63	2.33bcd	2.40bcd
-	1.6	-	7 – 9	15	1.33cd	1.43cdefghij
-	1.8	-	10 – 12		1.50cd	1.00fghij
-	2.0	-	-	-	-	-
-		0.2	-	-	-	-
-		0.4	8 – 10	80	2.33bcd	2.50bc
-		0.6	8 – 12	88	3.00abcd	1.60cdefghi
-		0.8	-	-	-	-
-		1.0	7 – 9	80	1.67bcd	0.97ghij
-		1.2	8 – 9	69	3.00abcd	1.06efghij
-		1.4	9 – 11	66	2.67bcd	0.30ij
-		1.6	-	-	-	-
-		1.8	10 – 12	63	1.33cd	2.20bcdefg
-		2.0	7 – 9	50	2.00bcd	2.13bcdefg
MS+ 2% sucrose		-	8 – 10	42	3.33abcd	2.26bcdef
½ MS+2% sucrose		-	6 – 7	100	4.14abcd	4.28a
½ MS+ 2% sucrose+ growth regulators						
0.5	-	-	11 – 15	90	4.17abcd	0.60hij
1.0	-	-	8 – 12	100	5.83a	0.51ij
2.0	-	-	12 – 15	57	4.00abcd	0.46ij
-	0.5	-	10 – 12	90	4.40abc	0.40ij
-	1.0	-	10 – 15	28	1.33cd	0.26bcdefg
-	2.0	-	8 – 12	57	3.75abcd	0.37ij
-	-	0.5	10 – 15	42	3.67abcd	1.20defghij
-	-	1.0	10 – 12	42	3.33abcd	0.46ij
-	-	2.0	8 – 12	57	4.75ab	0.42ij
¼ MS+ 2% sucrose			13 – 15	42	2.67bcd	2.96b

Data pooled from three independent experiments each with 10 replicates per treatment. Data presented of 4 weeks old cultures. Mean values within column followed by the same letter are not significantly different ($p<0.05$; Duncan's Multiple Range Test)

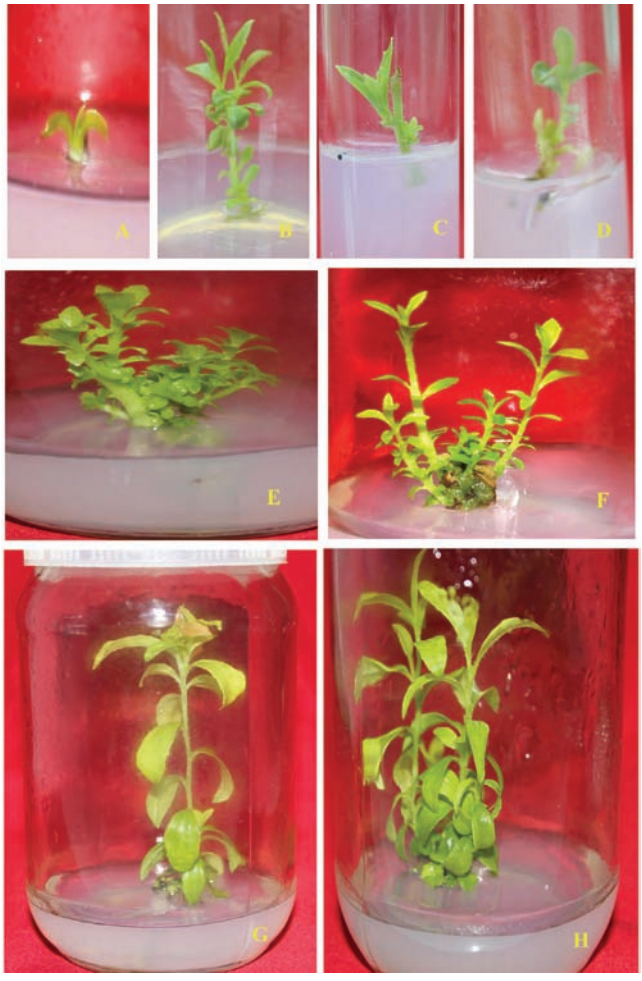

Fig. 1. Multiple shoot induction of *Blepharispermum subsessile* DC., A. Bud break in shoot tip in MS+BAP (1.5mg/l) + IAA (0.2mg/l); B. Shoot initiation in shoot tip in MS+BAP (1.5mg/l) + IAA (0.2mg/l) after 6 days; C. Bud break in nodal segment in MS+BAP (2.5mg/l); D. Shoot initiation in nodal segment in MS+BAP (2.5mg/l); E & F. Multiple shoot proliferation and G & H. Shoot elongation.

Fig. 2. Root induction of *Blepharispermum subsessile* DC., In *in vitro* rooted shoot in A. MS+IBA (0.6mg/l); B. MS+NAA (1.4mg/l); C. % MS+IAA (1.0mg/l); D. *In vitro* rooted shoots; E. An acclimatised plantlet in the polypot after 2 weeks of transfer and F. Acclimatised plantlets in root trainer.

ratio and maintained under greenhouse condition initially showed very slow growth. About 39% of micro-propagated plants survived hardening under greenhouse condition. It was observed that shoots with well-developed roots had a greater survival ability compared to those with shorter and slender roots (Fig.1H).

4. Conclusion

The results presented here suggest an efficient, reproducible and easy-to-handle protocol for *in vitro* regeneration of *Blepharispermum subsessile* using secondary cultures developed from shoot tips and nodes of primary shoots. The method has practical significance and the process has to be successfully exploited for large-scale production of cloned plants of this endangered medicinal plant species. The micro-propagation technique now developed will be helpful in producing planting materials in large-scale for raising commercial plantations and augmenting existing germplasm of this important and rare medicinal plant species in natural habitats.

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Micropropagation of *Syzygium cumini* (Linn.) Skeels through *in vitro* culture of seedling derived shoot tips

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ABSTRACT

Shoot tip explants from *in vitro* grown seedlings were cultured on Murashige and Skoog's (MS) medium supplemented with different cytokinins alone or in combination with auxins for multiple shoot induction. Benzylamino purine (BAP, 1.0mg/l) was most effective for multiple shoot proliferation. *In vitro* regenerated shoots were best rooted on ½ MS medium supplemented with 1.4 mg/l Indole-3-butyric acid (IBA). About 95 % of *in vitro* derived plantlets were successfully acclimatized, hardened and established in natural condition.

1. Introduction

Syzygium cumini, commonly known as Jamun in Hindi and black plum in English, is a member of the family Myrtaceae. It has great importance in the food as well as wood industry and is useful in social forestry programme (Anonymous, 1992). The tree has a great economic importance since most of the parts including the bark, leaves, seed and fruits are used as alternative medicine to treat various diseases (Chaudhary and Mukhopadhyay, 2012). In traditional system of medicine it is extensively used against diabetes and sore throat (Schossler *et al*., 2004). The tree (aerial parts like stem, bark, leaves, flower, fruit, seed) is rich in phytochemicals like glycoside jambolin, anthocyanins, tannins, terpenoids, gallic acid and various minerals (Chaudhary and Mukhopadhyay, 2012). Its bark is used as astringent in dysentery; seeds are antidiabetic; fruits are used to treat cough, diabetes, dysentery, inflammation (Swami *et al*., 2012).

Syzygium cumini suffers from very low seed viability and poor germination in its natural habitat (Dent, 1948). Multiplication in Jamun is also carried out through budding and grafting, but to obtain a scion or bud it requires a fresh shooting period and also population maintained is very low and budding showed less success (Choudhri *et al*., 2013). Tissue culture has emerged as a science with a vast potential for human welfare ranging from large-scale plant production in horticulture and forestry, human health, plant protection as well as environmental protection (Anis and Ahmad, 2016). However woody plants are generally not easy to culture due to constraints of episodic growth pattern, their recalcitrant nature, complex vegetative life cycle, and phenolic exudation. Furthermore, their regeneration and multiplication is not easy under *in vitro* condition, especially when explants are taken from mature trees. They often secrete substances into the medium in response to wounding or excision which inhibits the growth and development of explants *in vitro* (Naaz *et al*., 2014). A few tissue culture works has been done in *Syzygium cumini*. Hence in the present study attempts has been made for mass multiplication of this plant using shoot tip explants from *in vitro* seedlings.

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2. Materials and Methods

Seeds of *Syzygium cuminii* were collected during July. Then they were brought to the laboratory and thoroughly washed with running tap water for about an hour. The seeds treated with 5% teepol (v/v) for 5 minutes and then washed with distilled water. The seeds were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) solution for about 3-5 minutes followed by thorough wash with sterilized distilled water for 3-4 times to remove traces of $HgCl_2$. Seeds were then inoculated on half and full strength of Murashige and Skoog's (1962) basal medium (MS) with and without agar (0.8%). Shoot tips from two weeks old seedlings were excised aseptically and cultured on MS medium alone or supplemented with different concentrations (0.2 to 1.5 mg/l) and combinations of growth regulators (6 benzylamino purine or BAP, Kinetin or Kn, Indole-3-acetic acid or IAA, Indole-3-butyric acid or IBA, α-Napthalene acetic acid or NAA). The *in vitro* grown shoots were transferred on to half and full strength of MS basal medium without or supplemented with auxins (1.0 -1.8 mg/l of IBA, IAA or NAA) for root induction. All the cultures were maintained at 25 $\pm$ 2^0C with 16 h photoperiod at 55% relative humidity. For proper growth and development the cultures were frequently transferred onto fresh medium at fifteen days intervals.

For each treatment, ten replicates were taken and each experiment was carried independently and repeated thrice. The cultures were kept under regular observation and data were recorded at 7-day interval. The percentage of response, mean number of shoots, mean shoot length, mean number of roots and standard error of mean (S.E.M) for each treatment were calculated.

3. Results

3.1 In vitro seed germination

Germination of seeds was observed in all the media tested but the best response (90%) was observed on ½ MS liquid medium. The seeds took 5-6 days for germination and after two weeks 4 shoots (due to polyembryony) developed (Fig. 1).

3.2 Multiple shoot induction

The shoot tips were carefully excised from *in vitro* grown seedlings and transferred to MS media devoid of any growth regulators or MS supplemented with different concentrations and combinations of growth regulators. The explants failed to show any morphogenetic response on growth regulator free MS medium. Shoot tips cultured on MS media supplemented with different concentrations of cytokinins (0.5 to 1.5 mg/l of BAP or Kn) alone or in combination with lower concentrations of auxins (0.2 and 0.5 mg/l of IAA, IBA or NAA) responded differently (Table 1).

Among the different concentrations and combinations of growth regulators tested, best response was observed on MS medium supplemented with 1.0 mg/l BAP (Table 1). BAP was found to be more effective than Kn. It was also observed that addition of auxins to the cytokinin supplemented medium reduces the percentage of response and number of shoots per explant. Shoot buds started developing after one week of inoculation on MS medium supplemented with 1.0 mg/l BAP (Fig. 2) and more number of shoot buds were developed after two weeks (Fig. 3). Elongation of shoots and development of more shoots were observed after three weeks of culture (Fig. 4). An average of 14.4 ± 0.2 shoots having an average of 2.2 ± 0.1 cm shoot length per explant was recorded after four weeks of culture (Table - 1, Fig. 5).

3.3 In vitro rooting

The *in vitro* regenerated shoots (more than 2cm) were carefully separated and transferred to rooting medium. Full strength of MS medium with or without auxins and ½ MS medium without auxins did not respond for rhizogenesis but showed callusing at the base. After four weeks of culture it was noticed that ½ MS basal media supplemented with IBA produced better response than the media supplemented with IAA or NAA. Among all the concentrations tested 1.4 mg/l IBA showed best response for rooting (Table – 2). Half - strength MS medium supplemented with 1.4 mg/l IBA produced an average of 4.6 ± 0.2 roots with an average length of 4.2 ± 0.2 cms (Table 2; Fig. 6).

3.4 Acclimatization

After four weeks of maintenance in the rooting medium the plantlets with well developed roots were carefully removed from the culture tubes and washed thoroughly by sterilized double distilled water to remove any remains of medium. The well rooted plantlets were transferred to thermo cool glass containing a sterile mixture of garden soil, sand and compost in the ratio of 1:1:1 (Fig. 7). They were irrigated with MS liquid medium at two days interval for fifteen days and kept in the culture room at 25 ± 2^0 C with 16 h photoperiod at 55% relative humidity. Gradually they were irrigated with ½ MS liquid medium and then by plain water and transferred to less humid conditions in order to acclimatize the plants to normal atmospheric conditions. About 95 percent of the regenerated plants survived after hardening procedure (Fig. 8). No morphological difference was found in between the natural and *in vitro* grown plants.

Table 1

Effect of plant growth regulators on multiple shoot formation from shoot tip derived from *in vitro* seedling of *Syzygium cumini*

Plant growth regulators (mg/l)					% response	Shoots/explant	Shoot length in cm
BAP	Kn	IAA	IBA	NAA		Mean* ± S.E.M	Mean* ± S.E.M
0.5					63	9.2 ± 0.2	1.4 ± 0.1
1.0					80	14.4 ± 0.2	2.2 ± 0.1
1.5					66	11.3 ± 0.1	1.7± 0.2
	0.5				46	6.1 ± 0.1	1.0 ± 0.1
	1.0				66	9.6 ± 0.2	1.5 ± 0.2
	1.5				50	7.4 ± 0.2	1.2 ± 0.1
1.0		0.2			70	12.1 ± 0.1	1.8 ± 0.1
1.5		0.5			60	8.6 ± 0.2	1.3 ± 0.1
	1.0	0.2			56	7.3 ± 0.1	1.3 ± 0.2
	1.5	0.5			43	5.7 ± 0.1	1.1 ± 0.1
1.0			0.2		66	9.5 ± 0.2	1.2 ± 0.1
1.5			0.5		56	6.6 ± 0.1	1.0 ± 0.1
	1.0		0.2		53	5.8 ± 0.1	1.1 ± 0.2
	1.5		0.5		40	3.7 ± 0.2	0.9 ± 0.1
1.0				0.2	73	10.7 ± 0.3	1.6 ± 0.1
1.5				0.5	60	7.8 ± 0.2	1.4 ± 0.1
	1.0			0.2	56	6.2 ± 0.1	1.3 ± 0.1
	1.5			0.5	46	4.2 ± 0.1	1.0 ± 0.2

*Mean values given in the table are the average of three independent experiments each with 10 replicates with the standard error

Table 2

Effect of auxins on root induction of *in vitro* shoots

Auxins (mg/l)			% rooting	Number of roots/shoot	Root length in cm
IBA	IAA	NAA		Mean* ± S.E.M	Mean* ± S.E.M
1.0			80	2.8 ± 0.1	2.3 ± 0.2
1.2			83	3.7 ± 0.2	3.5 ± 0.3
1.4			96	4.6 ± 0.2	4.2 ± 0.2
1.6			90	3.2 ± 0.2	3.1 ± 0.2
1.8			73	2.4 ± 0.1	2.2 ± 0.1
	1.4		76	2.3 ± 0.1	3.2 ± 0.2
	1.6		83	2.7 ± 0.2	2.8 ± 0.1
	1.8		70	1.8 ± 0.1	1.8 ± 0.1
		1.4	70	2.2 ± 0.2	2.7 ± 0.1
		1.6	60	1.6 ± 0.1	2.1 ± 0.2
		1.8	53	1.2 ± 0.1	1.6 ± 0.1

*Mean values given in the table are the average of three independent experiments each with 10 replicates with the standard error

Fig. 1: Germination of seed on ½ MS medium after 2 weeks of culture, Fig. 2: Shoot tips developed after one week on MS + 1.0 mg/l BAP, Fig. 3: Multiple shoots regeneration from shoot tips after two weeks on the same medium, Fig. 4: Multiple shoots after three weeks of culture, Fig. 5: Multiple shoots proliferation after four weeks of culture on MS + 1.0 mg/l BAP, Fig. 6: Rooting on ½ MS medium + 1.4 mg/l IBA, Fig. 7: Regenerated plant acclimatized on a potting mixture of 1:1:1 of soil, sand and compost, Fig. 8: *In vitro* regenerated plants growing in natural condition.

4. Discussion

In the present study it was observed that MS medium supplemented with BAP induced multiple shoots in shoot tip explants derived from two weeks grown *in vitro* seedlings. In contrast Yadav *et al*. (1990) also studied *in vitro* micropropagation through culture of shoot tip and node from 10-15 day-old seedling of *Syzygium cumini* using MS medium supplemented with BA singly and in combination with NAA, IAA or IBA. Jain and Babbar (2000) studied multiple shoot development from epicotyl segments of *in vitro* seedling of *Syzygium cumini* using MS medium supplemented with 1.0 mg/l BA. In this study we also observed that MS medium supplemented with only BAP 1.0 mg/l was best for induction of multiple shoots from shoot tip and it corroborates the findings of Jain and Babbar (2000).

In the present study root induction was observed on ½ MS medium supplemented with IBA, IAA and NAA. However best response was observed on half strength of MS medium supplemented with 1.4 mg/l IBA. IBA was also reported to have favoured root induction in earlier reports of *S. cumini* (Remashree *et al*., 2007; Choudhri *et al*., 2013; Vidwans *et al*., 2015). IBA induced rooting was also reported in other fruit trees including pomegranate (Naik *et al*., 1999). Rathore *et al*. (2004) also reported best rooting on half-strength MS + 10.0 µM IBA. However addition of 0.1% activated charcoal to the medium was found to be essential. Contrary to our work Jain and Babbar (2000) and Jain and Babbar (2003) reported rooting on Knop's medium supplemented with 1.0 mg/l IAA.

5 Conclusion

The present work describes a protocol for mass multiplication of *Syzygium cumini*, an economically important species, using shoot tip explants from *in vitro* grown seedlings. This method of plant regeneration has the potential to meet the demand of quality planting materials of the species for commercial utilization.

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Herbal antidotes: A possible replacement for serum antivenom with effective lead compound using bioinformatics tools

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ABSTRACT

Anti snake venom serum is the only rescue method in health centerswhen there is a fatal snake bite condition. And these anti venoms derived from animal tissues are not properly designed so they have a lot of side effects like anaphylaxis and even a high dose can be fatal. So instead of serum anti venom, various plant products can be used which have less side effects. These plant extracts must be tested in silico with their structure for effective proper binding with the target venom protein. These herbal antidotes must be designed in such a way that it can target the venom protein. To know the exact lead compound which can act upon that protein, Bioinformatics tools are used as a starting point for in silico lead compound development. Toxic protein are downloaded from PDB site and are docked with probable lead compound and based on the docking respective lead compounds are screened. This is an attempt to show the probable lead compounds extracted from plants which can act upon various venom proteins and can be a probable cure for wide ranges of snake bites.

1. Introduction

More than 2,000,000 snake bites are reported in the India, and it is estimated that >50000 people die of snakebite each year. Still it is a surprise that snake bite poisoning is seldom mentioned as a priority for health research in the developing country like India (Barma *et al*., 2014) Snake envenomation is an important global health issue. Snakebite is declared as a "Neglected Tropical Disease" by the World Health Organization. As a result, this may be considered as a matter of global health concern for the people in general and the rural communities of the developing countries in particular. It constitutes an occupational hazard especially in field of agriculture for farmers, farm labours, villagers, migrating population and hunters. It is a major health hazard that leads to high mortality and great suffering in victims. Highest incidence and mortality due to snake bites is reported from South and Southeast Asian countries having extensive agricultural practices and diversity in snake specie (Gupta & Peshin, 2014). There are more than 3000 known species of snakes of which around 300 are poisonous. In India out of 216 species, approximately 53 are poisonous. It is estimated that in India alone, there are more than 2, 00,000 venomous bites per year, of which 35,000-50,000 are fatal. The estimates are arbitrary as majority of cases goes unreported. In rural areas, where most of the bites take place, the victims are mostly taken to traditional healers, who neither report them to the authorities nor document the cases, hence paucity of reliable epidemiological data. The factors mainly responsible for high mortality associated with scorpion bite are poor health services, difficult and untimely transportation facilities, wrong traditional beliefs, delay in anti-snake venom administration.

2. Snake venom proteins

Snake venoms are complex mixtures of small molecules and peptides/proteins, and most of them display

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certain kinds of bioactivities. They include neurotoxin, cytotoxic, cardio toxic, myotoxic, and many different enzymatic activities (Chan *et al*., 2016). Snake venom toxicity is due to the cumulative effect of various toxins present in the venom. Although most toxins are active individually, they will exert synergistic effects in combination. For example, neurotoxic components from different families of proteins act in concert and block the neuromuscular transmission. Venoms from Viperidae, Crotalidaecontains serine-proteases, hemorrhagins (fibrinogenases possessing high anti-thrombotic activity), fibrinolytic activators, metalloproteases and group II PLA2 isoenzymes, as well as non-enzymatic proteins (C-type lectins, CRISP and disintegrins) that activate or inhibit coagulant factors or platelets, or disrupt the endothelium.

Phospholipase A2 (PLA2) is probably the most thoroughly investigated toxins both in hemotoxic and presynaptic neurotoxic snake venoms. PLA2 has also been classified as a presynaptic neurotoxin, identified in the venoms of *Crotalidae*, *Elapidae*, *Hydrophiidae*and *Viperidae*snakes. PLA2 are ubiquitous intra- and extra-cellular enzymes hydrolyzing glycerophospholipids at the *sn*-2 position of the glycerol backbone releasing lysophospholipids and fatty acids, in turn arachidonate metabolites control inflammation and pain. PLA2 are responsible for the local inflammation following *viperid* snakebite envenomation. Though PLA2 has happened to be the most important compounds in major snake bite issues, it can be taken as the effective target protein.

3. Phospholipase A2 (Pla2) from *naja naja* (common cobra) venom

Cobra venom is rich in postsynaptic neurotoxins called alpha-bungarotoxin and cobratoxin (Fig. 1). Cardio- toxin content of cobra venom has direct action on skeletal, cardiac, smooth muscles, nerves and neuromuscular junction causes paralysis, circulatory, respiratory failure, cardiac arrhythmias, various heart block and cardiac arrest because the venom releases calcium ions from the surface membrane to the myocardium (Doley & Kini, 2009). Cobra venom is of smaller molecular size and rapidly absorbed into circulation. Cobra venom binds especially to Ach receptors, prevents the interaction between Ach and receptors on postsynaptic membrane result in neuromuscular blockade.

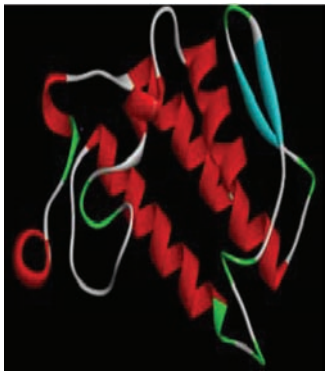

Fig.1: Three dimensional structure (PDB ID-IA3D) of Phospholipase A2 (PLA2) from *Naja naja* (Common Cobra) venom

4. Phospholipase A2 from common indian krait venom (PDB ID-1TC8)

Common Indian krait venom contains both presynaptic beta bungarotoxin and alpha bungarotoxin (Fig. 2). These toxins initially release Ach at the nerve endings, at neuromuscular junction and then damage it subsequently preventing the release of Ach. Irrespective of Krait, its venom is 10 times more lethal than cobra. But unfortunately unlike as in cobra bite, the victim reports too late due to delayed clinical manifestations. It injects the venom into skin or skin deep.

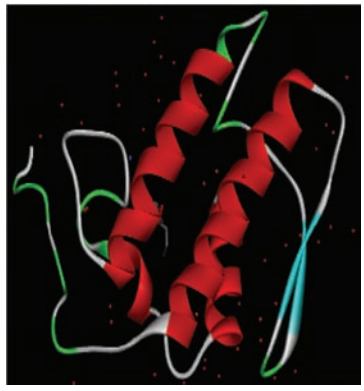

Fig.2: Three dimensional structure (PDB ID-ITC8) of Phospholipase A2 from Common Indian Krait venom

5. Phospholipase A2 from russel viper venom (PDBID-1VIP)

Viper venom interferes with blood clotting. Venoms contain serine proteases, metalloproteinases, C-type lectins, disintegrins, and phospholipases and it exhibits both anticoagulant and procoagulant effects on blood clotting mechanism resulting in defibrination syndrome or disseminated intravascular fibrino-coagulopathy (Bernard *et al*., 2001). Russell's venom is a rich source of enzymes that activates factor X to convert prothrombin to thrombin in presence of calcium factor V and platelets (Fig. 3). Haemorrhagins-1, 2 and metallo-endopeptidase causes acute rapid bleeding in brain, lungs, kidney, heart, and gastrointestinal tract. It causes severe vasoconstriction followed by vasodilatation of the microvessels.

6. Anti snake venom serum

The most effective antidote against snake venom is the anti snake venom. It is usually pepsin refined F (ab) fragments of IgG purified from the serum or plasma of a horse or sheep that has been immunized with the venom of

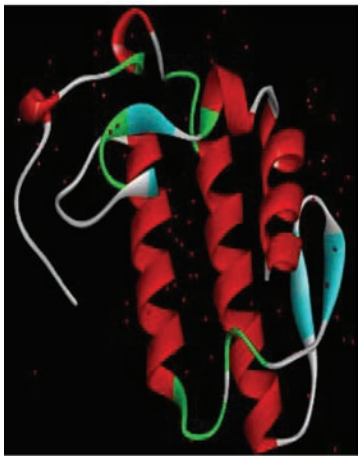

Fig.3: Three dimensional structure (PDB ID-1 VIP) of Phospholipase A2 from Russel Viper venom

one or more species of snakes (Kumarapppan *et al*., 2011). ASV neutralizes the venom of a particular species (monovalent/ monospecific) or various different species (polyvalent/polyspecific). The antibodies against a particular species may also neutralize the venom of a closely related species (paraspecific activity). In India, horses are hyper immunized against the venom of four common poisonous snakes the "Big Four" (Cobra, Krait, Russell's viper and Saw-scaled viper), to produce polyvalent anti snake venom.

7. Antivenom treatment of snake envenomation and its limitation

Antivenomes, in most countries are costly and may be in limited supply. Antivenomes for therapeutic use are often preserved as freeze-dried ampoules, but some are available only in liquid form and must be kept refrigerated. The majority of snake antivenomes are administered intravenously. The intramuscular route has been questioned in some situations as they are not uniformly effective. Antivenome should be given as quickly as possible so that the venom's side effects can be managed. It should be given only if the range of specificity is stated which includes the species known or through to have been responsible for the bite. Liquid antivenome that turned opaque should not be used because precipitation of protein indicates loss of activity which is directly proportional to increased risk of reactions. There are some critical issues with ASV. The main issues with ASV in actual clinical practice are species specificity, difficulty inavailability, affordability and ideal storage conditions. One of the principal drawbacks of the immunotherapy is the issue of specificity. There is a huge species variation with current taxonomy identifying one, four and eight species of Russell's viper, cobras and kraits, respectively. Two subspecies of saw-scaled vipers have also been identified. Russell's viper venom has also shown regional variation. So the variable composition and antigenic reactivity of the venom restricts the use of a particular ASV to a geographical area withrelevant specificity. Moreover, ASV cannot be raised against all species because the literature on distribution and diversity of venomous species is scarce. The concept of "Big Four" restricts the development of an effective ASV. Venom variation, low potency, bites by otherspecies could be responsible for the reported failure of polyvalent ASV in countering the venom effects in India.The liquid form requires cold chain. The production of monovalent ASV is a costly affair. In India the monovalent ASV is not produced. The other drawbacks with ASV therapy are the adverse reactions ranging from early reactions (pruritus, urticaria) to potentially fatal anaphylaxis. Few cases may also develop serum sickness. Endotoxin contamination could also lead to pyrogen reactions.Side effects of anti-venom therapy are anaphylactic reaction (difficulty in breathing and swallowing; hives; itching, especially of feet or hands, reddening of skin, especially around ears, swelling of eyes, face, or inside of nose, unusual tiredness or weakness, sudden and severe), serum sickness (enlargement of the lymph glands, fever, generalized rash and itching; inflammation of joints), pyrogen reaction-probably due to the action of high concentrations of non-immunoglobulin proteins present in commercially available hyper-immune anti-venom.

8. Herbal antidote: A possible choice

Thus, plants can be a possible choice as they have fewer side effects and have capacity to respond wide range of venom molecules. plant species were found to possess different herbal compounds (acids, alkaloids, steroids, enzymes, peptides, pigments, glycoproteins and glycosides, phenols, pterocarpanes, tannins, terpenoids, quinonoid xanthenes and other compounds) which are effective against snake envenomation by neutralizing different enzymes and toxins (procoagulent enzymes, haemorrhagins, cytolytic or necrotic toxins, phospholipases A2,B, C, D, hydrolases, phosphatises, proteases, esterases, acetylcholine esterase, transaminase, hyaluronidase, phosphodiesterase, nucleotidase, ATPase and nucleosidases) in venoms. Due to inadequate health care facilities especially in rural areas of India, people largely depend on alternative treatment by traditional healers who have knowledge based on ancient culture, ethnic practices and herbal antidotes (Makhija & Khamar, 2010). The plant kingdom provides an inexhaustible source of various herbal compounds with pharmacological potential which hold the key to antivenin activity. The plant kingdom has tremendous resources which have been thoroughly exploited by ethnic tribes in India.

Various phytochemicals with enzyme inhibiting and protein binding properties, active against snake envenomation include flavonoids, polyphenols, saponins, tannins,

terpenoids, xanthene etc. Phenolic, especially polyphenols, like some tannin bind proteins and act directly on venom components. They could also competitively block the receptors. Flavonoids like myricetin, quercetin, amenthoflavone have antihemorrhagic potential. Ursolic acid commonly found in many medicinal plants has enzyme inhibitory activity. Gallic acid (3,4,5-tri-hydroxy benzoic acid), on testing against the local toxicity of *Daboia russelli* venom and its purified hemorrhagic complex, showed inhibition of in-vitro proteolytic activity of both venom and hemorrhagic complex, without inhibiting phospholipase activity of venom. In-vivo experiments, showed inhibition of hemorrhage, edema forming, dermo- and myonecrotic activities of both the venom and the complex. 2- hydroxy-4-methoxy benzoic acid, salicylic acid and p-anisic acid have shown neutralization of phospholipase A2 activity of banded krait, which was superior to ASV neutralization. The terpenoids from the plant have antiproliferative effects and certain phenolic glycoside derivatives have demonstrated enzyme inhibitory activity against venom. Triterpenoids from the root extract of *Emblica officinalis* and *Vitex negundo* are suggested to significantly neutralize the venom induced effects of *Vipera russelii* and *Naja kauothia*. There is a huge collection of Indian medicinal plants used for treating snake bites.

9. Virtual screening of lead compound

Today *in silico* approaches have gained immense popularity and have become an integral part of the industrial and academic research, directing drug designing and discovery. However, bioinformatics tools have seldom been applied for analysis of plant-based medicines used in traditional systems of health care, particularly to find out anti-venom from phytochemicals (Fig. 4 & 5). Recently, efforts in this line have been made; for example, Pithayanukal *et al*. (2009) made an attempt for *in vitro* and molecular docking studies for revealing the anti-snake venom activity of seed kernel extract of *Mangifera indica* L. cv. 'Fahlum' (Thai mango). Prashar *et al*. (2015) studied anti-snake venom activities of ethanol and aqueous extracts of *Cassia hirsuta* against Indian cobra (*Naja naja*) venom induced toxicity. The species of medicinal plants having inhibitory properties against snake venoms has also been reviewed by Soares *et al*. (2005).

Phospholipase A2, a hemolytic protein which is found in the venom of all the five common poisonous snake species in India was selected as a target protein and plant derived molecules such as 2-dihydroxy 4-methoxy benzoic acid from *Hemidesmus indicus* R. Br. and 5-hydroxy 7,8-dimethoxy flavon from *Andrographis paniculata* (Burm. f.) Wall. ex Nees were selected as the ligand molecules and docking of these molecules were done (Nisha *et al*, 2014). The results indicate that both the plant derived compound have significant inhibitory effect. It is noteworthy to note that the crude root drug of *H. indicus* was reported to have anti-viper venom activity (Alam *et al*., 1994) but the docking results between Phospholipase A2 isolated from the five different snake species [*O. hannah Cantor* (King Cobra) - PDB ID : 1GP7, *N. naja L.* (Indian Cobra) - PDB ID :1A3D, D. *rusellii* Shaw & Nodder (Russell's Viper) - PDB ID : 1Q7A, *B. caeruleus* Schneider (Krait) - PDB ID: 1DPY and *E. carinatus* Schneider (Saw-scaled Viper) - PDB ID : 1OZ6] with 2-hydroxy –4-methoxy benzoic acid, the active compound from *H. indicus* revealed that the compound has more inhibitory effect to Indian Cobra venom. These investigations indicate the possibility of the discovery of novel lead molecules through virtual screening of plant derived drug molecules.

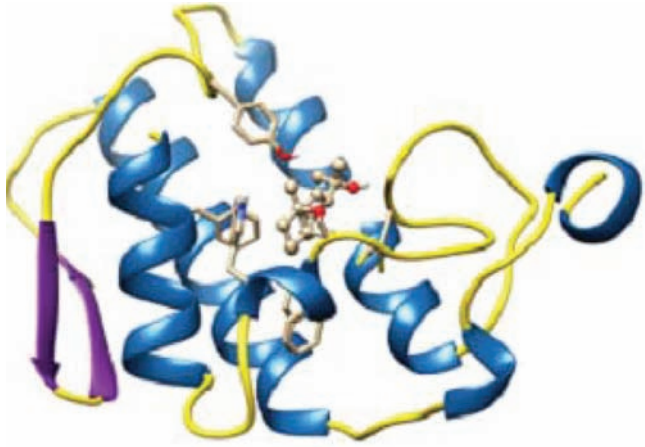

Fig. 4: Proximadiol isolated from *Acorus camalus* L. docked with cobra venome phospholipase A2 (PDB ID IA3D)

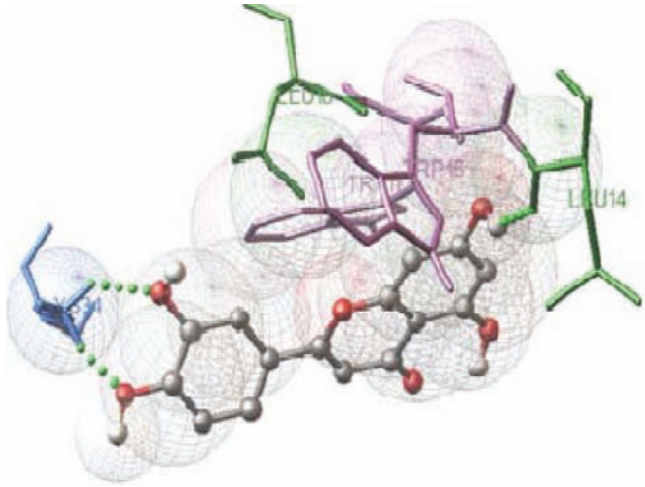

Fig. 5: Molecular interaction of Firamperemophillane reported from *Vitex negundo* L. with the active site residues of Serine protease in Cobra venome; hydrogen bonds are represented in green dots.

10. Conclusion

The use of plant extracts and isolated chemical compounds as antidotes for snake venom is a common practice in places where a prompt access to serum therapy is lacking, and is also used as a supplemental alternative to conventional antivenom serotherapy. Although there are a number of reports on plants from different geographical areas that are able to neutralize snake venoms, only a few chemical compounds have been isolated and identified as active components. In reviewing this area of research, one comes to the conclusion that many plants recorded as anti-snake venoms in popular use may display antidotal properties due to the great number of active compounds they contain. The isolated plant components or their mixtures offer a great potential to complement serotherapy, once their inhibitory effects and action mechanisms are explored and fully characterized. Screening studies of venom inhibition by vegetal extracts and components, which are conveniently performed using *in vitro* or pre-incubation assays, should be complemented by *in vivo* tests that more adequately evaluate their therapeutic potential as alternative or supplemental drugs against snake bites. The active principles of plants structurally resemble mammalian secondary metabolites and this similarity is the basis for their physiological action. The anti-venom activity of plant extracts may be due to the presence of enzymatic inhibitors, chemical inactivators or immune-modulator principles. The efficiency of the vegetal species as inhibitors of the toxic and pharmacological action of snake venoms may be attributed to the presence of multiple factors. The identified active substances are mostly low molecular weight compounds that exhibit more than one biochemical/pharmacological property in addition to antidotal effect. Further studies on the isolation, structural characterization and action mechanism of these natural inhibitors must be carried out in the future. Snake venom inhibitors from plants may become helpful alternative or supplemental tools for the treatment of envenoming, as well as important leads for the synthesis of new drugs of medical interest.

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Bryoflora of Bhubaneswar and its adjoining areas

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ABSTRACT

Thirty six species of bryophytes under 18 families and 20 genera were reported from different localities of Bhubaneswar and its adjoining areas which, encompass 26 species of mosses under 10 families and 13 genera, 8 species of liverworts under 5 families and 5 genera and 2 species of hornworts under 2 families and 2 genera during a study conducted for the period from January to September, 2016. In terms of species richness, the botanical garden of Regional Plant Resource Centre was found to be the most diverse as compared to Khandagiri and Udayagiri hill and Chandaka-Dampara wildlife sanctuary. Pottiaceae, Fissidentaceae, Bryaceae, Funariaceae and Ricciaceae were the dominant families in the study area. Three mosses such as *Archidium birmannicum* Mitt. ex Dixon, *Eurhynchium hians* (Hedw.) Sande Lac. and *Barbula indica* (Hook.) Spreng. were new distributional record for bryoflora of Odisha while *Physcomitrium pyriforme* (Hedwig) Hampe, *Ectropothecium cyperoides* (Hook. ex Harv.) A. Jaeger, *Dicranella macrospora* Gangulee, *Bryum junghugianum* Madhusoodanan and *Eurhynchium striatulum* (Spruce) Schimp. were new record for Eastern Ghats of India. The present study provides base line information on bryophytes for future bryological studies and bryomonitoring in Bhubaneswar.

1. Introduction

Bryophytes are the second largest group of plants after angiosperms with about 25,000 species worldwide (Buck and Goffinet, 2000). So far, 2489 taxa of bryophytes recorded from India, comprising 1786 species in 355 genera of mosses, 675 species in 121 genera of liverworts and 25 species in six genera of hornworts (Dandotiya *et al.,* 2011). In India they are distributed in Eastern and Western Himalayas, South India and Central India (Nath and Asthana, 2005). The state of Odisha is an abode for around 3000 species of plants which includes 138 species of Orchids, 170 species of pteridophytes, 10 species of gymnosperms, 71 species of mangroves and their associates and 7 species of sea grasses (Dash *et al.,* 2015). Apart from angiosperms, gymnosperms and pteridophytes, lower group of plants like algae, bryophytes and lichens also contribute substantially to the floral richness of the state. But the studies on cryptogams in general, and bryophytes in particular, are insufficient as compared to other groups of plants. Bryophytes of Odisha were documented by Dash *et al.* (2007), Dash and Saxena (2009), Dash *et al.* (2009), Dash and Saxena (2011) and Alam *et al.* (2013) from time to time and reported several species from some of the biodiversity rich sites of Odisha. Recently, Mishra *et al.* (2016) reported 149 species of Bryophytes from 12 district of Odisha with many new records for the state.

Bhubaneswar and its adjoining areas are surrounded by some biodiversity rich sites like Khandagiri and Udaygiri hills, Chandaka-Dumpara wildlife sanctuary, Botanical Garden of Regional Plant Resource Centre Campus, Botanical Garden of Nandankan, Sikharchandi hill and Dhauligiri hill where both wild and introduced plants are protected and conserved with an amalgamation of in-situ and ex-situ methods. Many researchers studied plant diversity in Bhubaneswar and its adjoining areas like Saxena and Brahmam (1996), Choudhury and Patnaik (1982), Biswal

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and Thatoi (2005) (Chandaka), Panda and Panda (2012) (Nandankanan zoological Park), Panda *et al*. (2014) (Regional Plant Resource Centre) etc. However the bryophytes have not received any attention from none of the researchers so far. We for the first time conduct a detailed survey and documentation on bryophytes of Bhubaneswar and its adjoining areas.

2. Materials and Methods

Field survey was conducted in Khandagiri and Udaygiri hills (KUH), Chandaka-Dumpara wildlife sanctuary (CDWLS), Botanical Garden of Regional Plant Resource Centre Campus (BGRPRC), Botanical Garden of Nandankanan Zoological Park (BGNZP), Sikharchandi hill (SCH) and Dhauligiri hill (DGH) located in Bhubaneswar for the period from January to September, 2016. Bryophyte samples like corticolous (growing on tree bark), rupicolous (on rocks), saxicolous (on stones and pebbles), lignicolous (fallen logs) and terricolous (on roadside cuts/on soil) forms were collected in the polythene bags and brought to the laboratory, washed and dried. Collected samples are kept in standard Herbarium packets in dried forms and were deposited in the newly created herbarium of Odisha Biodiversity Board (OBB), Bhubaneswar. Internal structures were studied and photographed with elaborate details using compound microscope. Whole plant photographs were taken to illustrate habit using digital camera (Nikon D-3300 with macro lens of 90mm). Identification of the specimens was done referring the literatures of Chopra and Kumar (1988), Kashyap (1929), Kashyap and Chopra (1932), Gangulee (1969 – 1980) and Singh (2014).

3. Result and discussion

In the present investigation, a total of 36 species of bryophytes under 17 families and 20 genera were reported from Bhubaneswar and its adjoining regions. This comprised 26 species of mosses under 10 families and 13 genera, 8 liverworts under 5 families and 5 genera and 2 hornworts under 2 families and 2 genera (Table 1).

Among mosses, genus *Fissidens* showed maximum diversity with 4 species followed by *Bryum* with 4 species and *Philonotis* with 2 species. In liverworts, *Riccia* with 3 species and *Marchantia* with 2 species were the dominant genera. Only two hornworts viz. *Anthoceros angustus*, and *Phaeoceros laevis*, were collected from the study sites. Out of 36 species, 24 species were collected from BGRPRC followed by 16 species from BGNZP, 13 species from KUH and 9 species from CDWLS. DGH and SCH contributed lesser number of species in comparison to other study sites, which may be attributed to the altered habitat conditions of the hills due to anthropogenic interventions. Termite mounts in the botanical garden of RPRC provides amicable environment for several species of *Fissidens* and three species of *Philonotis* to grow luxuriantly. Each species was studied in detail with its habitat type and substratum and the bryoflora of Bhubaneswar was categorised into terricolous, corticolous, saxicolous, rupicolous and lignicolous forms following their corresponding habitats. In total *25* terricoulous, 14 rupicolous, 4 corticolous, 4 saxicolous and 2 lignicolous forms were reported in this study.

Three species of mosses viz., *Archidium birmannicum* Mitt. ex Dixon, *Eurhynchium hians* (Hedw.) Sande Lac., *Barbula indica* (Hook.) Spreng. (Fig1.1) were recorded for the first time from Odisha while 5 mosses viz., *Physcomitrium pyriforme* (Hedwig) Hampe (Fig1.3), *Ectropothecium cyperoides* (Hook. ex Harv.) A. Jaeger ((Fig1.4), *Dicranella macrospora* Gangulee (Fig1.5), *Bryum junghugianum* Madhusoodanan (Fig1.2) and *Eurhynchium striatulum* (Spruce) Schimp. were recorded for the first time from Eastern Ghats of India. A thorough survey of literature (Chopra, 1975; Gangulee, 1969-80; Srinivasan 1974; Udar, 1976; Srivastava and Udar, 1979; Ellis, 1992; Sharma and

Figure 1: Bryoflora of Bhubaneswar and its adjoining areas: 1. *Barbula indica* (Hook.) Spreng. 2. *Bryum junghugianum* Madhusoodanan 3. *Physcomitrium pyriforme* (Hedwig) Hampe, 4. *Ectropothecium cyperoides* (Hook. ex Harv.) A. Jaeger, 5. *Dicranella macrospora* Gangulee

Table 1

Checklist of bryophytes of Bhubaneswar and its adjoining areas.

Name of the species	Family	Distribution	Growth Form	Specimen number
Hornworts				
Anthoceros angustus Steph.	Anthocerotaceae	CDWS, BGNZP	T	BoOH1Aa
Pheoceros laevis (L.) Prosk. subsp. *laevis*	Notothyladaceae	BGRPRC, KUH	T	BoOH2Pl
Liverworts			T	
Cyathodium cavernarum Kunze	Cyathodiaceae	All sites	T,R	BoOL1Cc
Aneura pinguis (L.) Dumort.	Aneuraceae	BGRPRC, BGNZP	T	BoOL2Ap
Heteroscyphus argutus (Nees)	Lophocoleaceae	KUH, CDWS	T,S,R	BoOL3Ha
Marchantia linearis Lehm. & Lindenb.	Marchantiaceae	BGRPRC, BGNZP	T	BoOL4Ml
Marchantia palmata Reinw., Nees & Blume	Marchantiaceae	BGRPRC	T	BoOL5Mp
Riccia crystallina L.	Ricciaceae	BGRPRC, BGNZP	T	BoOL6Rc
Riccia discolor Lehm. & Lindenb.	Ricciaceae	BGRPRC	T	BoOL7Rd
Riccia glauca L.	Ricciaceae	BGRPRC, BGNZP	T	BoOL8Rg
MOSSES				
Archidium birmannicum Mitt. ex Dixon	Archidiaceae	BGRPRC, BGNZP	T	BoOM1Ab
Barbula arcuata Griff.	Pottiaceae	SCH, DGH, BGNZP, CDWLS		R, S BoOM2Ba
Barbula consanguinea (Thwaites & Mitt.) A. Jaeger.	Pottiaceae	BGPRPC, SCH	R	BoOM3Bc
Barbula javanica Dozy & Molk.	Pottiaceae	All sites	R	BoOM4Bj
Barbula indica (Hook.) Spreng.	Pottiaceae	KUH, BGRPRC	R	BoOM5Bi
Bryum argenteum Hedw.	Bryaceae	BGNZP	R	BoOM6Ba
Bryum junghugianum Madhusoodanan	Bryaceae	BGRPRC	R	BoOM7Bj
Bryum capillare Hedw.	Bryaceae	BGRPRC	R	BoOM8Bc
Bryum coronatum Schwagr.	Bryaceae	All sites	R,C	BoOM9Bc
Dicranella macrospora Gangulee	Dicranaceae	BGRPRC	C	BoOM10Dm
Ectropothecium cyperoides Jaeger	Hypnaceae	BGNZP	C,L	BoOM11Ec
Entodon flavescens (Hook.) A. Jaeger	Entodontaceae	KUH	T,S,C	BoOM12Ef
Entodon plicatus Müll. Hal.	Entodontaceae	KUH	T	BoOM13Ep
Entodontopsis wightii (Mitt.) W.R. Buck & R.R. Ireland	Entodontaceae	KUH	T	BoOM14Ew
Eurhynchium hians (Hedw.) Sande Lac.	Brachytheciaceae	BGNZP	T	BoOM15Eh
Eurhynchium striatulum (Spruce) Schimp.	Brachytheciaceae	BGRPRCBGRPRC	T	BoOM16Es
Fissidens ceylonensis Dozy & Molk.	Fissidentaceae	BGRPRC, KUH	T	BoOM17Fc
Fissidens bryoides Hedw.	Fissidentaceae	BGRPRC	R,L,S	BoOM18Fb
Fissidens sylvaticus Griff.	Fissidentaceae	CDWLS, BGRPRC	T	BoOM19Fs

Fissidens taxifolius Hedw.	Fissidentaceae	BGNZP	T	BoOM20Ft
Funaria hygrometrica Hedw.	Funariaceae	KUH	T	BoOM21Fh
Funaria hygrometrica var. *calvescens* (Schwagr.) Mont.	Funariaceae	KUH	T	BoOM22Fhvc
Hyophila involuta (Hook.) A. Jaeger	Pottiaceae	All sites	R	BoOM23Hi
Philonotis mollis (Dozy & Molk.) Mitt.	Bartramiaceae	BGRPRC, BGNZP	R,T	BoOM24Pm
Philonotis hastata (Duby) Wijk & Margad.	Bartramiaceae	BGRPRC	R,T	BoOM25Ph
Physcomitrium pyriforme (Hedw.) Hampe	Funariaceae	BGRPRC	T	BoOM26Pp

Srivastava, 1993; Daniels and Daniel, 2003; Nair and Madhusoodanan, 2005; Kumar and Krishnamurthy, 2007; Dash *et al.,* 2007; Dash and Saxena, 2009; Dash *et al.,* 2009; Dash and Saxena, 2011; Alam *et al.,* 2013; Bansal and Nath, 2014; Mishra *et al.,* 2016) revealed that these species are not reported from here earlier and were considered as new distributional records for Odisha and Eastern Ghats, respectively.

Since no systematic data non bryophytes of Bhubaneswar are available till date, the current report can be considered as baseline data that would provide road map for future research towards biomonitoring. Generally the conservation measures are taken for higher group of organisms only. In the case of lower group of plants, less importance is given by the taxonomist as well as the policy makers. The greatest risks to them are poor and deteriorating air quality and loss of critical habitats due to unplanned developmental activities. These issues are mainly societal, and the fate of these special organisms is a factor to consider in the overall conservation process.

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Diversity and distribution of exotic and alien plant species in Angul district of Odisha, India

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ABSTRACT

An exhaustive floristic survey was carried out during 2013-2015 in the Angul district of Odisha state to assess the diversity and distribution of exotic and alien plant species. The study revealed the occurrence a total of 124 species of flowering plants belonging to 92 genera under 42 families. Of these, trees, shrubs, climbers and herbs are represented by 2, 14, 3 and 105 species respectively. Asteraceae was the most speciose family with 20 species followed by Amaranthaceae (9 spp.), Convolvulaceae (7 spp.), Euphorbiaceae (7 spp.), Caesalpiniaceae (7 spp.) and Fabaceae (7 spp.). *Chromolaena odorata*, *Hyptis suaveolens*, *Parthenium hysterophorus*, *Alternanthera philoxeroides*, *Eichhornia crassipes* and *Lantana camara* were found to the most dominant and aggressive alien weeds of the district. Most of the exotics and alien weeds reported from the district were found to be native of Tropical America, and Tropical Africa and only few from the Asiatic region.

1. Introduction

Invasive alien plant species are the species whose introduction or spread threatens the environment, the economy of society, human health and biodiversity of a region. Biological invasions by alien species are widely recognized as a significant component of human caused global environmental change which results a significant loss in the economic value, biological diversity and change in the functioning of invaded ecosystem beyond its home range that establishes, naturalizes and spreads (Williamson, 1996). Introduction of these species may occur accidentally or through their being imported for a limited purpose and subsequently escaping or deliberately on a large scale (Levine, 1989). Many people introduce exotic species into new habitats for economic reasons (McNealy, 2001) and most cases of invasiveness can be linked to the intended or unintended consequences of economic activities. In many continental areas 20% or more of the plant species are now non-indigenous. On many islands the proportion of non-indigenous plant species is 50% or more (Randall and Marinelli, 1997). Invasive alien plants are a problem of global significance, causing impacts running into billions of dollars annually. In South Africa, at least 161 species cause serious problems in natural and semi-natural systems (Henderson, 1995); these plants may spread and come to dominate large areas in just a few years. The loss due to invasive species in United States estimated to be ~$125-150 billion each year and 25% of U.S agriculture gross national product is lost due to foreign pests and weeds (McNeely et al., 2001). Exotic plants can spread rapidly because of our mobile society and the intentional transportation of ornamental and forage plants (Randall and Marinelli, 1997).

India is a biodiversity rich country with different climate, soil, topography and vegetation which encourages different alien plants to proliferate extensively. Inventory of exotics and invasive alien plants of India has been done by several workers in different parts of the country (Reddy, 2008; Wagh & Jain, 2015; Chandrasekhar, 2012; Singh and

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Mahammed, 2015). In Odisha, 131 species of alien plant species have been identified in Dhenkanal district of Odisha (Nayak and Satapathy, 2015) and 80 from Utkal University campus, Bhubaneswar (Satapathy *et al*., 2016). In the present study, an account of 124 species of alien plant species of Angul district of Odisha belonging to 92 genera and under 42 families has been provided.

2. Materials and methods

2.1 Study area

Angul, a centrally located district in the state of Odisha, covers a geographical area of 6232 sq. km and lies between 20° 31' N and 20° 40' N Latitude and 84° 15' E and 85° 23' E Longitude. The climate of the Angul district is hot and dry sub-humid type with an average annual rainfall of 1696 mm. The average minimum and maximum temperatures are 19.6°C and 33.3°C respectively. The the relative humidity generally varies from 31 to 88%. The forest coverage of the district is 3336.63 sq. km.(http://angul.nic.in/)

2.2 Methods

The present study was conducted in Angul district during 2013-15 to document the diversity and distribution of exotic and alien plant species including invasive ones. Interaction with local inhabitants was made to collect information regarding the uses of these Invasive Alien plant Species (IAPS) by them. Plant samples were collected and photographed from their natural habitats including agricultural lands, wetlands, roadside and forest areas of the district and identified in consultation with "The Flora of Orissa" (Saxena and Brahmam, 1994-1996) and other available literature. Collected plant specimens were preserved as herbarium specimens following standard field and herbarium methods in the Herbarium of the Post Graduate Department of Botany, Utkal University, Bhubaneswar.

Review of literature on invasive and alien plant species throughout the world (Mooney and Drake, 1987; Heywood, 1989; Cox, 1999; D'Antonio and Vitousek, 1992; Drake *et al*., 1989; Huxel, 1999; Mooney, 1999; Almeilla and Freitas, 2001; Cowie, 2001; McNeely *et al*., 2001; Reddy *et al*., 2008) was helpful to throw light on the history of invasion, spread and harmful effects of alien weeds especially those occurring in Angul district of Odisha. The country of origin of the species were determined based on the work of Vavilov (1951), Matthew (1969), Maheswari and Paul (1975), Nayar (1977), Hajra and Das (1982), Sharma (1984), Saxena (1984), Reddy and Raju (2002) and Reddy, and Reddy (2004).

3. Results and Discussion

The exhaustive floristic survey of Angul district revealed the occurrence of 124 invasive alien plant species belonging to 92 genera and under 42 families. Habit-wise analysis revealed that the herbs were represented by 105 species (84.6%) followed by shrubs (14), climbers (03) and trees (02) (Table 2 & Figure-1). Among the 42 families, Asteraceae was the dominant family with 20 (47.6%) species followed by Amaranthaceae (09), Convolvulaceae (07), Euphorbiaceae (07), Caesalpiniaceae (07), Fabaceae (07), Poaceae (05), Solanaceae (05), Cleomaceae (04), Mimosaceae (03), Asclepiadaceae (03), Cyperaceae (03), Lamiaceae (03), Malvaceae (03), Tiliaceae (02) etc. These top fifteen families contributed 90 species which is 72.58% of the total species listed. The genus *Cassia* has the maximum number of species (7) followed by *Ipomoea* (6 species), *Alternanthera* (4 species), *Cleome* (4 species), and *Ludwigia* (3 species) (Table-1).The most invasive and troublesome species recorded from the district were *Chromolaena odorata*, *Hyptis suaveolens, Parthenium hysterophorus*, *Alternanthera philoxeroides, Eichhornia crassipes* and *Lantana camara.* The Invasive Alien Plant Species (IAPS) of Angul district were found growing in all kinds of ecosystems like forests, agricultural fields, waste and fallow lands, gardens and road-sides. It was also noted that more than half of alien plant species are growing luxuriantly, spreading rapidly and getting naturalized to newer areas and habitats.

Out of the 124 species now listed, 27 were identified as invasive weeds of wetland habitats including *Aeschynomene americana, Alternanthera paronychioides, Alternanthera philoxeroides, Cassia alata, Cyperus difformis, Cyperus iria, Echinochloa colona, Echinochloa crusgalli., Eclipta prostrata., Eichhornia crassipes, Gnaphalium polycaulon*, *Grangea maderaspatana, Ipomoea carnea, Ludwigia adscendes, Ludwigia octovlavis, Ludwigia perennis, Monochoria vaginalis, Pistia stratiotes, Salvinia molesta Sesbania bispinosa* and *Typha angustata* (Table-1). Similarly, aliens like *Lantana camara, Chromolaena odorata, Parthenium hysterophorus, Alternanthera* species etc. have invaded the forest floors and wastelands badly affecting the growth of indigenous species. It was observed that fast growing exotic trees like *Acacia auriculiformis*, *Cassia siamea, Acacia mangium, Grevillea pteridifolia* etc. have been introduced in the locality to meet the increasing demand of firewood or as avenue plants.

Analysis of relevant literature on country of origin and distribution of alien plant species of Angul district, it is observed that that maximum number of species were from tropical America and tropical Africa (Table-2). These alien

Table 1
List of invasive alien plants of Angul district, Odisha

S.N.	Species	Family	Habit	Nativity
1.	*Acanthospermum hispidum* DC.	Asteraceae	Herb	Brazil
2.	*Aerva javanica* (Burm.f.) Juss. ex Schult.	Amaranthaceae	Herb	Trop. America
3.	*Aeschynomene americana* L.	Fabaceae	Herb	Trop. America
4.	*Ageratum conyzoides* L.	Asteraceae	Herb	Trop. America
5.	*Alternanthera paronychioides* St. Hill	Amaranthaceae	Herb	Trop. America
6.	*Alternanthera philoxeroides* (Mart.) Griseb.	Amaranthaceae	Herb	Trop. America
7.	*Alternanthera pungens* Kunth	Amaranthaceae	Herb	Trop. America
8.	*Alternanthera sessilis* (L.) DC.	Amaranthaceae	Herb	Trop. America
9.	*Amaranthus spinosus* L	Amaranthaceae	Herb	Trop. America
10.	*Antigonon leptopus* Hook. & Arn.	Polygonaceae	Climber	Trop. America
11.	*Argemone mexicana* L.	Papaveraceae	Herb	Trop. Central & South America
12.	*Bidens pilosa* L.	Asteraceae	Herb	Trop. America
13.	*Blainvillea acmella* (L.) Philipson	Asteraceae	Herb	Trop. America
14.	*Blumea lacera* (Burm. f.) DC.	Asteraceae	Herb	Trop. America
15.	*Blumea obliqua* (L.) Druce	Asteraceae	Herb	Trop. America
16.	*Borassus flabellifer* L.	Arecaceae	Tree	Trop. Africa
17.	*Calotropis gigantea* R. Br.	Asclepiadaceae	Shrub	Trop. Africa
18.	*Calotropis procera* (Ait.) R. Br.	Asclepiadaceae	Shrub	Trop. Africa
19.	*Cannabis sativa* L.	Cannabaceae	Herb	Central Asia
20.	*Cardiospermum halicacabum* L.	Sapindaceae	Climber	Trop. America
21.	*Cassia absus* L.	Caesalpiniaceae	Herb	Trop. America
22.	*Cassia alata* L.	Caesalpiniaceae	Shrub	West Indies
23.	*Cassia hirsuta* L.	Caesalpiniaceae	Herb	Trop. America
24.	*Cassia obtusifolia* L.	Caesalpiniaceae	Herb	Trop. America
25.	*Cassia pumila* Lam.	Caesalpiniaceae	Herb	Trop. America
27.	*Cassia tora* L.	Caesalpiniaceae	Herb	Trop. South America
28.	*Catharanthus pusillus* (Murr.) G. Don	Apocynaceae	Herb	Trop. America
29.	*Celosia argentea* L.	Amaranthaceae	Herb	Trop. Africa
30.	*Chenopodium album* L	Chenopodiaceae	Herb	Europe
31.	*Chloris barbata* Sw.	Poaceae	Herb	Trop. America
32.	*Chromolaena odorata* (L.) King & Robins.	Asteraceae	Herb	Trop. America
33.	*Chrozophora rottleri* (Geis.) Juss. ex Spreng.	Euphorbiaceae	Herb	Trop. Africa
34.	*Cleome gynandra* L.	Cleomaceae	Herb	Trop. America
35.	*Cleome monophylla* L.	Cleomaceae	Herb	Trop. Africa
36.	*Cleome rutidosperma* DC.	Cleomaceae	Herb	Trop. America
37.	*Cleome viscosa* L.	Cleomaceae	Herb	Trop. America
38.	*Conyza bipinnatifida* Wall.	Asteraceae	Herb	Trop. America
39.	*Corchorus aestuans* L.	Tiliaceae	Herb	Trop. America
40.	*Crotalaria pallida* Ait.	Fabaceae	Herb	Trop. America

41.	*Crotalaria retusa* L.	Fabaceae	Herb	Trop. America
42.	*Croton bonplandianum* Baill.	Euphorbiaceae	Herb	Temperate South America
43.	*Cryptostegia grandiflora* R.Br.	Asclepiadaceae	Herb	Madagascar
44.	*Cuscuta chinensis* Lam.	Cuscutaceae	Herb	Mediterranean
45.	*Cuscuta reflexa* Roxb.	Cuscutaceae	Herb	Mediterranean
46.	*Cynodon dactylon* (L.) Pers.	Poaceae	Herb	Trop. Africa
47.	*Cyperus difformis* L.	Cyperaceae	Herb	Trop. America
48.	*Cyperus iria* L.	Cyperaceae	Herb	Trop. America
49.	*Datura innoxia* Mill.	Solanaceae	Shrub	Trop. America
50.	*Datura metel* L.	Solanaceae	Shrub	Trop. America
51.	*Digera muricata* (L.) Mart.	Amaranthaceae	Herb	SW Asia
52.	*Echinochloa colona* (L.) Link	Poaceae	Herb	Trop. South America
53.	*Echinochloa crusgalli* (L.) Beauv.	Poaceae	Herb	Trop. South America
54.	*Eclipta prostrata* (L.) Mant.	Asteraceae	Herb	Trop. America
55.	*Eichhornia crassipes* (Mart.) Solms	Pontederiaceae	Herb	Trop. America
56.	*Emilia sonchifolia* (L.) DC.	Asteraceae	Herb	Trop. America
57.	*Euphorbia heterophylla* L.	Euphorbiaceae	Herb	Trop. America
58.	*Euphorbia hirta* L	Euphorbiaceae	Herb	Trop. America
59.	*Evolvulus nummularius* (L.) L.	Convolvulaceae	Herb	Trop. America
60.	*Fuirena ciliaris* (L.) Roxb.	Cyperaceae	Herb	Trop. America
61.	*Galinosoga parviflora* Cav.	Asteraceae	Herb	Trop. America
62.	*Gnaphalium pensylvanicum* Willd.	Asteraceae	Herb	Trop. America
63.	*Gnaphalium polycaulon* Pers.	Asteraceae	Herb	Trop. America
64.	*Gomphrena serrata* L.	Amaranthaceae	Herb	Trop. America
65.	*Grangea maderaspatana* (L.) Poir.	Asteraceae	Herb	Trop. South America
66.	*Hyptis suaveolens* (L.) Poit.	Lamiaceae	Herb	Trop. America
67.	*Impatiens balsamina* L.	Balsaminaceae	Herb	Trop. America
68.	*Indigofera astragalina* DC.	Fabaceae	Herb	Trop. America
69.	*Indigofera linnaei* Ali	Fabaceae	Herb	Trop. Africa
70.	*Ipomoea carnea* Jacq.	Convolvulaceae	Shrub	Trop. America
71.	*Ipomoea eriocarpa* R.Br.	Convolvulaceae	Herb	Trop. Africa
72.	*Ipomoea hederifolia* L.	Convolvulaceae	Herb	Trop. America
73.	*Ipomoea obscura* (L.) Ker-Gawl.	Convolvulaceae	Herb	Trop. America
74.	*Ipomoea pes-tigridis* L.	Convolvulaceae	Herb	Trop. East Africa
75.	*Ipomoea quamoclit* L.	Convolvulaceae	Herb	Trop. America
76.	*Jatropha curcas* L.	Euphorbiaceae	Shrub	Trop. America
77.	*Jatropha gossypifolia* L.	Euphorbiaceae	Shrub	Brazil
78.	*Lantana camara* L.	Verbenaceae	Herb	Trop. America
79.	*Leonotis nepetiifolia* (L.) R. Br.	Lamiaceae	Herb	Trop. Africa
80.	*Leucaena leucocephala* (Lam.) de Wit	Mimosaceae	Tree	Trop. America
81.	*Ludwigia adscendes* (L.) Hara	Onagraceae	Herb	Trop. America
82.	*Ludwigia octovlavis* (Jacq.) Raven	Onagraceae	Herb	Trop. Africa

83.	*Ludwigia perennis* L.	Onagraceae	Herb	Trop. Africa
84.	*Malachra capitata* (L.) L.	Malvaceae	Herb	Trop. America
85.	*Martynia annua* L.	Martyniaceae	Herb	Trop. America
86.	*Mecardonia procumbens* (Mill.) Small	Scrophulariaceae	Herb	Trop. North America
87.	*Melilotus alba* Desv.	Fabaceae	Herb	Europe
88.	*Melochia corchorifolia* L.	Sterculiaceae	Herb	Trop. America
89.	*Mikania micrantha* Kunth	Asteraceae	Climber	Trop. America
90.	*Mimosa pigra* L.	Mimosaceae	Shrub	Trop. North America
91.	*Mimosa pudica* L.	Mimosaceae	Herb	Brazil
92.	*Mirabilis jalapa* L.	Nyctaginaceae	Herb	Peru
93.	*Monochoria vaginalis* (Burm. f.) Presl.	Pontederiaceae	Herb	Trop. America
94.	*Nicotiana plumbaginifolia* Viv.	Solanaceae	Herb	Trop. America
95.	*Ocimum canum* Sims	Lamiaceae	Herb	Trop. America
96.	*Opuntia stricta* (Haw.) Haw.	Cactaceae	Herb	Trop. America
97.	*Oxalis corniculata* L.	Oxalidaceae	Herb	Europe
98.	*Parthenium hysterophorus* L.	Asteraceae	Herb	Trop. North America
99.	*Passiflora foetida* L.	Passifloraceae	Herb	Trop. South America
100.	*Pedalium murex* L.	Pedaliaceae	Herb	Trop. America
101.	*Peperomia pellucida* (L.) Kunth	Piperaceae	Herb	Trop. South America
102.	*Pistia stratiotes* L.	Araceae	Herb	Trop. America
103.	*Portulaca oleracea* L.	Portulacaceae	Herb	Trop. South America
104.	*Portulaca quadrifida* L.	Portulacaceae	Herb	Trop. America
105.	*Prosopis juliflora* (Sw.) DC.	Mimosaceae	Shrub	Mexico
106.	*Ruellia tuberosa* L.	Acanthaceae	Herb	Trop. America
107.	*Saccharum spontaneum* L.	Poaceae	Herb	Trop. West Asia
108.	*Salvinia molesta* D. S. Mitch.	Salviniaceae	Herb	Brazil
109.	*Scoparia dulcis* L.	Scrophulariaceae	Herb	Trop. America
110.	*Sesbania bispinosa* (Jacq.) Wight	Fabaceae	Shrub	Trop. America
111.	*Sida acuta* Burm.f.	Malvaceae	Herb	Trop. America
112.	*Solanum torvum* Sw.	Solanaceae	Shrub	West Indies
113.	*Solanum viarum* Dunal	Solanaceae	Herb	Trop. America
114.	*Sonchus asper* Hill	Asteraceae	Herb	Mediterranean
115.	*Spermacoce hispida* L.	Rubiaceae	Herb	Trop. America
116.	*Stachytarpheta jamaicensis* (L.) Vahl	Verbenaceae	Herb	Trop. America
117.	*Synadenium grantii* Hook. f.	Euphorbiaceae	Shrub	Trop. America
118.	*Synedrella nodiflora* (L.) Gaertn.	Asteraceae	Herb	West Indies
119.	*Tribulus terrestris* L.	Zygophyllaceae	Herb	Trop. America
120.	*Tridax procumbens* L.	Asteraceae	Herb	Trop. Central America
121.	*Triumfetta rhomboidea* Jacq.	Tiliaceae	Herb	Trop. America
122.	*Typha angustata* Bory. & Choub.	Typhaceae	Herb	Trop. America
123.	*Urena lobata* L.	Malvaceae	Shrub	Trop. Africa
124.	*Xanthium indicum* L.	Asteraceae	Herb	Trop. America

Table 2

Regions of nativity of invasive alien plant species of Angul district

Sl. No.	Region of Nativity	No. of Species
1.	Tropical America	76
2.	Tropical Africa	13
3.	Tropical South America	08
4.	Brazil	04
5.	Europe	03
6.	Tropical North America	03
7.	West Indies	03
8.	Mediterranean	03
9.	Central America	01
10.	Tropical Central South America	01
11.	Tropical East Africa	01
12.	Peru	01
13.	Mexico	01
14.	Temperate South America	01
15.	Madagascar	01
16.	West Asia	01
17.	South West Asia	01
18.	Tropical West Asia	01
19.	Central Asia	01

Table 3

Habit-wise distribution of invasive alien plant species in Angul district of Odisha

Sl. No.	Habit	No. of Species
1	Herb	105
2	Shrub	14
3	Climber	3
4	Tree	2

species were mostly from Tropical America (76 spp.), Tropical Africa (13 spp.),Tropical South America (8 spp.), Brazil (4 spp.), Europe (3 spp.), Tropical north America (3 spp.), West Indies (3 spp.) and Mediterranean region (3 spp.). However, one species each was native to Tropical Central America, Mexico, Tropical Central South America, Tropical East Africa, Peru, Temperate South America, Madagascar, West Asia, South West Asia, Central Asia and Tropical West Asia. (Table-2)

4. Conclusion

The invasive alien plant species of Angul district of Odisha pose great threat to the indigenous flora of the region. Due to their competitive advantage over native species in terms of seed dispersal and plant establishment, colonization in new and hostile habitats, ecological adaptability to a range of ecosystems and micro-climates, they have rapidly invaded

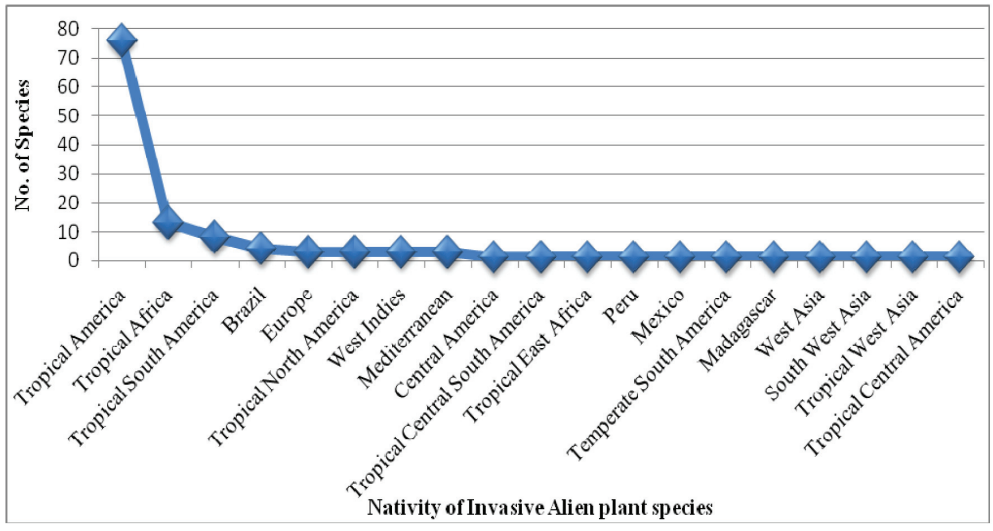

Fig. 1: Origin of invasive alien plants in Angul district of Odisha

the wastelands, crop fields, forest floors, village thickets, road sides and eliminated the native elements at several habitats. Weed invasion in agricultural fields is a matter of concern leading to considerable loss to the farmers in terms of requirement of additional manpower for weed control and reduction in crop productivity. However, many of the alien species are economically important as source of food, feed, medicine and could be used as valuable resources. Importantly, the ecological impact of exotics and alien weeds on local biodiversity and human health need to be assessed at regional and national level.

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Lichen family *Graphidaceae* in Arunachal Pradesh, India

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ABSTRACT

The present paper enumerates a total of 78 species of lichen family *Graphidaceae* (Graphidoid *Graphidaceae*) spread over 16 genera from the Indian state of Arunachal Pradesh, which is part of Himalaya Biodiversity Hotspot. Of these, one species, *Thalloloma hypoleptum* (Nyl.) Staiger is reported as new distributional record for India.

1. Introduction

Arunachal Pradesh, the land of rising sun, formerly known as NEFA (North Eastern Frontier Agency), is situated in the extreme north-eastern corner of India and lies between $91^0$30' E to $97^0$30' E longitude and $26^0$28' N to $29^0$30' N latitude. It is a part of Himalaya Biodiversity Hotspot, covering an area of 83,743 sq. km with the altitude ranging from 100 m to 7,090 m above mean sea level. As a part of the revisionary studies on family *Graphidaceae* in India, about 270 voucher specimens of family *Graphidaceae* collected from Arunachal Pradesh and deposited in various Indian herbaria (BSA, ASSAM, LWG) were studied by the author during December 2007 and March 2012. Based on the study, previously 65 species of *Graphidadeae* were reported from the state (Singh and Swarnalatha 2011a, b; Singh *et al*., 2011). Extension of the earlier work revealed that some more taxa were identified and now 78 species of Graphidoid *Graphidaceae* belonging to 16 genera are known from Arunachal Pradesh. The species of Graphidoid *Graphidaceae* studied from Arunachal Pradesh in this work are enumerated in this paper; the names have been taxonomically reviewed/validated, with the distribution being updated. The species, *Thalloloma hypoleptum* (Nyl.) Staiger, is now reported as new for India. All the identified taxa are enumerated here citing the complete list of specimens examined.

2. Materials and Methods

This study is based on the specimens collected from different localities of Arunachal Pradesh and deposited in herbarium at Botanical Survey of India, Eastern Regional Centre, Shillong (ASSAM), Botanical Survey of India, Central Regional Centre, Allahabad (BSA) and National Botanical Research Institute, Lucknow (LWG). All the specimens cited in this paper were studied at the Lichen Laboratory, Botanical Survey of India, Central Regional Centre, Allahabad, during December 2007 to March 2012.

The morphological features of the thalli and ascocarps were studied under stereo zoom microscopes (Olympus SZ51 and Nikon SMZ1500) and anatomical characters were examined with compound microscopes (Magnüs MLX-Tr and Leica DM2500). All anatomical measurements were made in water mounts. Colour spot tests were performed by using K, C and PD reagents. The lichen substances were identified by Thin Layer Chromatography (TLC) by using solvent system A, following White and James (1985). All specimens were examined under UV light (365 nm). The methodology and the concept of Staiger (2002) and Lücking and Rivas Plata (2008) have been followed here.

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3. Enumeration of Taxa

Carbacanthographis marcescens (Fée) Staiger & Kalb, Biblioth. Lichenol. 85: 109. 2002.

Specimens examined: Arunachal Pradesh: Upper Subansiri District, Daporizo-Taliha Road, 6 km from Daporizo, alt. *c.* 300 m, 15 Jan. 1983, *K.P. Singh* 3043B (ASSAM); West Siang District, Kabu forest near Kabu village, 14 Mar. 2006, *U. Dubey* 07-009098 (LWG).

Diorygma hieroglyphicum (Pers.) Staiger & Kalb, in Staiger, Biblioth. Lichenol. 85: 151. 2002.

Specimens examined: Arunachal Pradesh: Tirap District, Near Dirok river, 14 km Deomals, 24 Feb. 1982 *K.P. Singh* 834 (ASSAM); Upper Subansiri District, Daporizo-Taliha Road, 6 km from Daporizo, alt. *c.* 300 m, 15 Jan. 1983, *K.P. Singh* 3043 (ASSAM); Daporizo-Zero Road, 2 km from Daporizo, on *Terminalia* tree, alt. *c.* 300 m, 16 Jan. 1983, *K.P. Singh* 3094B (ASSAM); Lohit District, Namsai, Near Peyong village, alt. *c.* 180 m, 30 Dec. 1983, *K.P. Singh* 4160B (ASSAM); Namsai, Lathauo Coffee Plantations, on *Dipterocarpus* tree, 30 Dec. 1983, *K.P. Singh* 4187A & B (ASSAM); West Kameng District, Near Dedza bridge, Bahlukpong-Tanga Road, alt. *c.* 1216 m, 03 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4854 (BSA); West Kameng District, 10.5 km to Nechiphu, Tanga-Bhalukpong Road, alt. *c.* 1184 m, 07 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5168 (BSA).

Diorygma junghuhnii (Mont. & Bosch) Kalb, Staiger & Elix, Symb. Bot. Upsal. 34(1): 157. 2004.

Specimens examined: Arunachal Pradesh: Upper Subansiri District, Daporizo-Zero Road, 2 km from Daporizo, on *Terminalia* plantations, 16 Jan. 1983, *K.P. Singh* 3087 (ASSAM); Taliha-Nachoo foot track, alt. *c.* 400 m, 19 Jan. 1983, *K.P. Singh* 4082 (ASSAM); Lohit District, Namsai, Near Peyong village, alt. *c.* 180 m, 30 Dec. 1983, *K.P. Singh* 4160 & 4170 (ASSAM); Lower Subansiri District, Near Kimin, alt. *c.* 400 m, 21 Jan. 1984, *K.P. Singh* 5067 (ASSAM); Lower Dibang Valley District, Mehao Wild Life Sanctuary, Sally lake, alt. *c.* 500 m, 31 Oct. 2002, *P.K. Dixit* 279D (BSA); Mehao Wild Life Sanctuary, Sally lake, alt. *c.* 390–400 m, 07 Nov. 2002, *K.P. Singh & P.K. Dixit* 451B & 461A (BSA); Mehao Wild Life Sanctuary, Abengo-Cornuway, alt. *c.* 500 m, 08 Nov. 2002, *K.P. Singh & P.K. Dixit* 495A (BSA); West Kameng District, Bhalukpong, Kameng river bank, alt. *c.* 220–229 m, 01 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4628, 4629 & 4632 (BSA); Tipi Orchidarium, alt. *c.* 171 m, 02 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4732, 4733 & 4734 (BSA); Tipi, Near Neychiung festival ground, alt. *c.* 160 m, 02 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4736, 4763 & 4754 (BSA); Near Dedza bridge, Bahlukpong-Tanga Road, alt. *c.* 1216 m, 03 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4834 & 4836 (BSA); 10.5 km to Nechiphu, Tanga-Bhalukpong Road, alt. *c.* 1184 m, 07 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5152 & 5163 (BSA); Tipi forest rest house campus, alt. *c.* 202 m, 08 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5186, 5187, 5207, 5208, 5213, 5220, 5221, 5239 & 5240 (BSA).

Diorygma longilirellatum B.O. Sharma & Makhija, Mycotaxon 109: 212. 2009.

Specimens examined: Arunachal Pradesh: West Kameng District, near Dedza bridge, 03 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4825 & 4845 (BSA).

Diorygma megasporum Kalb, Staiger & Elix, Symb. Bot. Upsal. 34(1): 160. 2004.

Specimens examined: Arunachal Pradesh: Lower Dibang Valley District, Mehao Wild Life Sanctuary, Sally Lake, alt. *c.* 500 m, 12 Nov. 2000, *K.P. Singh & P. Bujarbaru*a 10878 (ASSAM); Mehao Wild Life Sanctuary, Roing Mini Zoo Area, 20 Nov. 2000, *K.P. Singh & P. Bujarbarua* 11082 (ASSAM); Mehao Wild Life Sanctuary, Mehao Lake, alt. *c.* 1550–1700 m., 15 Nov. 2002, *K.P. Singh & P.K. Dixit* 580D, 598C & 605A (BSA); West Kameng District, Sessa Orchid Sanctuary, alt. *c.* 1033–1170 m, 01 Mar. 2009, *K.P. Singh & G. Swarnalatha*, 4699, 4720 & 4727 (BSA); West Kameng, Near Dedza bridge, Bahlukpong-Tanga Road, alt. *c.* 1216 m, 03 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4846, 4847, 4852A/B/C, 4856, 4857 & 4860 (BSA); 10.5 km to Nechiphu, Tanga-Bhalukpong Road, alt. *c.* 1184 m, 07 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5164, 5167, 5169 & 5170 (BSA); Tipi forest rest house campus, alt. *c.* 202 m, 08 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5184 (BSA).

Diorygma pachygraphum (Nyl.) Kalb, Staiger & Elix, Symb. Bot. Upsal. 34(1): 163. 2004.

Specimen examined: Arunachal Pradesh: West Kameng District, Bhalukpong to Tenga road, near dedza bridge, alt. *c.* 1216 m, 03 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4831 (BSA).

Diorygma pruinosum (Eschw.) Kalb, Staiger & Elix, Symb. Bot. Upsal. 31(1): 166. 2004.

Specimen examined: Arunachal Pradesh: Upper Subansiri, Daporizo-Zero Road, 2 km from Daporizo, on *Terminalia* plantations, alt. *c.* 300 m, 16 Jan. 1983, *K.P. Singh* 3094A (ASSAM).

Diorygma radiatum (D.D. Awasthi & S.R. Singh) Kr.P. Singh & Swarnalatha, Indian J. Forest. 32 (1): 179. 2009.

Specimen examined: Arunachal Pradesh: Lower Dibang Valley District, Mehao Wild Life Sanctuary, Roing mini zoo, 24 Apr. 2000, *K.P. Singh* 11087 (ASSAM).

Dyplolabia afzelii (Ach.) A. Massal., Neagen. Lich.: 6. 1854b.

Specimen examined: Arunachal Pradesh: Lohit District, Namsai, Near Peyong basti, alt. *c.* 150 m, 30 Dec. 1983, *K.P. Singh* 4174 (ASSAM).

Fissurina subcontexta (Nyl.) Nyl., Lich. Nov. Zeland.: 125. 1888a.

Specimen examined: Arunachal Pradesh: Lower Debang Vally District, Roing, Near ITI, alt. *c.* 400 m, 07 June 1984, *D.K. Upreti* L81796 (LWG).

Glyphis cicatricosa Ach., Syn. Meth. Lich.: 107. 1814.

Specimens examined: Arunachal Pradesh: West Siang District, Along, Bahadur Reserve forest alt. *c.* 375 m, 2 Jan. 1983, *K.P. Singh* 2596 (ASSAM); Lohit District, Hayuliang Inspection Bunglow Compound, alt. *c.* 600 m, 04 Jan. 1984, *K.P. Singh* 4279A/C (ASSAM); Lower Subansiri District, fozali to fanhu road, alt. *c.* 1000 m, 22 Jan. 1984, *K.P. Singh* 5153B (ASSAM); West Kameng District, Near Dedza bridge, Bahlukpong-Tanga Road, alt. *c.* 1216 m, 03 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4861 (BSA); Near Dedza bridge, Bahlukpong-Tanga Road, alt. *c.* 1095 m, 03 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4798 (BSA); Near Dedza bridge, Bahlukpong-Tanga road, alt. *c.* 1216 m, 03 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4863 (BSA); Dirang, 2 km to Santhi Vally, alt. *c.* 1601 m, 06 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5046 (BSA); Dirang, Near Khopathar bridge, alt. *c.* 1575 m, 06 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5072, 5075 (BSA); 10.5 km to Nechiphu, Tanga-Bhalukpong Road, alt. *c.* 1184 m, 07 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5171 (BSA); Dirang, Near NRCY, alt. *c.* 1700–2000 m, 04 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5359 (BSA).

Glyphis scyphulifera (Ach.) Staiger, Biblioth. Lichenol. 85: 175. 2002.

Specimens examined: Arunachal Pradesh: Lohit District, Hayuliang Inspection Bunglow Conpound, alt. 600 m, 04 Jan. 1984, *K.P. Singh* 4279B (ASSAM); West Kameng District, Bhalukpong, Kameng river bank, alt. *c.* 220–229 m, 01 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4625 (BSA); Tipi forest rest house campus, alt. *c.* 202 m, 08 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5242C (BSA).

Graphis ajarekarii Patw. & C.R. Kulk., Norweg. J. Bot. 26(1): 45. 1979.

Specimen examined: Arunachal Pradesh: West Kameng District, Tipi forest guest house campus, alt. *c.* 202 m, 08 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5257 (BSA).

Graphis caesiella Vain., Acta Soc. Fauna Flora Fenn. 7 (2): 122. 1890.

Specimens examined: Arunachal Pradesh: Lower Subansiri District, fozali to fanhu road, alt. *c.* 1000 m, 22 Jan. 1984, *K.P. Singh* 5153B (ASSAM); West Kameng District, Dirang, Near Khopathar Bridge, alt. *c.* 1575 m, 06 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5068 (BSA); Tipi forest rest house campus, alt. *c.* 202 m, 08 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5242A (BSA).

Graphis capillacea Stirt., Proc. Roy. Soc. Glasgow 11: 315. 1879.

Specimens examined: Arunachal Pradesh: East Kameng District, Phakui Wild life Sanctuary, Dichu-Julley Nala, alt. *c.* 300–440 m, 15 Apr. 1999, *K.P. Singh* 10035 (ASSAM); Upper Subansiri District, L.c. the way of Lebe to Nuk Basti, 25 Mar. 2000, *K.P. Singh* 10696B (ASSAM).

Graphis chlorotica A. Massal., in Kremp., Verh. K. K. Zool. -Bot. Ges. Wein. 21: 865. 1871.

Specimens examined: Arunachal Pradesh: Lower Debang Vally District, Roing-Anini Road, alt. *c.* 1350–1650 m, 12 Jan. 1984, *K.P. Singh* 4659 (ASSAM); Mehao Wild Life Sanctuary, Sally lake, alt. *c.* 500 m, 31 Oct. 2002, *P.K. Dixit* 278 E (BSA); Mehao Wild Life Sanctuary, Diti lake, alt. *c.* 2500–2750 m, 04 Nov. 2002, *K.P. Singh & P.K. Dixit* 365 (BSA); Mehao Wild Life Sanctuary, Sally lake, alt. *c.* 390–400 m, 07 Nov. 2002, *K.P. Singh & P.K. Dixit* 469B (BSA); West Kameng District, Shergaon forest, alt. *c.* 1950–2050 m, 24 Nov. 1984, *K.P. Singh* 5647 (ASSAM).

Graphis cincta (Pers.) Aptroot, in Archer, Fl. Australia 57: 651. 2009.

Specimens examined: Arunachal Pradesh: Changlang District, Namdapha, 43 mile, 22 Feb. 1982, *K.P. Singh* 823 (ASSAM); Upper Subansiri District, on the way of Dumporizo to Daporizo, 27 Mar. 2000, *K.P. Singh* 10689 & 10695 (ASSAM).

Graphis contortuplicata Müll. Arg., Linn. Soc., Bot. 29: 225. 1892.

Specimen examined: Arunachal Pradesh: Upper Subansiri District, Daporizo-Zero Road, 2 km from Daporizo, alt. *c.* 300 m, 16 Jan. 1983, *K.P. Singh* 4015 (ASSAM).

Graphis crebra Vain., Hedwigia 38: 256. 1899.

Specimen examined: Arunachal Pradesh: Upper Subansiri

District, on the way of Leb to Nuk basti, *K.P. Singh* 10696 D (ASSAM).

Graphis cycasicola A.W. Archer & Elix, Australas. Lichenol. 61: 18. 2007.

Specimen examined: Arunachal Pradesh: West Kameng District, Shergaon forest, alt. *c.* 1950–2050 m. 24 Nov. 1984, *K.P. Singh* 5683 (ASSAM).

Graphis daintreensis (A.W. Archer) A.W. Archer [as '*daintriensis*'], Telopea 11 (1): 72. 2005.

Specimens examined: Arunachal Pradesh: West Kameng District, Bhalukpong, Kameng river bank, *K.P. Singh & G. Swarnalatha* 4640 & 4642 (BSA).

Graphis dimidiata Vain., Acta Soc. Fauna Flora Fenn. 7 (no. 2): 108. 1890.

Specimen examined: Arunachal Pradesh: West Kameng district, Dirang, Rama Camp, Chug forest, *K.P. Singh* 2011 (ASSAM).

Graphis duplicata Ach., Syn. Meth. Lich.: 81. 1814.

Specimens examined: Arunachal Pradesh: West Siang District, Nigam Basti towards Basar, alt. *c.* 680 m, 31 Dec. 1982, *K.P. Singh* 2537 (ASSAM); Lohit District, Namsai, Near Peyong village, alt. *c.*180 m, 30 Dec. 1983, *K.P. Singh* 4172 (ASSAM); West Kameng District, Near Dedza bridge, Bahlukpong-Tanga road, alt. *c.* 1095 m, 03 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4808B (BSA); Dirang, 2 km to Santhi Vally, alt. *c.* 1601 m, 06 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5049 (BSA).

Graphis farinulenta Müll. Arg., Bull. Soc. Roy. Bot. Belg. 30: 80. 1891.

Specimens examined: Arunachal Pradesh: Lower Dibang Valley District, Mehao Wild Life Sanctuary, Sally lake, alt. *c.* 500 m, 31 Oct. 2002, *P.K. Dixit* 282C, 282D & 282F (BSA).

Graphis filiformis Adaw. & Makhija, Mycotaxon 99: 314. 2007.

Specimens examined: Arunachal Pradesh: West Kameng District, Near Dedga bridge, Bhalukpong-Tanga Road, alt. *c.* 1216 m, 03 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4872 & 4866 (BSA).

Graphis furcata Fée, Essai Crypt. Ecorc.: 40. 1824.

Specimens examined: Arunachal Pradesh: West Siang District, Along, Jamola Basti forest, along the riverside, alt. *c.* 400 m, 03 Jan. 1983, *K.P. Singh* 2612 (ASSAM); West Kameng District, Tipi–Bhalukpong Road, Dijiling forest, alt. *c.* 400 m, 18 Apr. 1982, *K.P. Singh* 1116B (ASSAM); West Kameng district, 10.5 km to Nechiphu, Tanga-Bhalukpong Road, alt. *c.* 1184 m, 07 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5158 (BSA).

Graphis glaucescens Fée, Essai Crypt. Ecorc.: 36. 1824.

Specimen examined: Arunachal Pradesh: West Kameng District, Bhalukpong-Tipi Road, alt. *c.* 136 m, 01 Mar. 2009, *K.P. Singh & G. Swarnalath*a 4646 (BSA).

Graphis glauconigra Vain., Ann. Acad. Sci. Fenn., ser. A, 15(6): 242. 1921.

Specimen examined: Arunachal Pradesh: West Kameng District, Bhalukpong-Tipi road side forest, alt. *c.* 136 m, 01 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4660 (BSA).

Graphis handelii Zahlbr., in Hand. -Mazz., Symb. Sin. 3: 39 & 40. 1930.

Specimens examined: Arunachal Pradesh: Mehao Wild Life Sanctuary, Mayudia, alt. *c.* 2250 m, 06 Nov. 2002, *K.P. Singh & P.K. Dixit* 442A (BSA).

Graphis intermediella Stirt., Proc. Roy. Soc. Glasgow 11: 316. 1879.

Specimen examined: Arunachal Pradesh: Changlang District, Miao, 15 Feb. 1982, *K.P. Singh* 659 (ASSAM).

Graphis japonica (Müll. Arg.) A.W. Archer & Lücking, in Lücking, Archer & Aptroot, Lichenologist 41 (4): 437. 2009.

Specimens examined: Arunachal Pradesh: Upper Subansiri District, Daporizo-Zero Road, 2 km from Daporizo, alt. *c.* 300 m, in *Terminalia* plantation, 16 Jan. 1983, *K.P. Singh* 3069 (ASSAM); West Kameng District, Sessa Orchid Sanctuary, alt. *c.* 1033–1170 m, 01 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4716 (BSA); Tipi forest rest house campus, alt. *c.* 202 m, 08 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5247 & 5251 (BSA).

Graphis kousyuensis (Horik. & M. Nakan.) Lücking, in Lücking, Archer & Aptroot, Lichenologist 41 (4): 437. 2009.

Specimens examined: Arunachal Pradesh: West Kameng District, on the fallen tree in Kameng River, 08 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5313, 5294 (BSA).

Graphis librata C. Knight., Trans. New Zealand Inst. 16: 404. 1884.

Specimens examined: Arunachal Pradesh: Lower Dibang Valley District, Mehao Wild Life Sanctuary, Roing Mini Zoo Area, 09 Nov. 2002, *K.P. Singh & P. K. Dixit* 516 (BSA); West Kameng District, Tipi forest rest house campus, alt. *c.* 202 m, 08 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5215, 5242B & 5253 (BSA).

Graphis longiramea Müll. Arg., J. Linn. Soc., Bot. 29: 225. 1892.

Specimens examined: Arunachal Pradesh: Tirap District, Khonsa, Khela forest, 27 Feb. 1982, *K.P. Singh* 967 (ASSAM); Lohit District, Madhuban Reserver forest, alt. *c.* 250 m, 30 Dec. 1983, *K.P. Singh* 4203 (ASSAM); Lower Dibang Vally, Roing, Deopani forest, along the stream, alt. *c.* 700 m, 04 June 1984, *D.K. Upreti & B.C. Upreti* L81756 (LWG); Mehao Wild Life Sanctuary, Diti hill, alt. *c.* 2550–2750 m, 04 Nov. 2002, *K.P. Singh & P.K. Dixit* 373F/2 (BSA); Mehao Lake, alt. *c.* 1550 m, 14 Nov. 2002, *K.P. Singh & P.K. Dixit* 547 & 559A (BSA); West Kameng District, Sessa Orchid Sanctuary, alt. *c.* 1033–1170 m, 01 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4714 (BSA).

Graphis longispora D.D. Awasthi & S.R. Singh, Norweg. J. Bot. 24: 3. 1977.

Specimen examined: Arunachal Pradesh: Lower Debang Valley District, Riyali-Annini foot track, 10 km from Riyali, alt. *c.* 900 m, 13 Jan. 1984, *K.P. Singh* 4786 (ASSAM).

Graphis marginata Raddi., Raddi, G., Mem. Soc. Ital. Sc. 18: 344, tab. 3, fig. 2. 1820.

Specimen examined: Arunachal Pradesh: West Siang District, Along, Jamola Basti forest, along the riverside, alt. *c.* 400 m, 03 Jan. 1983, *K.P. Singh* 2611 (ASSAM).

Graphis nanodes Vain., Ann. Acad. Sci. Fenn. 15(6): 209. 1921.

Specimens examined: Arunachal Pradesh: Lower Dibang Valley District, Mehao Wild Life Sanctuary, 9 Oct. 2001, *P.K. Dixit* 55 (BSA); West Kameng District, on fallen tree in Kameng river, 08 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5305 (BSA).

Graphis parilis Kremp., Flora 59: 422 & 445. 1876.

Specimens examined: Arunachal Pradesh: East Kameng District, Phakui Wild life Sanctuary, Dichu-Julley Nala, alt. *c.* 300–440 m, 15 Apr. 1999, *K.P. Singh* 10084 (ASSAM); West Kameng District, 10.5 km to Nechiphu, Tanga-Bhalukpong Road, alt. *c.* 1184 m, 07 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5151B & 5172 (BSA).

Graphis pertricosa (Kremp.) A.W. Archer, Telopea 11: 73. 2005.

Specimens examined: Arunachal Pradesh: West Kameng District, Dirang, Yang forest, alt. *c.* 1625–1800 m, 05 May. 1982, *K.P. Singh* 1897 (ASSAM); Dirang, near National Research Center on Yak, 04 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4918A & 4918B (BSA); 10.5 km to Nechiphu, Tanga-Bhalukpong Road, alt. *c.* 1184 m, 07 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5151B & 5159 (BSA).

Graphis pinicola Zahlbr., in Hand.-Mazz., Symb. Sin. 3: 40 & 43. 1930.

Specimen examined: Arunachal Pradesh: West Kameng District, Tipi forest rest house campus, alt. *c.* 202 m, 08 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5244 (BSA).

Graphis proserpens Vain., Bot. Tidsskr. 29(2): 132. 1909.

Specimens examined: Arunachal Pradesh: East Kameng District, Mehao Wild Life Sanctuary, Diti lake, alt. *c.* 2500–2750 m, 04 Nov. 2002, *K.P. Singh & P.K. Dixit* 370B, 376A & 390B (BSA); West Kameng District, 15 km to Sange, Dirang-Sange Road, alt. *c.* 2306–2344 m, 05 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5003 (BSA).

Graphis pyrrhocheiloides Zahlbr., Cat. Lich. Univ. 2: 321. 1923.

Specimens examined: Arunachal Pradesh: Lohit District, Hayuliang Inspection Bungalow Compound, alt. *c.* 600 m, 04 Jan. 1984, *K.P. Singh* 4277A/B/C/D (ASSAM).

Graphis scripta (L.) Ach., Kgl. Vetensk. -Akad. Nya Handl.: 145. 1809.

Specimens examined: Arunachal Pradesh: East Kameng District, Phakui Wild Life Sanctuary, (Dichu-Julley Nala), alt. *c.* 300–440 m, 15 Apr. 1999, *K.P. Singh* 10023 (ASSAM); West Kameng District, Bomdila-Sepper Road, 2 km from Bomdila, alt. *c.* 2272–2300 m, 03 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4816 (BSA).

Graphis sitapurensis Makhija & Adaw., Mycotaxon 91: 378. 2005.

Specimen examined: Arunachal Pradesh: Lower Dibang Valley District, Mehao Wild Life Sanctuary, Mahao Lake, alt. *c.* 1550 m, 14 Nov. 2002, *K.P. Singh & P.K. Dixit* 551A/2 (BSA).

Graphis streblocarpa (Bél.) Nyl., Flora 49: 133. 1866.

Specimens examined: Arunachal Pradesh: Changlang District, Namdapha, 36th mile, 20 Feb. 1982, *K.P. Singh* 796 (ASSAM); West Kameng District, Tipi, alt. *c.* 400 m, 16 Apr. 1982, *K.P. Singh* 1039 (ASSAM); Upper Subansiri District, Daporizo-Zero Road, 2 km from Daporizo, on *Terminalia* plantations, alt. *c.* 300, 16 Jan. 1983, *K.P. Singh* 3075 (ASSAM); Upper Subansiri District, Daporizo-Zero Road, 2 km from Daporizo, on *Terminalia* plantations, alt. *c.* 300, 15 Jan. 1983, *K.P. Singh* 3092 (ASSAM); Debang Vally District, Rayali-Kronli foot track, alt. *c.* 400–900 m, 14 Jan. 1984, *K.P. Singh* 4835 (ASSAM); Lower Subansiri

District, Yazali, Near Yazali bridge, alt. *c.* 750 m, 22 Jan. 1984, *K.P. Singh* 5105 (ASSAM); West Kameng District, Sessa Orchid Sanctuary, alt. 1150–1250 m, 9 Oct. 2001, *P.K. Dixit* 09 (BSA).

Graphis striatula (Ach.) Spreng., Syst. Veg. Lich. 4: 250. 1827.

Specimens examined: Arunachal Pradesh: East Kameng District, Seppa, Kucchinalakaku kau, alt. *c.* 550–1050 m, 11 May 1982, *K.P. Singh* 2103 (ASSAM); West Kameng District, Near Dedga bridge, 15 km to tanga, Bhalukpong-Bomdila Road, alt. *c.* 1094 m, 03 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4787 (BSA).

Graphis subassimilis Müll. Arg., Flora 65: 333. 1882.

Specimen examined: Arunachal Pradesh: Upper Subansiri District, Daporizo-Leko basti post, alt. *c.* 300–500, 14 Jan. 1983, *K.P. Singh* 3002 (ASSAM).

Graphis subserpentina Nyl., Acta Soc. Sci. Fenn. 7: 465. 1863.

Specimens examined: Arunachal Pradesh: West Kameng District, Dirang, Yang forest, alt. *c.* 1625–1800 m, 05 May 1982, *K.P. Singh* 1900A (ASSAM); Dirang, Near Apple garden, alt. *c.* 2123 m, 06 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5130 (BSA).

Graphis tenella Ach., Syn. Meth. Lich.: 81. 1814.

Specimen examined: Arunachal Pradesh: Lower Dibang Valley District, Mehao Wild Life Sanctuary, Mahao lake, alt. *c.* 1550–1700 m, 15 Nov. 2002, *K.P. Singh & P.K. Dixit* 608A (BSA).

Graphis cf. valparaiensis Adaw. & Makhija, Mycotaxon 99: 323. 2007.

Specimens examined: Arunachal Pradesh: Changalang District, Namdapha, 21 Mar. 1981, *K. P. Singh* 310 (ASSAM); Debang Valley, Rayali-Kronli foot track, alt. *c.* 400"900 m, 14 Jan. 1984, *K.P. Singh* 4826 (ASSAM); Mehao Wild Life Sanctuary, Diti lake, alt. *c.* 2500-2750 m, 04 Nov. 2002, *K.P. Singh & P.K. Dixit* 379 (BSA); Lower Dibang Valley District, Mehao Wild Life Sanctuary, Diti hill, alt. *c.* 2500-2750 m, 04 Nov. 2002, *K.P. Singh & P.K. Dixit* 383B (BSA); Mehao Wild Life Sanctuary, Mehao lake, alt. *c.* 1550 m, 14 Nov. 2002, *K.P. Singh & P.K. Dixit* 548D, 562B & 565D (BSA); Mehao lake, alt. *c.* 1550–1700 m, 15 Nov. 2002, *K.P. Singh & P.K. Dixit* 548D & 565D (BSA); Mehao lake, alt. *c.* 1550-1700 m, 15 Nov. 2002, *K.P. Singh & P.K. Dixit* 607E (BSA); Mehao Wild Life Sanctuary, Baudhi camp to Mehao lake, alt. *c.* 1400-1500 m, 16 Nov. 2002, *K.P. Singh & P.K. Dixit* 636A (BSA); Mahao lake to Devpani track, 17 Nov. 2002, *K.P. Singh & P.K. Dixit* 648D (BSA); West Kameng District, Tipi forest rest house campus, alt. *c.* 202 m, 08 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5180, 5250 & 5255 (BSA).

Graphis vittata Müll. Arg., Flora 65: 335. 1882.

Specimens examined: Arunachal Pradesh: Changlang District, Namdapha, *K.P. Singh* 331 (ASSAM); West Kameng District, Tipi-Bhalukpong Road, Dijiling forest, alt. *c.* 400 m, 18 Apr. 1982, *K.P. Singh* 1116A/C/D, 1122, 1133 & 1143 (ASSAM); Lower Dabang Valley District, Mehao Wild Life Sanctuary, Mahao lake, alt. *c.* 1550–1700 m, 15 Nov. 2002, *K.P. Singh & P.K. Dixit* 603A (BSA).

Hemithecium amboliense Makhija & Dube, in Makhija, Dube, Adawadkar & Chitale, Mycotaxon 93: 367. 2005.

Specimen examined: Arunachal Pradesh: Lower Dibang Valley District, Mehao Wild Life Sanctuary, Roing Mini Zoo, 09 Nov. 2002, *K.P. Singh & P.K. Dixit* 504F (BSA).

Hemithecium aphaneomicrosporum Makhija & Adaw., Mycotaxon 91: 348. 2005.

Specimen examined: Arunachal Pradesh: West Kameng District, Tipi, Neychning festival ground, near during bridge, alt. *c.* 160 m, 02 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4757 (BSA).

Hemithecium aphanes (Mont. & Bosch) M. Nakan. & Kashiw., Bull. Natl. Sci. Mus. Tokyo, B, 29(2): 88. 2003.

Specimens examined: Arunachal Pradesh: Debang Valley, Hunli-Desali foot track, alt. *c.* 400–900 m, 15 Jan. 1984, *K.P. Singh* 4901 (ASSAM); East Kameng District, Phakui Wild Life Sanctuary, (Dichu–Julley Nala), alt. *c.* 300–440 m, 15 Apr. 1999, *K.P. Singh* 10023 (ASSAM); Lower Dibang Valley District, Mehao Wild Life Sanctuary, Mehao lake to Mini Mehao lake, 23 Nov. 2000, *K.P. Singh & P. Bujarbarua* 11141 (ASSAM); 10 Oct. 2001, *P.K. Dixit* 56 & 57 (BSA); West Kameng District, Bhalukpong, Kameng river bank, alt. *c.* 220–229 m, 01 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4635 (BSA); Bhalukpong–Tipi Road, alt. *c.* 136 m, 01 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4649, 4670, 4671 (BSA); Sessa Orchid Sanctuary, alt. *c.* 1033–1170 m, 01 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4719 (BSA); Tipi forest rest house campus, alt. *c.* 202 m, 08 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5189, 5190 (BSA); Bhalukpong–Tipi Road, alt. *c.* 136 m, 01 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5322B (BSA).

Hemithecium balaghatensis Adaw. & Makhija, Mycotaxon 92: 388. 2005.

Specimen examined: Arunachal Pradesh: West Kameng

District, Tipi forest rest house campus, alt. *c.* 202 m, 08 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5203 (BSA).

Hemithecium nagalandicum (Kr.P. Singh & G.P. Sinha) Adaw. & Makhija, Mycotaxon 92: 390. 2005

Specimens examined: Arunachal Pradesh: Lower Dibang Valley District, Mehao Wild Life Sanctuary, Mehao lake to Gahori Camp, 25 Nov. 2000, *K.P. Singh & P. Bujarbarua* 11225 (ASSAM); 09 Oct. 2001, *P.K. Dixit* 30 (BSA); Mehao Lake, alt. *c.* 1550 m, 14 Nov. 2002, *K.P. Singh & P.K. Dixit* 554E & 556B (BSA); Mehao Wild Life Sanctuary, Baudhi Camp to Mehao Lake, alt. *c.* 1400–1550 m, 16 Nov. 2002, *K.P. Singh & P.K. Dixit* 628A (BSA).

Hemithecium nakanishianum (Patw. & C.R. Kulk.) Makhija & Dube, in Makhija, Dube, Adawadkar & Chitale, Mycotaxon 93: 370. 2005.

Specimen examined: Arunachal Pradesh: West Kameng, Bhalukpong-Tipi Road, alt. *c.* 136 m, 01 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4652 (BSA).

Hemithecium norsticticum Makhija & Dube, in Makhija, Dube, Adawadkar & Chitale, Mycotaxon 93: 371. 2005.

Specimen examined: Arunachal Pradesh: West Kameng District, Tipi-Bhalukpong road side forest; alt. *c.* 250–300 m, 10 Oct. 2001, *G.P. Sinha & T.A.M. Jagadeesh* 11292 (ASSAM).

Leiorreuma exaltatum (Mont. & Bosch) Staiger, Biblioth. Lichenol. 85: 298. 2002.

Specimen examined: Arunachal Pradesh: Debang Valley, Rayali–Kronli foot track, alt. *c.* 400–900 m, 14 Jan. 1984, *K.P. Singh* 4886 (ASSAM).

Leiorreuma lyellii (Sm.) Staiger, Biblioth. Lichenol. 85: 301. 2002.

Specimen examined: Arunachal Pradesh: Lower Dibang Valley District, Mehao Wild Life Sanctuary, Sally lake, alt. *c.* 500 m, 12 Nov. 2000, *K.P. Singh & P. Bujarbarua* 10870 (BSA).

Leiorreuma subpatellulum Dubey, Upreti & Nayaka, Lichenologist 42 (6): 711. 2010.

Specimen examined: Arunachal Pradesh: Upper Siang District, Jengging, near circut house, 28°54' N 95°06' E, alt. 900 m, on bark, 30 Oct. 2007, *U. Dubey* 07-011897 (holotype-LWG!).

Pallidogramme chrysenteron (Mont.) Staiger, Kalb & Lücking, in Lücking, Chaves, Sipman, Umaña & Aptroot, Fieldiana, Bot. 46: 9. 2008.

Specimen examined: Arunachal Pradesh: Lower Subansiri District, on the way of Tazang, 2 km from Ziro, 25 Sep. 1981, *K.P. Singh* 620 (ASSAM).

Phaeographis caesioradians (Leight.) A.W. Archer, Telopea 11: 75. 2005.

Specimen examined: Arunachal Pradesh: Upper Subansiri District, Daporizo, alt. *c.* 300–600 m, 15 Jan. 1983, *K.P. Singh* 4053 (ASSAM).

Phaeographis dendroides (Leight.) Müll. Arg., Flora, 65: 336. 1882b.

Specimen examined: Arunachal Pradesh: West Siang District, Doji, 16 km from Along towards Basar, 26 Mar. 2006, *U. Dubey* 06-006342 (LWG)

Phaeographis divaricoides Räsänen, in Arch. Soc. Zool. Bot. Fenn. Vanamo 5: 31. 1950.

Specimens examined: Arunachal Pradesh: Mehao Wild Life Sanctuary, Mehao to Ghauri Camp, alt. *c.* 1500–1550 m, 16 Nov. 2002*, K.P. Singh & P.K. Dixit* 615B (BSA); Mehao Wild Life Sanctuary, Baudhi to Mehao lake, alt. *c.* 1400–1550 m, 16 Nov. 2002, *K.P. Singh & P.K. Dixit* 636C (BSA).

Phaeographis intricans (Nyl.) Staiger, Biblioth. Lichenol. 85: 329. 2002.

Specimen examined: Arunachal Pradesh: Lower Dibang Valley District, Mehao Wild Life Sanctuary, Mehao Lake, alt. *c.* 1550–1700 m., 15 Nov. 2002, *K.P. Singh & P.K. Dixit* 581D (BSA).

Phaeographis scalpturata (Ach.) Staiger, Biblioth. Lichenol. 85: 345. 2002.

Specimen examined: Arunachal Pradesh: Changlang District, Namdapha, 21 Mar. 1981, *K.P. Singh* 308 (ASSAM).

Phaeographopsis indica (Patw. & Nagarkar) Sipman & Aptroot, in Aptroot, Diederich, Sérusieux & Sipman, Biblioth. Lichenol. 64: 129. 1997

Specimen examined: Arunachal Pradesh: West Kameng District, Tipi forest rest house campus, alt. *c.* 202 m, 08 Mar. 2009, *K.P. Singh & G. Swarnalatha* 5182 (BSA).

Platygramme caesiopruinosa (Fée) Fée, Bull. Soc. Bot. France 21: 30. 1874.

Specimen examined: Arunachal Pradesh: West Kameng District, Dirang, Yarg forest, alt. *c.* 1625–1800 m, 05 May 1982, *K.P. Singh* 1900B (ASSAM).

Platygramme discurrens (Nyl.) Staiger, Biblioth. Lichenol. 85: 362. 2002.

Specimens examined: Arunachal Pradesh: West Siang District, Bame to Daborijo Road, Near RMP camp, alt. *c.*

900 m, 30 Dec. 1982, *K.P. Singh* 2481C (ASSAM); Lower Subansiri District, on the way of Piti from Yazali, up to 300 km, 21 Mar. 2000, *K.P. Singh* 10451 (ASSAM).

Platygramme wattiana (Müll. Arg.) V. Tewari & Upreti, Indian J. Forest. 31(3): 458. 2008.

Specimens examined: Arunachal Pradesh: Near Lailyngkot, 19 Jan. 1981, *K.P. Singh* 145 (ASSAM); Lower Subansiri District, Ganga nagar, 22 Sep. 1981, *K.P. Singh* 344 (ASSAM); West Siang District, Bame to Daborijo Road, 30 Dec. 1982, *K.P. Singh* 2481 (ASSAM).

Sarcographa glyphiza (Nyl.) Kr.P. Singh & G.P. Sinha, Indian Lichens: An Annotated Checklist, 13. 2010.

Specimens examined: Arunachal Pradesh: East Kameng District, Seppa, Kucchinalakaku kau, alt. *c.* 550–1050 m, 11 May 1982, *K.P. Singh* 2111 (ASSAM); Basar Nigam, alt. *c.* 680 m, 31 Dec. 1982, *K.P. Singh* 2545C (ASSAM); Upper Subansiri District, Daporizo-Zero Road 2 km from Daporizo, on *Terminalia* tree, 16 Jan. 1983, *K.P. Singh* 3068 (ASSAM); Upper Subansiri District, Daporizo, Donbasti forest, alt. *c.* 300–600 m, 17 Jan. 1983, *K.P. Singh* 4045A (ASSAM); East Kameng District, Phakui Wild Life Sanctuary, Dichu–Julley Nala, alt. *c.* 300-440 m, 15 Apr. 1999, *K.P. Singh* 10090 (ASSAM).

Sarcographa labyrinthica (Ach.) Müll. Arg., Mem. Soc. Phys. Genève 29(8): 62. 1887.

Specimens examined: Arunachal Pradesh: Changlang District, 36th mile Nandapha, 20 Feb. 1982, *K.P. Singh* 761 (ASSAM); Tirap District, Khonsa, Khela forest, 27 Feb. 1982, *K.P. Singh* 977, 1001 (ASSAM); Lohit District, Madhuban Reserver forest, alt. *c.* 250 m, 30 Dec. 1983, *K.P. Singh* 4203 (ASSAM); Upper Subansiri District, Daporizo, zero Road 2 km from Daprizo, alt. *c.* 300 m, 16 Jan. 1983, *K.P. Singh* 4003 (ASSAM); Daporizo, Donbasti forest, alt. *c.* 300–600 m, 17 Jan. 1983, *K.P. Singh* 4045B/C (ASSAM); Lower Dibang Valley District, Mehao Wild Life Sanctuary, Mahao Lake north bank, 24 Nov. 2000, *K.P. Singh & P. Bujarbarua* 11181 (ASSAM); West Kameng District, Tipi, Near Neychiung festival ground, alt. *c.* 160 m, 02 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4748 (BSA).

Sarcographa medusulina (Nyl.) Müll. Arg., Flora, Regensburg 70: 77. 1887c.

Specimen examined: Arunachal Pradesh: West Siang District, Basar Nigam, alt. *c.* 680 m, 31 Dec. 1982, *K.P. Singh* 2545A (ASSAM).

Sarcographa tricosa (Ach.) Müll. Arg., Mém. Soc. Phys. Genève 29 (8): 63. 1887c.

Specimens examined: Arunachal Pradesh: Tirap District, Khonsa, Khela forest, 27 Feb. 1982, *K.P. Singh* 969 (ASSAM); West Siang District, Basar Nigam, alt. *c.* 680 m, 31 Dec. 1982, *K.P. Singh* 2545B (ASSAM); Debang Valley, Rayali-Anniri foot track, alt. *c.* 900 m, 13 Jan. 1984, *K.P. Singh* 4752 (ASSAM); Debang Vally, Rayali–Krouls foot track, alt. *c.* 400–900 m, 14 Jan. 1984, *K.P. Singh* 4848 (ASSAM); Debang Vally, Hunh–Desali foot track, alt. *c.* 400–900 m, 15 Jan. 1984, *K.P. Singh* 4945 (ASSAM); Debang Vally, Hunh–Desali foot track, alt. *c.* 400–900 m, 15 Jan. 1984, *K.P. Singh* 4945 (ASSAM); Lower Debang Valley District, Roing–Anini Road, foot track, 16 Jan. 1984, *K.P. Singh* 5019C (ASSAM); Mehao Wild Life Sanctuary, Mehao Lake, alt. *c.* 1550 m, 14 Nov. 2002, *K.P. Singh & P.K. Dixit* 547 & 559A (BSA); Lower Subansiri District, Near Kimin, alt. *c.* 400 m, 21 Jan. 1984, *K.P. Singh* 5073 (ASSAM).

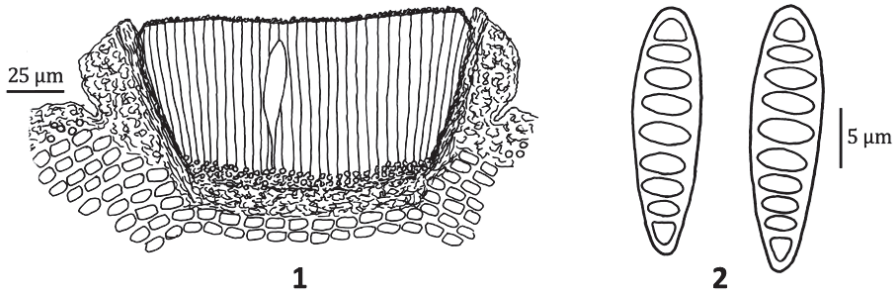
Schistophoron indicum Kr. P. Singh & Swarnal. Lichenologist 43(3): 209. 2011.

Specimens examined: Arunachal Pradesh: East Siang District, Gette Basti, alt. *c.* 500–700 m, 8 Jan. 1983, *K.P. Singh* 2858 (holotypus - BSA!; isotypus - ASSAM!).

Thalloloma hypoleptum (Nyl.) Staiger, Biblioth. Lichenol. 85: 437. 2002.

Graphis hypolepta Nyl., Acta. Soc. Sci. Fenn. 7: 472. 1863a. - Type: Colombia (Nova Granata), Bogot, 2400 m, Leg. *Lindig nr.* 715. (isotype - M).

Thallus corticolous, epiphloeodal, 42–48 µm thick above the bark, 5–8.4 cm diam., continuous, delimited by blackish-brown prothallus; surface yellowish white, farinose. Thallus in section lacking upper cortex and has numerous calcium-



Figs. 1-2. *Thalloloma hypoleptum*(Nyl.) Staiger : 1. V.S. of ascomata, 2. Ascospores.

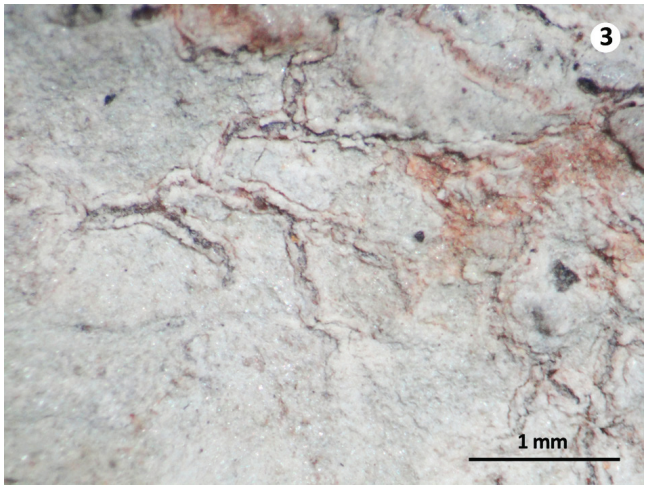


Fig. 3. *Thalloloma hypoleptum* (Nyl.) Staiger

oxalate crystals. Ascomata lirelliform, concolorous with thallus, delicate, simple to bifurcate or irregularly branched, straight to curved, immersed to erumpent, with lateral thalline margin, 0.8–2.5 mm long and less than 0.1–0.2 mm broad, acute at the ends, thalline margin separated from thallus by a slit; disc slit-like to moderately open, slightly concave, reddish brown, with white pruina; excipulum complete, uncarbonized, golden-brown, laterally covered by thalline margin; labia entire, divergent; epihymenium brown to dark-brown, 6–8 µm high; hymenium hyaline, not inspersed, 58–73 µm high, I–; subhymenium hyaline, 16–21 µm high; paraphyses unbranched to branched and anastomosing, *c.* 1.5 µm thick, tips branched, brown walled. Asci ellipsoid, 43–52 × 14.5–17.5 µm. Ascospores 8 per ascus, biseriate to aggregate, ellipsoid, transversely septate, 18–26 × 6–8 µm, 5–9 locular, hyaline, I+ blue-violet.

Chemistry: Thallus K–, C–, KC–, P–; UV+ yellow; lichexanthone present.

Remarks: *Thalloloma hypoleptum* is characterised by its yellowish white, farinose, ecorticate thallus; immersed to erumpent, lirelliform ascomata; moderately open, reddish-brown disc with white pruina; uncarbonized excipulum with entire, divergent labia; hyaline, transversely septate, 18–26 µm long ascospores and presence of lichexanthone (thallus UV+ yellow). *T. hypoleptum* somewhat resembles *T. anguinaeforme* in external morphology but latter species differs by having muriform ascospores and lacking lichexanthone (thallus UV–). Further, *T. deplanatum* is another morphologically similar species but *T. deplanatum* distinguished by its larger (50–65 × 10–13 µm and 10–15 locular) ascospores.

Distribution: Borneo, Japan, Papua New Guinea, North and South America; it is now reported here from India

Specimen examined: Arunachal Pradesh: West Kameng District, Sessa Orchid Sanctuary, alt. 1033–1170 m, 01 Mar. 2009, *K.P. Singh & G. Swarnalatha* 4717 (BSA).

Remarks: In the study area the species has been collected from tree barks in evergreen subtropical forests of West Kameng District. This is phyto-geographically interesting as the only other species of the genus recorded in India is *Thalloloma patulum* (A. W. Archer) Adaw. & Makhija.

Thecaria quassiicola Fée as '*quassiaecola',* Meth. Lichenogr. Gen.: 92, tab. 1, fig. 16. 1824.

Specimens examined: Arunachal Pradesh: Tirap District, Khonsa, Khela forest, 27 Feb. 1982, *K.P. Singh* 939 (ASSAM); West Kameng District, Tipi–Bhalukpong Road, Desiling forest, 18 Apr. 1982, *K.P. Singh* 1141 (ASSAM); West Siang District, Along, Jamola Basti forest, along the riverside, alt. *c.* 400 m, 03 Jan. 1983, *K.P. Singh* 2605 (ASSAM); Upper Subansiri District, Daporizo to Leko basti forest, alt. *c.* 300–500 m, 14 Jan. 1983, *K.P. Singh* 2979 (ASSAM); Lower Dibang Valley District, Roing Anini Road, 16 Jan. 1984, *K.P. Singh* 5026 (ASSAM); Roing, Near Damduck bifurcation, alt. *c.* 450 m, 17 Jan. 1984, *K.P. Singh* 5034 (ASSAM).

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Chromatographic and spectroscopic fingerprint analysis of *Hedychium coronarium* rhizome extracts through HPLC, GC-MS and FTIR

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ABSTRACT

Hedychium coronarium J. koenig. is an important medicinal and aromatic plant of Zingiberaceae. Bioactive constituents present in the extract have created a lot of interest because of its tremendous biological activity. The biggest challenge lies in the authentic identification of the drug, hence it is of utmost importance to develop spectral and chromatographic fingerprint that represents pharmacologically active and chemically characteristic components of *H. coronarium*. The present study was initiated to carry out chemical fingerprinting using HPLC, FTIR and GC-MS. A total of twelve absorption peaks were present in the IR spectra which can be used to characterize the extract. This study also attempts to develop HPLC fingerprint of the rhizame extract. Observation on HPLC spectra shows the presence of twelve distinct peaks. The IR spectra show the presence of peak at frequencies between ranges from 467.65 to 3327.57 cm^{-1}. FTIR analysis confirmed the presence of alkane, alkene, alcohol, amines, acid and halogens in the extract. A total of 21 phytoconstituents was identified in the methanolic extract from the rhizome extract of the plant by GC-MS. These chemical fingerprinting would be of commercial significance for quality control and authentication of *H. coronarium* extracts.

1. Introduction

Herbal medicines and their preparations are being used extensively throughout the world for combating various diseases. Inferior quality of herbal drug is a hindrance for its globalization and modernization. Hence ensuring their quality is of utmost importance. Quality control of herbal drug is more challenging since it involves a high level of chemical complexity due to diverse bioactive components. Furthermore, loss of principal active components may result in loss of pharmacological action of the plant. Therefore, significant efforts have been made for qualitative and quantitative characterization of samples using fingerprint technologies. (Lin *et. al.*, 2001). WHO also stresses the importance of analytical techniques for standardization of herbal drugs.

Hedychium coronarium J. Koenig (family *Zingiberaceae*) commonly known as Butterfly ginger is a rhizomatous herb widely cultivated in tropical and subtropical regions (Ray *et. al*., 2016). Rhizome extracts of *H.* coronarium are used as drug in traditional herbal medicine for the treatment of tonsillitis, infected nostrils, tumor, and fever. It is also used as a febrifuge, tonic, excitant and anti-rheumatic in the Ayurvedic system of Indian medicine (Jain *et. al*., 1995). The essential oil extracted from leaves, flowers and rhizome of this plant have potent antimicrobial, antifungal, anti-inflammatory, antibacterial and analgesic effects. Coronarin D, a labdane diterpenoid present in *H. coronarium* possess diverse pharmacological activities supported by various investigations. Coronarin D has shown to inhibit NF-Kβ leading to induction of apoptosis (Kunnumakkara *et. al*, 2008) and inhibit the release of *β*-hexosaminidase from RBL-2H3 cells (Morikawa *et.al*, 2002).Pharmacological actions reported are antibacterial (Reuk-ngam *et. al.*, 2014) and antifungal activities (Kaomongkolgit *et. al.*, 2012). In the present study chemical fingerprint has been developed using HPLC, FTIR and GCMS to control the quality of herbal drug of *H. coronarium*.

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2. Materials and methods

2.1. Plant source and extract preparation

Rhizomes of *H. coronarium* was collected in October 2015, at the flowering stage in the Ushabali valley (latitude 19° 56' 31.3" N, longitude 83° 39' 22.3" E), Phulbani Territorial Forest Division, at an elevation of 745 m above the sea level. The plant was authenticated by Dr. P.C. Panda, Principal Scientist, (Taxonomy and Conservation Division, RPRC, Bhubaneswar) and voucher specimen of plant (9741) was housed in the herbarium of Regional Plant Resource Centre, Bhubaneswar. Rhizomes were shade dried, powdered and subjected to extraction in solvent methanol using soxhlet apparatus for 8 h. The extract was filtered and the solvent was removed in a rotary evaporator at 50°C. Extracts were stored at 4°C for future analysis.

2.2. Chromatographic conditions of HPLC analysis

HPLC system for chromatographic analysis consisted of a separation module (Waters 600E) equipped with Empower software (Waters) and comprising of quaternary pump, an in-line vacuum degasser and a photodiode array detector (Waters 2996). The chromatographic separation was carried out on a Cosmosil C_8 column (250 × 4.6 mm, 5 ìm i.d.) using an isocratic elution. The mobile phase consisted of a mixture of solvent acetonitrile (80%, A) and water (20%, B). The solvent flow rate was 1.0 ml/min. The injection volume was 10 µl and the column temperature was ambient. The photo diode array detector wavelength was set at 254 nm.

2.3. Fourier Transform Infrared Spectrophotometer analysis

Dried powder of methanolic extract was used for FTIR analysis. 10 mg of the extract powder was encapsulated in 100 mg of KBr pellet, in order to prepare sample disc. The powdered sample was loaded in FTIR spectrophotometer (Jasco, FTIR-6000 series), with a scan range from 400 to 4000 cm^{-1}.

2.4. Gas Chromatography mass spectrometry analysis

GC-MS analysis of this extract was performed using THERMO TRACE 1300 gas chromatography equipped with THERMO TSQ 8000 mass detector. 0.1 µl of the extract was injected in split less mode using helium as the carrier gas at a constant flow rate of 1 ml/min. The column used was TG 5MS (30 m x 0.25 mm, 0.25 µm) silica capillary column. For GC-MS detection, an electron ionization energy system with ionization energy of 70eV was used. Ion source temperature is maintained at 230°C. The injector temperature is maintained at 250°C whereas transfer line temperature is maintained at 280°C. The mass spectrometer was scanned from a mass range of 50-600 amu. Compounds were identified using the NIST Mass Spectral Database.

3. Results and discussion

In this work, a method based on reverse phase HPLC separation combined with PDA detection has been developed for analysis in *H. coronarium*. An isocratic elution was chosen since it is simple, requires only one pump and minimizes the variation of baseline and ghost peaks. For RP-HPLC, various columns are available, but a Cosmosil C_8 column (250 × 4.6 mm, 5 ìm i.d.) was preferred because its peak shape and resolution were better. Among the different mobile phases employed, acetonitrile and water (80:20 v/v) was found to be suitable for analysis. Further, a flow rate of 1 ml/min and an injection volume of 10 ìl along with UV detection at 254 nm provided the optimal conditions for analysis of the compounds. The results from HPLC crude extract profile for rhizome extract of *H. coronarium* showed the presence of 12 peaks at the retention time between 0 to 12 minutes (Fig. 1).

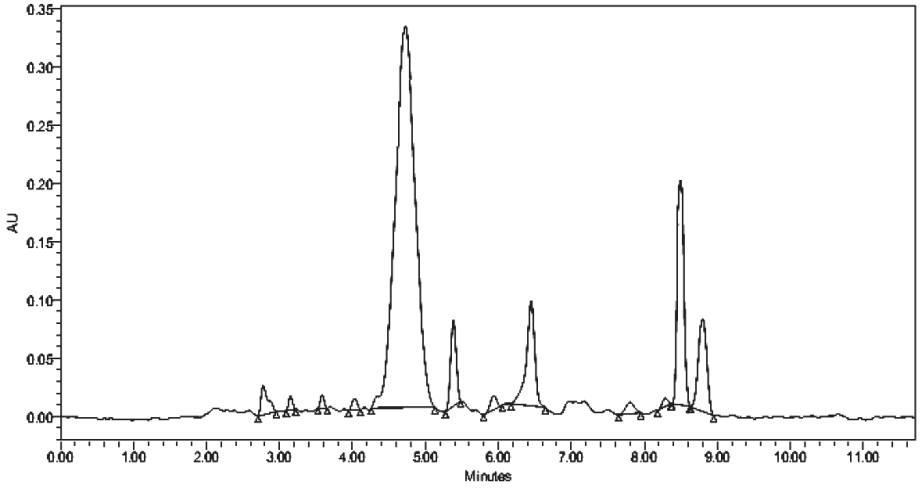

Fig. 1: HPLC Chromatogram of *H.coronarium* rhizome methanolic extract.

The peak spectrum of rhizome extract showed the highest peak spectrum at 4.703, 8.491 min. while, at 6.448, 8.797, 5.382 and 2.777 min showed a moderate peak spectrum (Table 1).

Table 1

HPLC results of rhizome extract of *H.coronarium*

Peak Name	RT	Area	Area %	Height
Peak 1	2.777	168911	1.70	25204
Peak 2	3.150	44555	0.45	12223
Peak 3	3.585	41272	0.41	11492
Peak 4	4.029	48879	0.49	9717
Peak 5	4.703	6206929	62.32	327504
Peak 6	5.382	394219	3.96	73869
Peak 7	5.930	73511	0.74	12230
Peak 8	6.448	723480	7.26	90439
Peak 9	7.802	82169	0.82	9808
Peak 10	8.282	37710	0.38	7243
Peak 11	8.491	1496133	15.02	297653
Peak 12	8.797	642461	6.45	79280

RT: Retention time in minutes (min).

Table 2

FTIR spectral peak values and functional groups of rhizome extracts of *H. coronarium*

S.No	Peak values	Functional groups
1	3327.57	Amine
2	2919.70	Alkane
3	1650.77	Alkene
4	1385.60	Alkane
5	1265.07	Carboxylic acid
6	1175.40	Amine
7	1057.76	Alcohol
8	827.31	Aromatic alkene
9	775.24	Aromatic alkene
10	698.11	Aromatic alkene
11	625.79	Aromatic alkene
12	467.65	Halogen

Peak values are expressed in wave number (cm^{-1})

Results of FTIR spectroscopic analysis in the methanolic extracts of *H. coronarium* have revealed the existence of various chemical constituents (Fig. 2). The absorption bands, the wave number (cm^{-1}) of prominent peaks obtained from absorption spectra are described in Table 2.

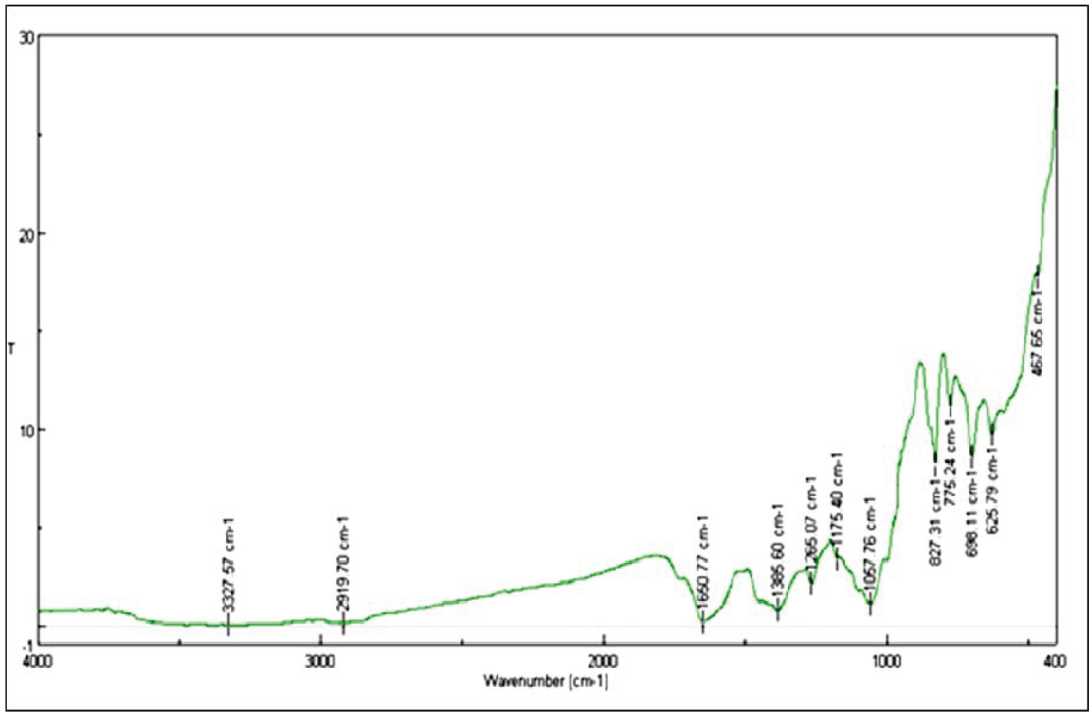

Fig. 2: FTIR Spectra of *H. coronarium* rhizome methanolic extract

The IR spectrum of methanolic extract reveals structural information about major and minor constituents. The functional group identification is attributed to the stretching and bending vibrations of the compound.

GC-MS chromatogram analysis of the methanolic extract of *H. coronarium* showed 21 peaks which indicating the presence of twenty one phytochemical constituents (Fig. 3).

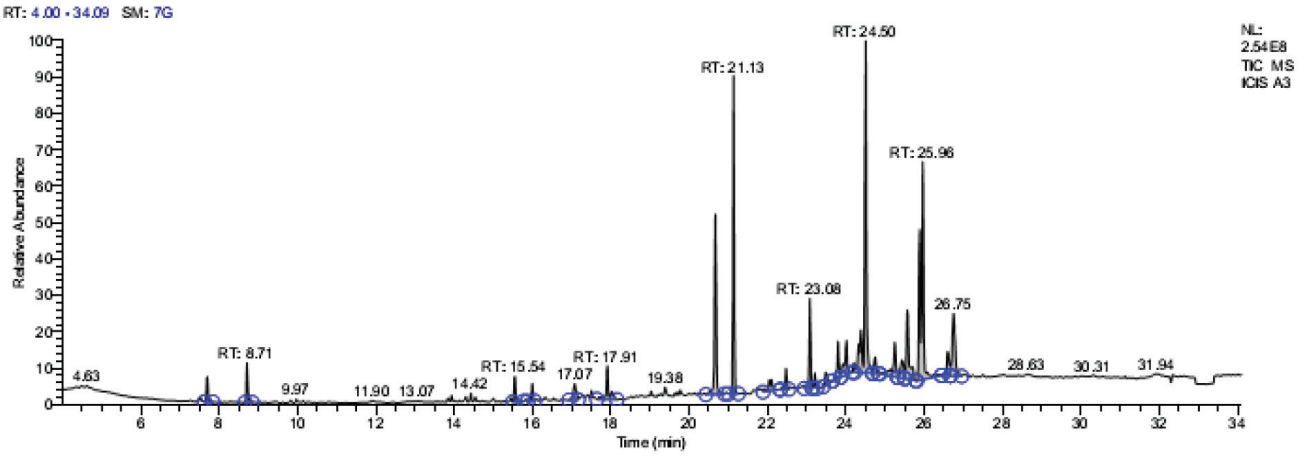

Fig. 3: GC-MS chromatogram of *H. coronarium* methanolic rhizome extract

On comparison of the mass spectra of the constituents with the NIST library, the phytocompounds were characterized and identified. Of the compounds identified, the most prevailing compounds were Spiro [furan-2(5H), 2'(1'H)-naphtho [2,1-b] furan]-5-one (19.89%), Retinoyl-â-glucuronide 6',3'-lactone (18.21%), 9-cis-Retinal (12.07%) and ã-Carboethoxy-ã-butyrolactone (9.56%).

4. Conclusion

The combinative approach of these chemical fingerprinting techniques would help in evaluating quality consistency of herbal drug of *H. coronarium*.

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Molecular auditing of some selected Indian mangoes (*Mangifera indica* L.)

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ABSTRACT

This study describes phenotypic variation in respect of leaf and pomological traits and portrays genetic relationships among 12 selected mango genotypes of India using randomly amplified polymorphic DNA (RAPD) marker technique. The genotypes were phenotypically diverse in respect of 20 different (11 qualitative and 9 quantitative) traits. Traits like sugar/acid ratio, ripe fruit weight and total soluble solids demonstrated higher variations. Many of these traits had two or more than two phenotypic classes with economic importance and thus could be used in breeding to enhance fruit yield and quality. Twenty-three RAPD markers yielded a total of 307 amplified DNA fragments, of which 85.99% were polymorphic, indicating a high degree of genetic diversity. Primers OPA 8, OPA 19, OPG 9 and RPI-10 exhibited 100% polymorphism. Polymorphic information content (PIC) value for RAPD primers ranged from 0.38-0.82 with an average of 0.60. The resolving power varied from 4.83 to 23.5 with an average of 14.35. The average values for Na, Ne, I, He and uHe were calculated from the RAPD data as 1.82, 1.46, 0.41, 0.27 and 0.28 respectively. Twenty-one unique bands were generated which enabled identification of 9 different genotypes. The pair-wise Jaccard's similarity coefficient ranged between 0.55 and 0.81 indicating that the genotypes represent genetically diverse populations. The closest were two hybrids namely 'PKM-1' and 'PKM-2' and the most distant genotypes were 'Pusa Surya', 'Dashehari', 'Neeleshan Gujrat' and 'Sai Sugandh'. UPGMA dendrogram grouped the genotypes into four clusters basing on genetic relatedness/distance which was corroborated in 2D and 3D plots generated from principal component analysis. The study provides information to facilitate marker assisted breeding aimed at genetic improvement of this important fruit crop.

1. Introduction

Mango (*Mangifera indica* L., Family: Anacardiaceae, Order: Sapindales), is one the most important tropical fruit crops of the tropical and subtropical areas of the world. It has been under cultivation since 4,000 years in the Indian subcontinent and its cultivation is as old as Indian civilization (De Candolle, 1884). Representing the largest mango gene pool in the world encompassing over 1000 mango varieties endowed with a high degree of diversity, India is considered to be the center of origin of mango (Mukherjee, 1972; Ravishankar *et al*., 2000). Its place of importance can be understood from its being referred to as 'King of fruits' in the tropical world (Purseglove, 1972).

An ideal mango variety should be dwarf and a regular bearer with medium size fruit (250-300 g). Additionally, it should be highly tolerant of various fungal and bacterial diseases, stable pleasant flavor, attractive colour combined with good keeping quality. Conventional breeding of woody perennial fruit crops like mango based on selection for agro-horticultural attributes is difficult owing to long juvenile phase, self-incompatibility, high degree of cross-pollination and heterozygous nature, polyembryony, meager information on inheritance of important quantitative traits, etc. Existing diverse varieties available in India are not adequate for commercialization; outstanding new varieties in combination with desired superior trait could cater to national and

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international market demand. Furthermore, early and unambiguous identification of plant material is essential for effective germplasm characterization, which is helpful for plant breeders in selecting material for development of new crosses, in solving disputes related to patenting and intellectual property rights and to check bio-piracy, adulteration, etc. (Krishna and Singh, 2007).

Success of crop improvement program depends on proper varietal identification, characterization in combination with nature and magnitude of genetic variability. There is a relatively poor understanding of the pedigree and genetic relatedness of many mango cultivars. There is a considerable confusion in their nomenclature because many of them have unique local and regional names and the spelling and name variants have been translated to the Roman alphabet and that makes tracing their origins and ancestry difficult. Also, the performance of varieties varies under different climatic conditions (Singh, 1978). As in other fruit tree species, mango cultivars are currently identified on the basis of morphological traits based on descriptors (IPGRI, 1989, 2006). Over the last two decades, efforts were made in understanding the extent of variability of mango germplasm based on morpho-physiological traits (Rajwana *et al*., 2011; Bhuyan *et al*., 2007). Undoubtedly, phenotypic characterization forms the basis for germplasm characterization but, it is inaccurate due to the influence of the environment and often limiting number of discriminating traits. Moreover, this mode of identification is complicated with environmental effects on these characters and parallel selection for similar desired traits has often been misleading, labour intensive and time consuming. Many of these complications in characterizing plant germplasm based on phenotype and biochemical analysis can be overcome through direct identification of genotypes using DNA-based diagnostic assay. Compared to morphological markers, DNA markers are unaffected by environmental factors, highly heritable, polymorphic and unlimited in number; hence, they are extremely useful tool for depiction of genetic variability, genome fingerprinting, mapping, evolution, gene localization, population genetics, taxonomy and plant breeding.

Among the techniques used for genetic assessment, randomly amplified polymorphic DNA (RAPD) markers is most frequently used technique for genetic diversity analysis (Gupta and Rustgi, 2004) and proved to be as efficient as other molecular markers based on amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR) and inter simple sequence repeats (ISSR) for a range of plant species. Regardless of advances in DNA marker techniques, RAPD offers speed, simplicity, low cost, whole genome coverage for identification and classification of plants using small amount of DNA (Williams *et al*., 1990). In mangoes a few earlier studies have been carried out using RAPD profiles (Bajpai *et al*., 2008; Jena *et al*., 2010; Rajwana *et al*., 2011; Abou-Ellail *et al.,* 2014). Thus, RAPD could be an efficient technique for molecular auditing of Indian mangoes and to unravel the intraspecific relationships amongst different genotypes of mango. The present investigation is, therefore, aimed at accurate identification and estimation of the genetic divergence among 12 selected mango genotypes from different geographical locations of India using leaf, pomological and RAPD markers.

2. Materials and methods

2.1 Plant material

In the present investigation, we have used twelve promising mango genotypes encompassing commercial, local as well as hybrid genotypes obtained from orchards of Orissa University of Agriculture & Technology (O.U.A.T.) and Central Horticultural Experimentation Station (C.H.E.S.), Bhubaneswar, Orissa representing 4 different geographical locations (Eastern, Western, Northern and Southern zones of India) (Table 1). The plants were selected on the basis of their consistency in behavior for the last six years at their growing region for morphological observations as well as for collection of leaf samples for molecular characterization.

2.2 Morpho-biochemical trait evaluation

A total of 20 (11 qualitative plus 9 quantitative) different morphological and biochemical traits pertaining leaf, mature fruit and ripe fruit were assessed for the 12 studied mango genotypes. The qualitative traits were related to leaf (blade shape. leaf margin), mature fruit (skin colour, depth of stalk cavity, presence of neck) and ripe fruit (skin colour, flesh colour, juiciness, table quality, storage life, maturity time) (Table 1). The traits were recorded as per descriptor list (IPGRI, 1989; 2006) and DUS (Distinctness, Uniformity, Stability) guidelines (PPV & FRA, 2008). Evaluation of pomological characters were carried out on samples of 10 randomly chosen ripe fruits per genotype. All leaf related traits were documented from fully expanded mature leaves. Quantitative traits comprised fruit length and width (cm), fruit weight (g), physiological loss (g), peel, pulp and stone (%), total soluble solids (%) and sugar/acid ratio recorded from 10 randomly selected ripe fruits (Table 2). Traits like total soluble solids (TSS) and sugar/acid ratio were calculated using standard methods (Ranganna, 1986; A.O.A.C, 1990).

2.3 Genomic DNA extraction

Emerging young leaves of each of the 12 mango genotypes were collected from which genomic DNA was

isolated, individually frozen in liquid nitrogen and stored at - 80°C until processed. DNA was extracted following CTAB method as originally described by Doyle and Doyle (1990) with minor modifications (Jena *et al*. 2010). The quantity and quality of extracted DNA were determined as per Jena *et al*. (2010).

2.4 Primer screening and RAPD amplification

Initially, a total of 70 RAPD primers were screened with six mango genotypes of which 23 primers revealing clear, distinct, polymorphic and reproducible amplicons were included in the present study for further PCR analysis (Table 3). RAPD primers (OPA, OPC, OPG, and RPI-C Series) were purchased from Operon Technologies (Alameda, California, USA) and Bangalore Genei Pvt. Ltd. (India). RAPD-PCR amplification on each DNA sample was performed in a 25 µl reaction volume containing 1× Taq PCR buffer, 2 mM $MgCl_2$, 200 µM dNTP each, 10 pmoles of single primer, 1U of Taq DNA polymerase (Bangalore Genei, India), 30 ng of template DNA, and the rest sterile nuclease and protease-free water. Reactions without DNA were used as negative control. DNA amplification was carried out in a thermocycler (Applied Biosystems, USA) programmed at an initial pre-denaturation at 94 °C for 3 min followed by 44 cycles of denaturation at 94 °C for 1 min, annealing at 37 °C for 1 min, extension at 72 °C for 2 min with a finally at 72 °C for 7 min.

2.5 Agarose gel electrophoresis

All amplified products were loaded in wells (18 µl of sample + 3 µl of 6 × loading dye) and resolved on 1.5% agarose gel in 1× TBE buffer by electrophoresis at 70 V for 2 h followed by staining with ethidium bromide (1.0 µg/ml). The amplified fragments were photographed using gel documentation system (Bio-Rad, USA) and stored as digital pictures. Low range DNA ruler plus (Bangalore Genei, India) was used as molecular size standard to estimate the size of the fragments.

2.6 Data analysis

For the studied 9 quantitative traits, their descriptive statistics like the maximum, minimum, range, mean, standard error (SE), standard deviation (SD) and coefficients of variation (CV%) were computed using the SPSS® (Statistical Package for Social Studies) software version 17. The results were investigated for statistical significance by one way analysis of variance (ANOVA).

All distinct amplicons were scored visually as discrete variables using 1 for presence and 0 for absence separately for each marker and a binary matrix was obtained for RAPD with final data sets including both polymorphic and monomorphic bands. All amplifications were repeated thrice and only reproducible and unambiguous bands were considered for analysis. From the band patterns obtained with each primer, the genotype-specific bands (if any) along with their sizes were recorded. To determine the suitability and informative ability of RAPD markers, the performance of marker systems was measured using six parameters: polymorphism information content (PIC), effective multiplex ratio (EMR), marker index (MI), resolving power (Rp), genotype index (GI) and Shannon's information index (I). The PIC value for each primer was calculated using the formula PICi =2fi (1 - fi) (Roldan-Ruiz *et al*., 2000). Effective multiplex ratio was evaluated using formula; EMR $= \eta \times \beta$, where η is the average number of fragments amplified by genotype to a specific marker system (multiplex ratio) and β was estimated from the number of polymorphic bands (NPB) and the number of monomorphic bands (NMB); β = NPB/(NPB +NMB). Marker index (MI), which provides an estimate of marker utility for each primer, was calculated using the formula: MI = EMR×PIC. The resolving power (RP) of each primer was calculated as RP = ΣIb, Where $Ib = 1 - (2 \times |0.5 - p_i|)$, p_i being the proportion of genotypes containing the ith band (Prevost and Wilkinson, 1999). Genotype index (GI) represents proportion of genotypes actually distinguished by the primer i.e. the number of genotypes with unique fingerprints divided by total number of genotypes fingerprinted (Sehgal and Raina, 2005). The basic parameters for genetic diversity such as observed number of alleles (Na), effective number of alleles (Ne), Shannon's information index (I), expected heterozygosity (He) and unbiased expected heterozygosity (uHe) were calculated using GenAlEx 6.502 software (Peakall and Smouse, 2012). Pairwise-similarity matrices were generated by calculating Jaccard's similarity coefficient (Jaccard, 1908) to accomplish genetic similarity between the genotypes using NTSYS-pc software version 2.02 (Rohlf, 1993). These similarity coefficients were then subjected for construction of dendrogram by the unweighted pair group method with arithmetic average (UPGMA) and cluster analysis with NTSYS-pc. To further support the clustering results and to obtain a graphical picture of molecular variability 2D and 3D plots were generated from principal component analysis using NTSYSY-pc (version 2.02) (Rohlf, 1993). To estimate the robustness and validity of dendrogram typology and clustering bootstrap analyses were performed of 1000 bootstrap samples using the software WINBOOT (Yap and Nelson, 1996). Mantel's matrix correspondence test (Mantel, 1967) was performed by using MXCOMP algorithm of the NTSYS to compute the cophenetic correlation coefficient, *r*, which determines how well the dendrogram represents the similarity data.

Table 1

Characterization of mango genotypes based on leaf and pomological features[1]

Sl. No.	Genotype and its Geographic distribution		Leaf		Mature Fruit			Ripe Fruit					
			Blade shape	Margin	Skin colour	DSC	Presence of neck	PCS	Flesh colour	Juiciness	Table quality	Storage life	TFM
1	Pusa Surya	NI	Ovate	Wavy	Only green	Shallow	Slightly prominent	Orange	Light yellow	Medium	Excellent	Good	Late
2	Pusa Arunima	NI	Elliptic	Wavy	Green & red	Absent	Absent	Orange	Light orange	Medium	Excellent	Good	Late
3	Ambika	NI	Elliptic	Wavy	Green & purple	Shallow	Slightly prominent	Red & purple	Medium yellow	Medium	Excellent	Very Good	Late
4	PKM 1	SI	Oblong	Wavy	Green & red	Shallow	Slightly prominent	Green & yellow	Light orange	Medium	good	Good	Late
5	Janardhan Pasand	SI	Elliptic	Entire	Green & pink	Absent	Slightly prominent	Yellow & red	Light yellow	High	Excellent	Good	Early
6	PKM 2	SI	Oblong	Wavy	Only green	Shallow	Prominent	Green & yellow	Light yellow	Medium	Interme-diate	Good	Medium
7	Neeleshan Gujrat	WI	Ovate	Entire	Only green	Absent	Very prominent	Green & yellow	Medium yellow	Medium	Good	Good	Late
8	Mahmud Bahar	EI	Elliptic	Entire	Only green	Shallow	Absent	Yellow	Light yellow	Medium	Good	Good	Medium
9	Baramasi	EI	Elliptic	Entire	Only green	Shallow	Slightly prominent	Yellow	Medium yellow	Medium	Poor	Interme-diate	Very late
10	Sai Sugandh	WI	Oblong	Entire	Green & red	Absent	Prominent	Yellow & red	Light yellow	Medium	Excellent	Good	Late
11	Dashehari	NI	Ovate	Wavy	Only green	Absent	Absent	Yellow green	Orange	Medium	Interme-diate	Good	Medium
12	Arka Neelkiran	SI	Oblong	Wavy	Green & red	Medium	Absent	Yellow	Light Orange	Medium	Interme-diate	Very good	Late

EI: Easten India WI: Western India, NI: Northern India, SI: Southern India, DSC: Depth of Stack cavity, PCS: Predominant colour of skin, TFM: Time of fruit maturity.

[1]Features recorded in accordance with DUS (Distinctness, Uniformity, Stability) test guideline (PPV and FRA, 2008) and IPGRI guidelines (1989, 2006)

Table 2

Physico-chemical analysis of mango genotypes based on ripe fruit characteristics

Sl. No.	Genotypes	Fruit length (cm)	Fruit width (cm)	Ripe fruit weight (g)	Physiological loss (g)	Peel (%)	Stone(%)	Pulp (%)	Total Soluble Solids (%)	Sugar: Acid ratio
1	Pusa Surya	11.7	9.1	350.4	12.8	13.87	12.94	73.19	29	82.85
2	Pusa Arunima	11.2	8.2	235.8	18.4	13.18	13.14	73.68	19.8	40.0
3	Ambika	11.0	7.9	318	17.5	10.38	12.26	77.36	24	58.53
4	PKM 1	9.8	7.5	310.5	13.7	15.46	15.78	68.76	31	62
5	Janardhan Pasand	9.95	7.05	246.2	11	15.22	13.76	71.02	14.6	24.4
6	PKM 2	8.1	6.7	177	15.5	13.50	18.62	67.87	27	52.94
7	Neeleshan Gujrat	10.7	9.1	288.7	22.8	11.73	17.03	71.24	18.2	38.1
8	Mahmud Bahar	11.1	8.1	310	19.7	10.24	18.56	71.20	21	47.72
9	Baramasi	8.4	8.3	167.1	13.2	7.94	22.36	69.70	19	42.22
10	Sai Sugandh	15.4	9.2	389.6	28.4	10.49	18.31	71.20	18.6	39.57
11	Dashehari	8.5	5.9	175.3	13.2	12.04	15.76	72.20	27.2	75.55
12	Arka Neelkiran	8.45	7	210.2	20.6	16.41	13.56	70.03	17	37.77
	SE	0.59	0.30	21.30	1.46	0.73	0.88	0.72	1.45	4.88
	SD	2.03	1.03	73.77	5.05	2.52	3.06	2.50	5.02	16.91
	CV %	19.64	13.16	27.85	29.33	20.12	19.11	3.50	22.43	33.68

Table 3

Description and band amplification profile of RAPD primers used in the present study

Sl. No.	Primer ID	Sequence (5'-3')	% GC	Tm[1] (°C)	TNB	NPB	NMB	Range (bp)	PB (%)
1	OPA 6	GGTCCCTGAC	70	34	15	13	2	200-2000	86.66
2	OPA 7	GAAACGGGTG	60	32	16	14	2	100-2500	87.5
3	OPA 8	GTGACGTAGG	60	32	12	12	0	100-2500	100
4	OPA 9	GGGTAACGCC	70	34	21	20	1	100-2000	95.24
5	OPA 12	TCGGCGATAG	60	32	18	17	1	100-2000	94.44
6	OPA 17	GACCGC TTGT	60	32	11	10	1	200-2000	90.91
7	OPA 19	CAAACGTGGG	60	32	17	17	0	100-2000	100
8	OP C 2	GTGAGGCGTC	70	34	4	3	1	600-1500	75
9	OP C 5	GATGACCGC C	70	34	9	5	4	300-2000	55.55
10	OP C 8	TGGACCGGT G	70	34	9	8	1	150-2500	88.88
11	OP C 11	AAAGCTGCG G	60	32	12	9	3	300-3000	75
12	OP C 15	GACGGATCA G	60	32	12	10	2	100-2500	83.33
13	OP C 18	TGAGTGGGT G	60	32	10	8	2	300-1815	80
14	OP C 20	ACTTCGCCA C	60	32	12	10	2	300-2000	83.33
15	OPG 2	GGCACTGAGG	70	34	11	9	2	100-2500	81.82
16	OPG 9	CTGACGTCAC	60	32	14	14	0	200-2000	100
17	OPG 15	ACTGGGACTC	60	32	15	12	3	200-2000	80
18	OPG 17	ACGACCGACA	60	32	16	12	4	100-2000	75
19	RPI 1	AAAGCTGCGG	60	32	17	14	3	200-2500	82.35
20	RPI 2	AACGCGTCGG	70	34	15	14	1	300-3000	93.33
21	RPI 4	AATCGCGCTG	60	32	11	10	1	200-2500	90.91
22	RPI 7	ACATCGCCCA	60	32	14	7	7	200-3000	50
23	RPI 10	ACGATGAGCG	60	32	16	16	0	200-2000	100
TOTAL				307	264	43			
AVERAGE					13.35	11.48	1.87		85.99

[1]Tm: Melting temperature, TNB: Total number of bands; NPB: Number of polymorphic bands; NMB: Number of monomorphic bands; PB (%): Polymorphic band percentage

Table 4

Amplification performance and diversity parameters of mango genotypes as revealed by RAPD markers

Sl. No.	Primer ID	PIC	EMR	MI	Rp	GI	Na	Ne	I	He	uHe
1	OPA 6	0.53	5.73	3.04	18.33	0.08	1.87	1.53	0.46	0.31	0.32
2	OPA 7	0.66	5.50	3.41	18.67	0.08	1.88	1.53	0.46	0.31	0.32
3	OPA 8	0.75	6.58	4.08	13.17	0.00	1.83	1.69	0.54	0.37	0.39
4	OPA 9	0.82	4.38	3.29	17.33	0.03	1.95	1.47	0.48	0.28	0.30
5	OPA 12	0.66	5.17	3.14	17.50	0.05	1.83	1.50	0.45	0.30	0.31
6	OPA 17	0.63	5.36	2.77	11.83	0.00	1.91	1.51	0.47	0.31	0.32
7	OPA 19	0.77	6.29	3.90	17.83	0.03	2.00	1.59	0.50	0.33	0.35
8	OP C-02	0.58	4.25	2.47	4.83	0.00	1.75	1.49	0.44	0.29	0.31
9	OP C-05	0.49	1.56	0.76	10.33	0.00	1.56	1.16	0.17	0.10	0.10
10	OP C-08	0.62	3.11	2.40	6.67	0.00	1.89	1.31	0.34	0.21	0.22
11	OP C-11	0.60	3.17	1.90	12.33	0.03	1.75	1.33	0.31	0.20	0.21
12	OP C-15	0.52	5.67	2.95	15.33	0.00	1.83	1.60	0.44	0.33	0.34
13	OP C-18	0.51	4.30	2.19	11.17	0.00	1.40	1.31	0.28	0.19	0.19
14	OP C-20	0.66	3.83	2.53	11.67	0.00	1.83	1.41	0.38	0.25	0.26
15	OPG 2	0.58	5.09	2.95	13.33	0.00	1.83	1.41	0.40	0.26	0.27
16	OPG 9	0.62	4.50	3.69	10.50	0.03	2.00	1.50	0.47	0.31	0.32
17	OPG 15	0.63	3.27	2.83	14.17	0.00	1.80	1.31	0.30	0.19	0.20
18	OPG 17	0.38	5.81	2.87	23.50	0.00	1.75	1.53	0.43	0.29	0.31
19	RPI 1	0.49	5.76	2.85	22.33	0.03	1.82	1.55	0.46	0.31	0.33
20	RPI 2	0.63	5.67	3.38	16.17	0.05	1.93	1.63	0.51	0.35	0.36
21	RPI-4	0.57	4.91	2.74	11.00	0.03	1.91	1.52	0.46	0.30	0.32
22	RPI-7	0.44	1.71	2.70	18.00	0.05	1.50	1.19	0.20	0.12	0.13
23	RPI 10	0.73	5.25	2.65	14.00	0.08	2.00	1.57	0.48	0.32	0.33
	TOTAL	13.87	106.88	65.46	329.99	0.55	41.83	33.63	9.42	6.24	6.51
	AVERAGE	0.60	4.65	2.85	14.35	0.02	1.82	1.46	0.41	0.27	0.28

PIC: Polymorphism information content; EMR: Effective multiplex ratio; MI: Marker index; Rp: Resolving power; GI: Genotype index, Na: Observed number of alleles, Ne: Effective number of alleles, I: Shannon's information index, He: Expected Heterozygosity, uHe: Unbiased Expected Heterozygosity

Table 5

Genotype-specific RAPD markers for mango genotypes audited

Sl.No.	Marker[1]	Presence (+) / Absence (-)	Genotype identified	Sl.No.	Marker[1]	Presence (+) / Absence(-)	Cultivar identified
1	OPA – 6_{300}	(+)	Pusa Surya	12	OPG – 9_{840}	(-)	Baramasi
2	OPA – 6_{560}	(+)	Sai Sugandh	13	RPI – 1_{2000}	(-)	Ambika
3	OPA – 6_{1420}	(+)	PKM 2	14	RPI – 2_{650}	(+)	Mahmud Bahar
4	OPA – 7_{250}	(-)	PKM 2	15	RPI – 2_{2500}	(+)	Sai Sugandh
5	OPA – 7_{1000}	(+)	Sai Sugandh	16	RPI – 4_{2500}	(+)	Baramasi
6	OPA – 7_{1520}	(+)	Dashehari	17	RPI – 7_{500}	(-)	Ambika
7	OPA – 9_{500}	(+)	PKM 2	18	RPI – 7_{700}	(+)	Arka Neelkiran
8	OPA – 12_{150}	(+)	Neeleshan Gujrat	19	RPI – 10_{450}	(+)	Pusa Surya
9	OPA – 12_{1650}	(+)	Arka Neelkiran	20	RPI - 10_{550}	(+)	Pusa Surya
10	OPA – 19_{1000}	(+)	PKM 2	21	RPI – 10_{900}	(+)	Pusa Surya
11	OPC – 11_{630}	(+)	Ambika				

[1]Each RAPD marker is represented by the primer number and the band size (bp)

Table 6

Jaccards Similarity Matrix generated by RAPD primers for mango genotypes audited

	Pusa Surya	Pusa Arunima	Ambika	PKM 1	Janardhan Pasand	PKM 2	Neeleshan Gujrat	Mahmud Bahar	Baramasi	Sai Sugandh	Dashehari	Arka Neelkiran
Pusa Surya	1.00											
Pusa Arunima	0.58	1.00										
Ambika	0.67	0.72	1.00									
PKM 1	0.72	0.66	0.68	1.00								
Janardhan Pasand	0.63	0.72	0.68	0.71	1.00							
PKM 2	0.77	0.70	0.72	0.81	0.77	1.00						
Neeleshan Gujrat	0.64	0.70	0.72	0.75	0.77	0.78	1.00					
Mahmud Bahar	0.60	0.73	0.68	0.72	0.75	0.74	0.78	1.00				
Baramasi	0.68	0.70	0.66	0.70	0.61	0.68	0.63	0.68	1.00			
Sai Sugandh	0.66	0.61	0.60	0.71	0.60	0.68	0.55	0.60	0.63	1.00		
Dashehari	0.55	0.68	0.63	0.69	0.68	0.72	0.74	0.72	0.63	0.57	1.00	
Arka Neelkiran	0.60	0.69	0.67	0.74	0.79	0.79	0.74	0.75	0.64	0.68	0.70	1.00

3. Results and discussion

3.1 Leaf and pomological diversity based on morpho-biochemical traits

Description of mango germplasm through morpho-pomological parameters was a necessary prelude to biochemical or molecular characterization (Litz, 2004). In the present investigation twelve mango genotypes of India were evaluated with respect to 11 qualitative and 9 quantitative traits to determine the genotypic diversity through morphological and biochemical traits. A high degree of variation was observed among genotypes based on qualitative and quantitative characters related to leaf and fruit (Table 1, Table 2). Most of the qualitative traits as given for the leaf, mature fruits and ripe fruits were polymorphic showing more than two phenotypes in mango genotypes. The leaf blade shape was highly polymorphic among mango genotypes; ovate in 'Pusa Surya', 'Neeleshan Gurat' and 'Dashehari', elliptic in 'Pusa Arunima', 'Ambika, 'Janardhan Pasand', 'Mahmud Bahar' and 'Baramasi' while oblong in four hybrid genotypes ('PKM-1','PKM-2','Sai Sugandh' and Arka Neelkiran'). Two phenotypic classes for leaf margin were recorded; 'Janardhan Pasand', 'Neeleshan Gujrat','Mahmud Bahar', 'Baramasi' and 'Sai Sugandh' were with entire margin, whereas for the rest of the genotypes the leaf margin were wavy. A high variability in leaf characteristics was reported in indigenous mangoes of Pakistan (Rajwana *et al*., 2011).

Mature fruit skin colour was a highly polymorphic trait, varied from green to greenish red, purple and pink. 'Pusa Surya',' PKM 2', 'Neeleshan Gujrat', 'Mahmud Bahar', 'Baramasi' and 'Dashehari' had green skin colour, 'Sai Sugandh', 'Arka Neelkiran', 'Pusa Arunima' and 'PKM 1' had 'green and red' skin colour, 'Ambika' and 'Janardhan Pasand' had greenish purple and greenish pink skin colours respectively. Stalk cavity were absent in 'Pusa Arunima', 'Janardhan Pasand', 'Neeleshan Gujrat', 'Sai Sugandh' and 'Dashehari' where as it was 'medium' in only one genotype 'Arka Neelkiran' and 'shallow' in the rest of six genotypes. Ripe fruit skin and flesh colour are consumer preference traits. High variations were noticed in ripe fruit skin colour which ranged from orange, yellow, 'green and yellow', yellow green, 'yellow and red', 'red and purple' at the ripening stage. Interestingly, fruits of the genotype 'Ambika' possesses very attractive and unique 'red and purple' skin colour. A significant variation in ripe fruit skin colour was reported in mangoes (Barholia and Yadav, 2014; Sennhenn *et al*., 2014).

Our results also revealed a significant variability in flesh colours of fruits with more than three phenotypic classes. Least variation was observed for juiciness; only one genotype 'Janardhan Pasand' was highly juicy whereas the remaining genotypes were with medium range juiciness. Higher peel percentage were recorded in 'Arka Neelkiran', 'Janardhan Pasand', 'PKM 1', 'Pusa Surya', 'Pusa Arunima', 'PKM 2' and 'Ambika'. A high percentage of peel may be responsible for good storage life of the fruits. Fruit storage life is also a very important trait for packaging and transportation; most mango collections under study had 'very good' to 'good' storage life except 'Baramasi' which had 'intermediate storage life. A single mango genotype namely, 'Janardhan Pasand' was early maturing; 'PKM 2', 'Mahmud Bahar' and 'Dashehari' had mid-season maturity whereas the remaining eight genotypes showed late-very late fruit maturity. As these categorical morpho-pomological characters are discrete these could be used for varietal discrimination.

Significant differences were also obtained for the all 9 quantitative ripe fruit traits. The recorded mean values for each of the quantitative traits with summary statistics at $p \leq 0.05$ are presented in Table 2. Traits such as sugar: acid ratio, physiological loss, ripe fruit weight and TSS displayed high CVs (>20%) while the traits like peel percentage, fruit length and stone percentage had intermediate CV values. The remaining traits such as fruit width and pulp percentage presented comparatively low CV values (<15%). Galvez-Lopez *et al.* (2010) reported a similar range of CV % for fruit length, width and weight for native mangoes of Mexico.

The fruit length varied from 8.1cm ('PKM-2') to 15.4 cm ('Sai Sugandh'); fruit width from 5.9 cm ('Dashehari') to 9.2 cm ('Sai Sugandh'); fruit weight from 167.1 g ('Baramasi') to 389.6 g ('Sai Sugandh'); physiological loss from 11 g ('Janardhan Pasand') to 28.4g ('Sai Sugandh'; peel percentage 7.94% ('Baramasi') to 16.41% ('Arka Neelkiran'); stone percentage from 12.26% ('Ambika') to 22.36% ('Baramasi'); pulp percentage from 67.87% ('PKM-2') to 77.36% ('Ambika'); TSS from 14.6% ('Janardhan Pasand') to 31% ('PKM-1') and sugar/acid ratio from 24.4 ('Janardhan Pasand') to 82.85 ('Pusa Surya'). The results on fruit length and diameter were in accordance with the variation level detected in Mexican mangoes reported by Galvez-Lopez *et al*. (2010). Variation range for traits like pulp and stone %, TSS and sugar/acid ratio etc. corroborated with the findings of Anila and Radha on Indian mangoes (2003)

Most of the traits studied like red blush on skin, orange to dark-orange coloured flesh, 'excellent' table quality, 'very good' storage life, medium sized fruit, low amount of stone and high pulp percentage, high TSS and sugar/acid ratio had potential economic interest especially those related to fruit quality. They could thus serve as target traits for mango

growers and breeders. In the present study, high substantial variance between the genotypes for above traits coupled with high CV values and the existence of two or more phenotypic classes for each qualitative traits shows that Indian mango germplasm is a rich source of genetic variation for characters of commercial interest. Our investigation revealed that the genotypes like 'Ambika', 'Dashehari', 'Janardhan Pasand', 'Sai Sugandh' and 'Pusa Surya', possessing excellent fruit quality characters of consumer's preference, could be considered as promising candidates for selection of parents for breeding program. Two of these genotypes namely 'Janardhan Pasand' and 'Dashehari', have already been used for the development of different mango hybrids while the remaining three warrant immediate attention.

The results from the current research support the view that leaf-pomological traits and biochemical contents in fruits can be used efficiently for cultivar discrimination as well as for estimating the genetic relationships across large and diverse groups of mango genotypes. These findings are in accordance with other studies indicating that both quantitative and qualitative traits are very helpful in the identification and evaluation of cultivars in mango germplasm (Sennhenn *et al*., 2014; Khan *et al.,* 2015).

3.2 Genetic polymorphism and RAPD patterns

The knowledge of genetic variation and the genetic relationship between plant individuals can be an important consideration for efficient rationalization and utilization of germplasm resources. Besides morphological traits, a high degree of polymorphism was also observed at the molecular level. Initial screening of 70 RAPD primers yielded 23 primers with clear and reproducible banding patterns (Table 3). A total of 307 distinct bands were produced in different size ranging from 100 to 3000 bp with an average of 13.35 bands per primer, of which 264 (85.99%) were polymorphic and only 43 (14.01%) were monomorphic (Table 3). The total number of bands was found to range from 4 (OPC 2) to 21 (OPA 9) and the number of polymorphic bands ranged from 3 (OPC 2) to 20 (OPA 9). The percentage of polymorphism ranged from 50% (RPI 7) to 100% (OPA 8, OPA 19, OPG 9 and RPI 10) with an average of 84.75% polymorphism per primer. The banding patterns of 12 mango genotypes using OPA 6, OPA 7 and RPI 10 primers are displayed in Fig. 1a, b, c. The results of the present study were close to findings from genetic diversity studies on Indian mango genotypes using RAPD (Bajpai *et al*., 2008; Karihaloo *et al*., 2003). However, compared to our results, a low to moderate level of polymorphism with RAPD primers was observed in earlier experiments in Indian mangoes (Ravishankar *et al*., 2000) and Egyptian mangoes (Abou-Ellail *et al*., 2014). This disagreement between various studies may be ascribed to differences in the number of primers and genotypes used along with their diverse genetic backgrounds. High PIC value of 0.82 (OPA 9) and low PIC value of 0.38 (OPG 17) with an average value of PIC per primer 0.60 were obtained (Table 4). The highest EMR value of 6.58 was observed with the primer OPA 8 and the lowest EMR 1.56 was observed with the primer OPC 5 with an average of 4.65 per primer. Marker index (MI), which reflects the overall usefulness of a given marker system was found to be highest with the primer OPA 8 (4.08) and lowest in the primer OPC 5 (0.76), with an average of 2.85 per primer. Resolving power (RP), the discriminatory potential of the primer, was the highest with the primer OPG 17 (23.5) and the lowest with the primer OPC 2 (4.83) with an average of 14.35 per primer. The genotype Index (GI) ranged from 0.00-0.08 with an average of 0.02. The genetic diversity values based on Shannon index ranged between 0.17 (OPC 5) to 0.54 (OPA 8) with a mean of 0.41. Average values of observed number of alleles (Na), effective number of alleles (Ne), expected heterozygosity (He) and unbiased expected heterozygosity (uHe) of 1.82, 1.46, 0.27 and 0.28 (Table 4) respectively was recorded with RAPD markers. Most informative RAPD markers based on PIC, marker index and Shannon's index were identified as OPA 7, OPA 8, OPA 9, OPA 19, RPI 2 and RPI 10.

3.3 Genotype specific diagnostic markers

Using RAPD technique a total of 21 unique bands were generated which identified 9 ('Pusa Surya', 'PKM-2', 'Sai Sugandh', 'Dashari', 'Neelshan Gujrat', 'Arka Neelkiran', 'Ambika', 'Mahmud Bahar' and 'Baramasi') out of the 12 total genotypes audited (Table 5). Two genotypes namely 'Pusa Surya' and 'PKM 2' were the unique genotypes each possessing maximum of four RAPD specific loci. Two most informative primers OPA 6 & OPA 7 were identified each of which were able to generate the highest number (3) of unique bands individually for identification of three different genotypes. It was interesting to note that even the absence of a specific band has capacity for discriminating genotypes 'PKM-2' (250 bp), 'Baramasi' (840 bp) and 'Ambika' (500, 2000 bp). These genotype specific amplicons identified with RAPD marker systems will play essentially important roles in characterization, conservation, and utilization of mango germplasm. Similar type of presence/absence of specific loci(s) were reported in a number of plants with RAPD for rice (Raghunathachari *et al*., 2000) and cashew (Jena *et al*., 2016). The unique amplicons can be developed to SCAR markers for marker-assisted selection and other trait-specific analysis. Based on the ability to detect unique bands, OPA 6, OPA 7 and RPI

10 were recognized as efficient primers which would be useful for detecting mixtures and duplicates of mango seedlings in the future. This kind of marker tagging will contribute to the efficient selection and hybridization in mango breeding programs as the source of new and novel alleles aiming at genetic improvement of this fruit crop.

3.4 Genetic similarity and cluster analysis

The genetic similarity coefficient was evaluated by calculating the Jaccard's similarity coefficient based on the proportion of shared bands. Jaccard's similarity coefficient ranged from 0.55 to 0.81 with a mean value of 0.68 (Table 6). The high values of cophenetic correlation coefficient, r = 0.85 between the similarity matrix and co-phenetic matrix obtained from UPGMA dendrogram indicated good illustration of relationships between genotypes in the cluster analysis. The most closely related genotypes were 'PKM 1' vs 'PKM 2' with the highest similarity index (0.81) closely followed by 'PKM 2' vs 'Arka Neelkiran' and 'Janardhan Pasand' vs 'Arka Neelkiran' (0.79). On the other hand, most distantly related genotypes were 'Dashehari' vs 'Pusa Surya' and 'Sai Sugandh' vs 'Neeleshan Gujrat', 'with the lowest similarity index (0.55) followed by 'Dashehari' vs 'Sai Sugandh' (0.57) and 'Pusa Surya' vs 'Pusa Arunima'(0.58) representing most diverse varieties (Table 6). The similarity range detected in the present study using RAPD markers was higher than those reported by Karihaloo *et al*. (2003).

Assigning a cut-off point of 0.70 the UPGMA clustering algorithm of RAPD marker analysis separated the 12 genotypes into four major clusters (I - IV) spanning an index length of 0.63-0.81 (Fig. 2). Cluster I, II and IV included 2, 2, and 1 genotype respectively where as Cluster III comprised maximum 7 number of genotypes. The two most diverse genotypes 'Pusa Surya' (North India) and 'Baramasi' (East India) were grouped together in Cluster I. 'Pusa Surya' is a selection from an exotic cultivar 'Elden' of Brazil and 'Baramasi' is a novel land race as it bear fruits throughout the year. In Cluster II, two hybrids namely 'Pusa Arunima' ('Amrapali' × 'Sensation') and 'Ambika' ('Amrapali' × 'Janardhan Pasand') were included with a coefficient similarity of 0.72. These two hybrids have 'Amrapali' as the Female parent. Cluster III was divided into two sub-clusters (IIIA and IIIB) with a similarity of 71%. Sub-cluster IIIA comprises six genotypes of which five are hybrids. Sub-cluster IIIA was further divided to two sub-sub clusters, $IIIA_1$ and $IIIA_2$, the former included four genotypes (3 hybrids, 1 selection). Interestingly in this, the three hybrid genotypes 'PKM-1' ('Chinnaswarnarekha' × 'Neelum'), 'PKM-2' ('Neelum' × 'Mulgoa') and 'Arka Neelkiran' ('Alphonso' × 'Neelum') shared commonalities in their pedigree i.e. having same parent 'Neelum' as well as morphological features namely oblong leaves with wavy margins, good storage life, fruit lengh and weigth, pulp and peel %. Clustering of mango genotypes with 'Neelum' as a parent was also reported by Vasanthaiah (2009). The second sub-sub cluster $IIIA_2$ contained two hybrids 'Neeleshan Gujrat', and 'Mahmud Bahar' sharing 0.78 % similarity. At a similarity coefficient of 0.71, single genotype 'Dashehari' formed a distinct sub cluster IIIB. Interestingly, a single genotype namely 'Sai Sugandh', a cross-bred of two diverse genotypes 'Kesar' × 'Totapuri', formed a separate cluster IV. It is a distinct variety with large and long fruit having deep sinus with good storage life and excellent eating quality character. 'Totapuri' is a regular South Indian variety with large fruit with low fruit quality whereas 'Kesar' is a Western Indian variety with best quality small fruit. The relationship established for all genotypes in the cluster analysis presented in form of dendrogram was also mirrored in the two dimensional (2D) and three dimensional (3D) principal component analysis (PCA) of the mango genotypes audited (Fig. 3 and 4).

Classification of diversity in germplasm collections is important for plant breeding. In this study, we investigated genetic diversity in mango genotypes based on leaf and pomological characteristics along with a DNA-based molecular marker. There is much environmental influence accounting for the morphological variability observed. Therefore, compared to molecular techniques, morphometric traits are relatively less reliable and inadequate for precise discrimination of closely related genotypes and analysis of their genetic relatedness/distance. Nevertheless, phenotypic variables are useful for preliminary, fast, simple, and inexpensive varietal identifications and can be used as a general approach for assessing gross genetic diversity among genotypes. Many traits recorded in this study are with high economic importance and, therefore, they serve as target traits for selection by mango growers and breeders. RAPD analysis has been shown to be an useful technique for providing information concerning the degree of polymorphism and diversity parameters of mango. In addition, this technique can be exploited for efficiently identifying and characterizing mango germplasm with respect to specific agro-pomological traits. Understanding and structuring of the genetic diversity among mango genotypes will be a major foot step to accelerate linkage analysis, association mapping, marker assisted selection and cross breeding programs, which would aid strategies aimed at germplasm characterization, management, conservation and improvement of this important fruit crop.

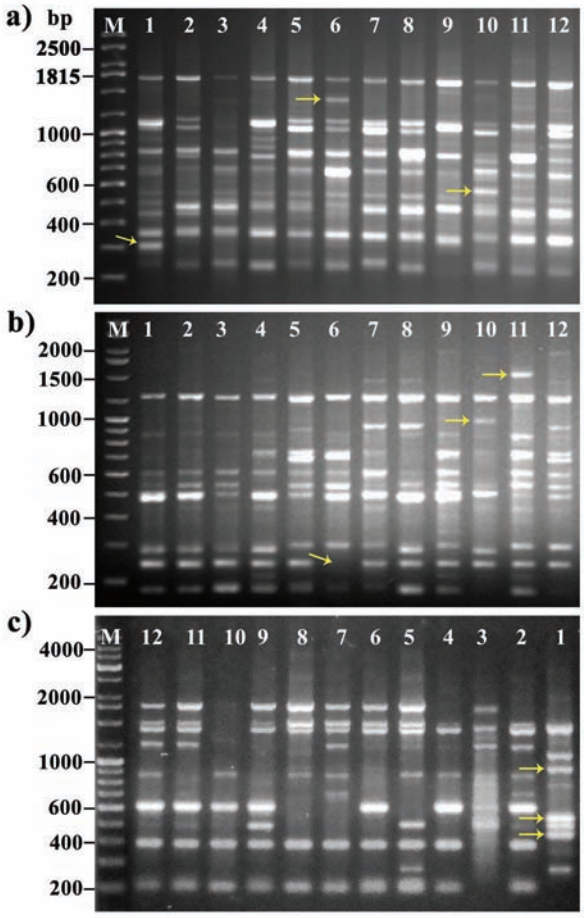

Fig. 1. RAPD profiling of 12 selected mango genotypes using primer OPA 6 (a), OPA 7 (b), and RPI 10 (c).Lane M : Low Range DNA Ruler Plus. Lanes 1-12 correspond to the mango genotypes (Listed in Table 1). *Arrows denote the presence of unique bands.*

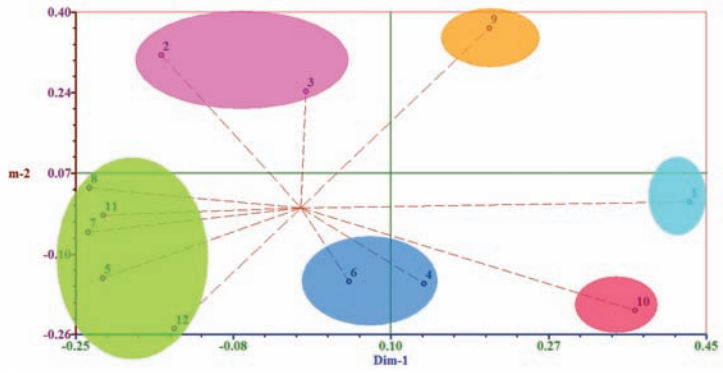

Fig. 3. Two dimensional plot generated from Principal Component Analysis (PCA) using RAPD markers. *(Numbers [1-12] plotted represent individual genotypes as listed in Table 1).*

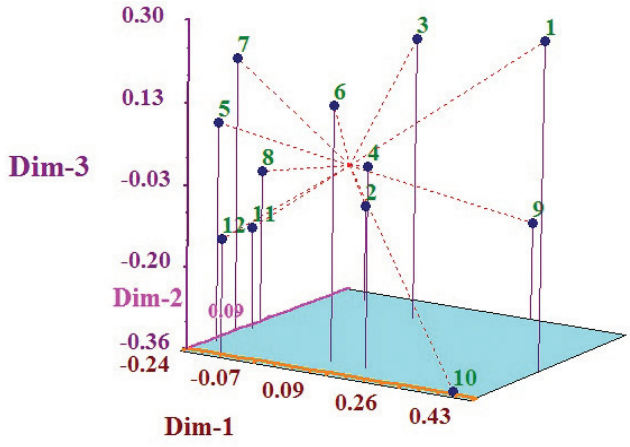

Fig. 4. Three dimensional distribution of mango genotypes based on PCA analysis based on RAPD markers. *(Numbers [1-12] plotted represent individual genotypes as listed in Table 1).*

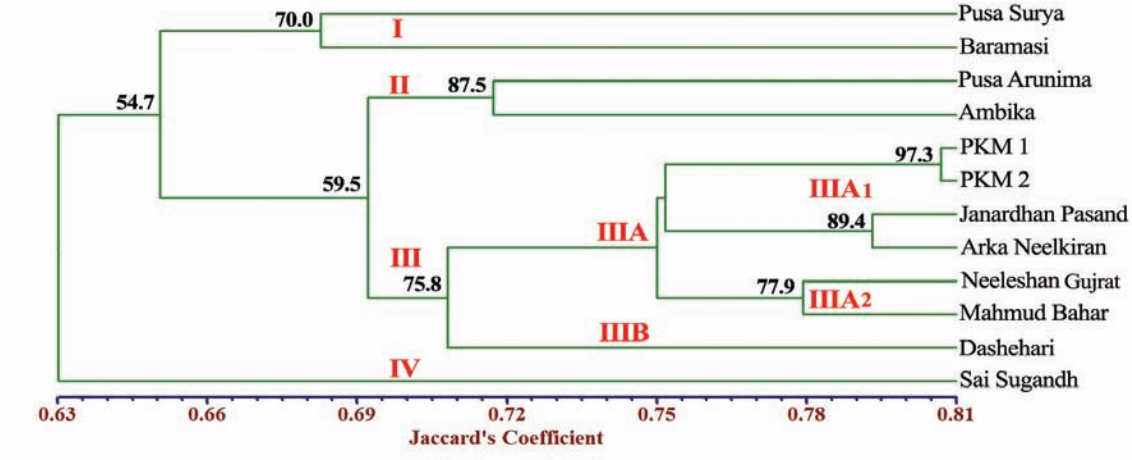

Fig. 2. UPGMA dendrogram constructed from RAPD based Jaccard's Similarity Coefficient depicting phylogenetic relationship among 12 selected mango genotypes. *(Values at the nodes correspond to bootstrap support [1000 replications])*

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Inter and intra-population genetic variability in *Calamus guruba* Buch.-Ham. (Arecaceae)-an economically important rattan species, using RAPD and ISSR molecular markers

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ABSTRACT

The genetic variability among nine natural populations and 45 accessions of *Calamus guruba* Buch.-Ham. (Arecaceae)- a dioecious and economically important Indian rattan species, was assessed using Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) molecular markers. Twenty RAPD and twenty ISSR primers were used to study the genetic diversity within and among populations of *C. guruba* collected from six districts of Odisha state. Out of 121 amplified loci with RAPD primers, 82 bands were polymorphic and 39 monomorphic in nature; no private band was detected. Similarly, with ISSR analysis, a total of 138 bands were generated which included 87 polymorphic and 51 monomorphic bands. The genetic similarity among all the accessions studies varied in the range of 65% to 99% indicating considerable variability within the species and close similarity among individuals of a particular population. Maximum genetic diversity was detected in the population collected from Chandaka Wildlife Sanctuary, Khurda district with an average polymorphism of 37.50% and minimum (18.98%) in the population from Khallikote, Ganjam district of Odisha. Use of *Calamus guruba* population from Chandaka with higher genetic variability as source of seed and other planting materials is suggested for maintaining the genetic stock and raising plantations under forestry programmes.

1. Introduction

Cane and rattans are spiny climbing palms belonging to the tribe Calameae of the subfamily Calamoideae under the family Arecaceae or Palmae (Uhl and Dransfield, 1987). They comprise about 600 species under 13 genera, which are distributed in equatorial Africa, South Asia, Southern China, the Malay Archipelago, Australia and the Western Pacific. Rattans and canes with solid stem are principal non-timber forest products in international trading and are highly valued and have social and economic importance because of their unique characteristics such as strength, durability, looks and bending ability. Due to overexploitation, habitat degradation and low regeneration capacity, the rattan resources of the world are under serious threat. About 117 species of rattans are considered as threatened to some degree (Walter and Gillett, 1998).

Of the 13 recognized genera of rattans and canes, *Calamus* is the most widespread, occurring in both the tropical and subtropical regions of Africa and south-east Asia (Uhl and Dransfield, 1987). A recent census suggests that the genus is represented by 374 species (Govaerts & Dransfield, 2005) in the world. Several species of canes are used for furniture, fancy items in cottage industries and basketry, and fruits of some are also consumed as a delicacy in parts of the world including India. There is extensive global demand for both raw and processed canes making it a valuable item for international trade.

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The species of *Calamus* are dioecious and wind-pollinated, with their phonological behaviour being influenced by climatic, topographical and edaphic factors. The low frequency of male plants and wastage of pollen during rains have led to decreased pollination efficiency and low seed set. It has been observed that adverse climatic factors influence the phenology of certain canes and cause variations in time and space of maturity and receptivity of stigma as well as production of pollen grains (Manohara *et al*., 2007). Overexploitation and forest fire pose serious threats to the survival of *Calamus* species in their natural habitats. Due to habitat degradation and overexploitation for trade purposes, the natural reserves of rattan are fast declining, causing genetic erosion of the existing resources.

In India, the genus *Calamus* is represented by 32 species (Karthikeyan *et al.,* 1989) and 22 species are reported to occur in Peninsular India alone (Anto *et al*., 2001). In Odisha, six species have been reported to occur in the wild till date (Mahapatra *et al*., 2012). Of these, *Calamus guruba* Buch.-Ham. ex Mart. (Kanta Beta) is the most common and economically important species occurring in several districts of Odisha. It's culms are used in making handicrafts, house construction, umbrella handles, basketry, ropes, mats etc. The fruits with fleshy pulp are edible and are often pickled. The tender shoots before emergence taste sweet and are eaten raw or after cooking as vegetable. Immature seeds are also used as beads. Besides, the roots have several medicinal uses.

In view of the extraction pressure and dwindling populations of *Calamus guruba* coupled with problems relating to reproductive biology and inherent genetic bottle-necks, understanding of genetic diversity is essential for developing appropriate conservation strategies. Different molecular markers have been used to assess the genetic variation and phylogenetic relationship of palms using PCR based markers and cpDNA studies (Wilson *et al*., 1990; Uhl *et al*., 1995; Baker *et al*., 1999; Asmussen *et al.,* 2000; Asmussen and Chase, 2001; Baker *et al*., 2000). The present study aims at evaluating the genetic diversity among and within populations of *Calamus guruba* occurring in Odisha using RAPD and ISSR markers with a view to identify populations with higher genetic variability for breeding and conservation programmes.

2. Materials and Methods

2.1. Plant materials

Forty five (45) individuals representing 9 different populations of *Calamus guruba* (CG1-CG45) were studied. Tender leaves from five different plants of each population were collected at random and were pooled together for genomic DNA extraction. The details of collection of samples are provided in Table 1**.**

2.2. Genomic DNA isolation

DNA was isolated from freshly collected young leaves by CTAB method as described by Doyle and Doyle (1990). RNA was extracted with RNaseA (Quiagen) treatment: @ 60 µg for 1 ml of crude DNA solution at 37 ^{0}C followed by two washings with phenol/chloroform/iso-amyl-alcohol (25:24:1 v/v/v) and subsequently two washings with chloroform/iso-amyl-alcohol (24:1 v/v). After centrifugation, the upper aqueous phase was separated, 1/10th volume of 3 M sodium acetate (pH 4.8) was added and DNA precipitated with 2.5 volume of pre-chilled absolute ethanol. The extracted DNA was dried and then dissolved in 10 mm Tris-HCl [tris(hydroxyl amino methane]/1 mm EDTA (ethylene diamine tetra acetic acid disodium salt) ($T_{10}E_1$ buffer, pH 8). Quantification was made by running the dissolved DNA in 0.8% agarose gel alongside uncut λ DNA of known concentration. The DNA was diluted to 25 ng/µl for RAPD or ISSR analysis**.**

Table 1

Details of accessions of *Calamus guruba* collected from different localities of Odisha with their geographical co-ordinates and identification code

Species Code	District	Location	Latitude	Longitude	Altitude
CG 1	Berhampur	Khallikote	N 19^0 36.503'	E 085^0 06.096'	141 ft
CG 2			N 19^0 36.505'	E 085^0 06.093'	142 ft
CG 3			N 19^0 36.500'	E 085^0 06.097'	140 ft
CG 4			N 19^0 36.507'	E 085^0 06.092'	139 ft
CG 5			N 19^0 36.509'	E 085^0 06.089'	145 ft
CG 6	Khurda	Barunei	N 20^0 09' 43.6"	E 085^0 39'01.7"	392 ft

CG 7			N 20^0 09' 43.4"	E 085^0 39’01.4"	390 ft
CG 8			N 20^0 09' 43.2"	E 085^0 39’01.6"	432 ft
CG 9			N 20^0 09' 43.4"	E 085^0 39’01.5"	435ft
CG 10			N 20^0 09' 43.0"	E 085^0 39’00.7"	437 ft
CG 11	Jagatsinghpur	Jasabantapur	N 20^0 13' 38.3"	E 086^0 09 ’35.2"	144 ft
CG 12			N 20^0 13' 38.1"	E 086^0 09 ’35.2"	145ft
CG 13			N 20^0 13' 37.9"	E 086^0 09 ’35.1"	141ft
CG 14			N 20^0 13' 38.1"	E 086^0 09 ’35.0"	136ft
CG 15			N 20^0 13' 38.3"	E 086^0 09 ’34.9"	133ft
CG 16	Cuttack	Odopada,	N 20^0 25’20.1"	E 086^0 04’05.8"	189 ft
CG 17		Kisannagar	N 20^0 25’20.2"	E 086^0 04’05.5"	159 ft
CG 18			N 20^0 25’20.0"	E 086^0 04’04.8"	160 ft
CG 19			N 20^0 25’20.0"	E 086^0 04’05.1"	156 ft
CG 20			N 20^0 25’20.6"	E 086^0 04’05.8"	157ft
CG 21	Khurda	Bhatapada,	N 19^0 49' 54.5"	E 085^0 01' 30.1"	346ft
CG 22		Balugaon Range	N 19^0 49' 54.8"	E 085^0 01' 30.0"	303ft
CG 23			N 19^0 49' 54.9"	E 085^0 01' 30.2"	280ft
CG 24			N 19^0 49' 55.1"	E 085^0 01' 30.1"	279ft
CG 25			N 19^0 49' 55.2"	E 085^0 01' 30.1"	280ft
CG 26	Khurda	Chandaka	N 20^0 18' 32.2"	E 085^0 48' 20.1"	294 ft
CG 27		Wildlife	N 20^0 18' 32.4"	E 085^0 48' 20.0"	297 ft
CG 28		Sanctuary	N 20^0 18' 33.0"	E 085^0 48' 20.6"	293 ft
CG 29			N 20^0 18' 33.5"	E 085^0 48' 20.2"	293 ft
CG 30			N 20^0 18' 34.7"	E 085^0 48' 20.6"	283 ft
CG 31	Keonjhar	Kodapada,	N 21^0 17' 15.5"	E 086^0 05' 21.8"	163 ft
CG 32		Anandapur	N 21^0 17' 15.2"	E 086^0 05' 21.5"	155 ft
CG 33		Wildlife Division	N 21^0 17' 15.4"	E 086^0 05' 21.4"	154 ft
CG 34			N 21^0 17' 15.5"	E 086^0 05' 21.0"	153 ft
CG 35			N 21^0 17' 15.4"	E 086^0 05' 21.2"	154 ft
CG 36	Cuttack	Nuapada,	N 20^0 31' 16.9"	E 085^0 49' 48.2"	119 ft
CG 37		Athagarh	N 20^0 31' 16.8"	E 085^0 49' 48.3"	120 ft
CG 38			N 20^0 31' 16.6"	E 085^0 49' 48.9"	122 ft
CG 39			N 20^0 31' 16.9"	E 085^0 49' 48.7"	121ft
CG 40			N 20^0 31' 16.7"	E 085^0 49' 48.8"	123 ft
CG 41	Bolangir	Gandhamardhan	N 20^0 50' 54.1"	E 082^0 51' 57.0"	1344 ft
CG 42		hills, Harishankar	N 20^0 50' 54.1"	E 082^0 51' 56.6"	1317 ft
CG 43			N 20^0 50' 53.9"	E 082^0 51' 56.7"	1310 ft
CG 44			N 20^0 50' 53.8"	E 082^0 51' 56.8"	1309 ft
CG 45			N 20^0 50' 53.9"	E 082^0 51' 56.9"	1305 ft

2.3 Random Amplified Polymorphic DNA (RAPD) analysis

Prior to polymerase chain reaction (PCR) for RAPD analysis random decamer Operon Primers (Operon Tech., Alameda, USA) were dissolved in double sterilized $T_{10}E_1$ buffer, pH 8.0 to the working concentration of 25 ng/ml. Out of Twenty five primers, twenty best selected primers as per the reproducibility and amplification pattern from A, C, D, N and AF series OPC-05, OPA-03, OPA-04, OPA-05, OPN-06, OPA-10, OPA-16, OPA-20, OPC-02, OPN-04, OPN-03, OPN-05, OPA-02 , OPN-08, OPAF-14, OPD-02, OPD-05, OPD-08, OPD-18& OPD-20 (Operon Tech. Alameda, CA) were used for RAPD analysis . 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 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

Inter Simple Sequence Repeats were used for PCR amplification. Out of twenty five primers screened, best twenty 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). Those primers were (PCP1, PCP2, PCP3, PCP5 ,PCP6, PCP7, PCP8, PCP9, PCP12, Oligo 1(b), Oligo 2(b), Oligo 3(b), Oligo 4(a), Oligo 4(b), Oligo 5(a), Oligo 5(b), Oligo 8(a), Oligo 9(a), Oligo 11(a) & Oligo 11(b)). 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 0.5U Taq DNA polymerase (Bangalore Genei, Bangalore, India). The amplification was carried out in a thermal cycler. The first cycle consisted of denaturation of template DNA at 94^{0}C for 5 min, primer annealing at specific temperature for particular primer 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^{0}C for 7 min.

The amplified products were resolved in 1.5% agarose gel stained with ethidium bromide . Standard DNA ruler (GeNei Medium Range DNA Ruler, Merk Millipore, Merk specialities Private Limited, Mumbai) was used. The electrophoresis was performed in a constant voltage at 60°C for two hours. The amplicons were visualized under the UV light and photographed. The gel was also documented by Gel Doc 2000(Bio Rad, USA) for scoring the bands. The size of the amplicons was determined by comparing them with that of ladder. The entire process was repeated at least three times to study the reproducibility.

2.5 Data analysis

The presence/absence of bands in RAPD and ISSR analysis was recorded in binary (0, 1) form. All the bands (polymorphic and monomorphic) were taken into account for calculation of similarity with a view to avoid over-/ underestimation of the distance (Gherardi *et al*., 1998). Jaccard's coefficient of similarity (Jaccard, 1908) was measured and a dendrogram based on similarity coefficients generated by the un-weighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973) and SHAN clustering. The statistical analysis was done using the computer package nNTSYS-PC (Rohlf, 1997). Resolving power (Rp) of the RAPD primer was calculated according to Prevost and Wilkinson (1999) as Rp = ΣIB, where IB (band informativeness) = 1 D [2 \ (0.5 D *P*)], *P* being the proportion of the 5 species containing the band.

3. Results

3.1 DNA isolation

The concentration of the total genomic DNA isolated from different species varied from 60 ng μl^{-1} to 1.8 µg μl^{-1} as can be seen from the agarose gel analysis (Fig. 1).

3.2 RAPD analysis

Out of 25 RAPD primers screened, 20 primers produced distinct reproducible bands (Table 2). A total of 121 amplified loci were generated including 82 polymorphic and 39 monomorphic bands. No unique band was observed. The RAPD banding patterns using three random primers

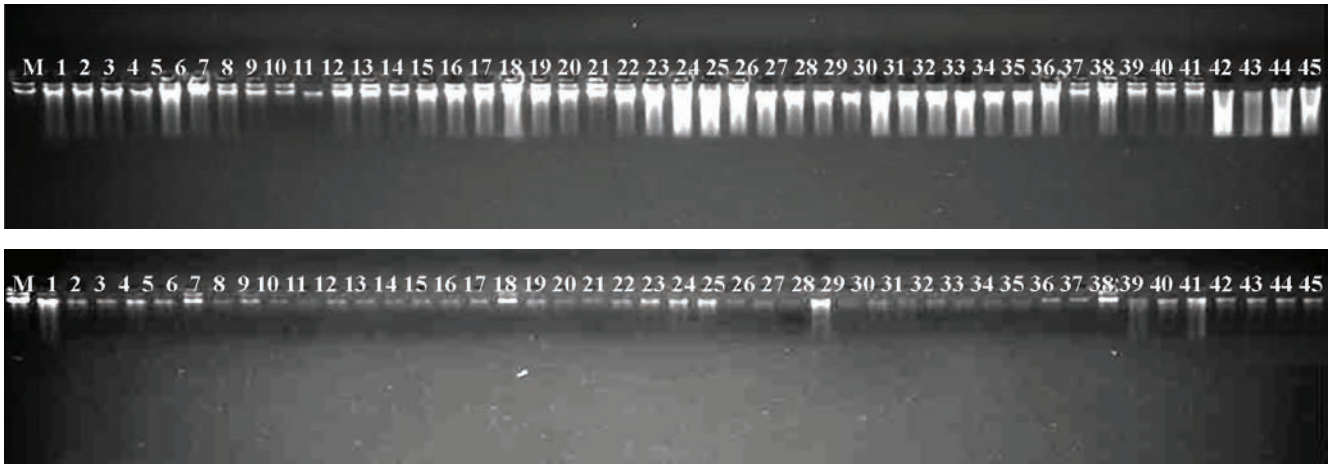

Fig. 1. Qualitative and quantitative analysis of total genomic DNA by 0.8% agarose gel. M, uncut phage λ DNA (600ng); lanes 1 to 45 *Calamus guruba* (CG1-CG45).

Table 2

Details of 20 RAPD primers used, bands amplified, percent polymorphism, primer index and resolving power

Sl No.	Primer Name	Primer Sequences	Range of amplicons in bp	Total No. of Bands	No. of Polymorphic Bands	No. of Monomorphic Bands	No. of Unique Bands	% Polymorphic Bands (PPB)	Resolving Power	Primer Index
1	OPC-05	5'-GATGACCGCC-3'	800-3000	4	2	2	0	50%	6.57	0.79
2	OPA-03	5'-AGTCAGCCAC-3'	1000-2500	6	3	3	0	50%	10.88	0.81
3	OPA-04	5'-AATCGGGCTG-3'	400-1200	4	2	2	0	50%	6.53	0.91
4	OPA-05	5'-AGGGGTCTTG-3'	580-2800	5	3	2	0	60%	8.62	0.97
5	OPA-10	5'-GTGATCGCAG-3'	380-3300	10	9	1	0	90%	13.64	2.87
6	OPN-06	5'-GAGACGCACA-3'	850-2300	5	3	2	0	60%	8.62	0.92
7	OPA-16	5'-AGCCAGCGAA-3'	680-3000	8	6	2	0	75%	12.71	1.67
8	OPA-20	5'-GTTGCGATCC-3'	600-2100	6	3	3	0	50%	10.88	0.83
9	OPC-02	5'-GTGAGGCGTC-3'	450-2600	6	5	1	0	83.33%	8	1.55
10	OPN-04	5'-GACCGACCCA-3'	580-2000	7	4	3	0	57.14%	12.35	1.24
11	OPN-03	5'-GGTACTCCCC-3'	700-2500	7	6	1	0	85.71%	7.86	2.45
12	OPN-05	5'-ACTGAACGCC-3'	750-2900	8	5	3	0	62.5%	13.2	1.55
13	OPA-02	5'-TGCCGAGCTG-3'	650-1900	9	8	1	0	88.88%	14.22	2.54
14	OPN-08	5'-ACCTCAGCTC-3'	500-2000	8	5	3	0	62.5%	11.37	1.68
15	OPAF-14	5'-GGTGCGCACT-3'	800-3000	5	4	1	0	80%	8.57	1.01
16	OPD-02	5'-GGACCCAACC-3'	980-1480	3	2	1	0	66.66%	4.84	0.77
17	OPD-05	5'-TGAGCGGACA-3'	400-1400	4	3	1	0	75%	5.77	0.84
18	OPD-08	5'-GTGTGCCCCA-3'	800-3000	6	3	3	0	50%	10.6	1.02
19	OPD-18	5'-GAGAGCCAAC-3'	560-2200	4	2	2	0	50%	6.31	0.64
20	OPD-20	5'-ACCCGGTCAC-3'	350-1820	6	4	2	0	66.66%	8.66	1.65

are represented in Fig. 2. The resolving power (Rp) of primers ranged from 1.81 (OPA3 and OPA 20) to 1.33 (OPC2) where as the primer index (Pi) varied from 0.35 (OPN3) to 0.13 (OPA3 and OPA20). The primers OPA10 produced highest number of amplified products (10), whereas OPD2 produced least number of amplified loci (03). Percent polymorphism with the primer OPA2 was found to be 88.88% and with OPC5, OPA3, OPA4, OPA20, OPD8, OPD18 the range of polymorphism varied between 50.00-85.71 %. The average number of bands and polymorphic bands per primer was 6.05% and 4.1%. The genetic similarity between accessions CG1 & CG45 and CG5 &CG7 was high (95%) with Jaccard's similarity coefficient (Jaccard, 1908) of 0.95. However, lowest similarity (52%) was observed between *Calamus guruba* accessions CG1 & CG45 and CG36 & CG 42. Average genetic similarity among all accessions was found to be 0.76.

The dendrogram constructed using RAPD data (Fig. 4) divided the 45 accessions of 9 populations of *Calamus guruba* into two clusters; one with 5 accessions collected from Harishankar, Bolangir district and the other with 40 accessions from 8 different populations. Both the clusters shared a node at 62% level of similarity. Similarly, all 5 accessions (CG 31-CG 35) from Anandapur, Keonjhar district formed a single clade and got separated from 2 accessions collected from Chandaka Wildlife Sanctuary, Bhubaneswar (CG 28 and CG 29) with 77% genetic similarity between them. In the dendrogram, all other 35 accessions did not form clear clusters according to the population to which they belonged; and were found intermingled with accessions of other populations.

3.3. ISSR analysis

For analyzing the genetic diversity of 45 accessions of *Calamus guruba* representing 9 widely distributed natural populations, 25 ISSR primers were screened, of which 20 primers responded well resulting in amplification of reproducible bands. A total 138 bands were amplified which include 87 polymorphic and 51 monomorphic ones (Table 3). The ISSR banding pattern is shown in Fig. 3. The primer PCP2 produced highest number of amplified loci (11), whereas least number (4) of bands were amplified with primers Oligo2 (b) and Oligo5(b). The primer PCP1 and PCP9 were responsible for amplification of highest number of polymorphic bands (88.88%). The primer PCP2 amplified maximum number of 5 monomorphic loci.

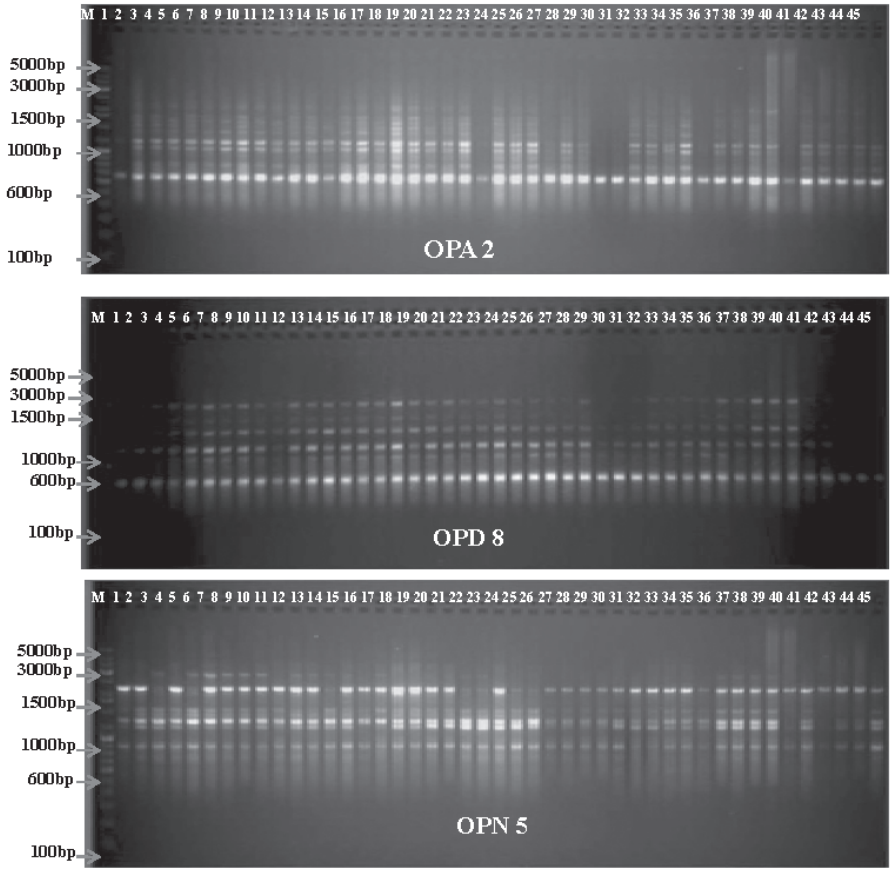

Fig.2. RAPD banding patterns of 45 accessions of *Calamus guruba* with primers OPA 2, OPD8 and OPN5; M-Genei ruler (Medium range) (100bp-5kb); Lanes 1 to 45, (CG1-CG45).

Table 3

Details of 20 ISSR primers used, bands amplified, percent polymorphism, primer index and resolving power

Sl No.	Primer Name	Primer Sequences	Range of amplicons in bp	Total No. of Bands	No. of Polymo-rphic Bands	No. of Mono-morphic Bands	No. of Unique Bands	% Polymo-rphic Bands (PPB)	Resolving Power	Primer Index
1	PCP-1	5'-GACGACGACG ACGAC-3'	200-1800	9	8	1	0	88.88%	14.48	2.35
2	PCP-2	5'- AGGAGGAGG AGGAGGAGG-3'	320-3050	11	6	5	0	54.54%	18.44	1.16
3	PCP-3	5'-GTCGGTGCGTG CGTGC-3'	300-1750	8	5	3	0	62.5%	9.86	1.16
4	PCP-5	5'-GAGAGAGAGAG AGAGAGAT-3'	620-1760	7	5	2	0	71.42%	10.62	1.24
5	PCP-6	5'-GACAGACAGA CAGACA-3'	620-1815	6	2	4	0	33.33%	10	0.16
6	PCP-7	5'-GGAGGAGGA GGA-3'	670-1950	6	5	1	0	83.33%	10.44	1.22
7	PCP-8	5'-GTGGTGGTGG TGGTG-3'	500-1830	7	3	4	0	42.85%	12.4	0.58
8	PCP-9	5'-GACACGACACG' ACACGACAC-3	250-1500	9	8	1	0	88.88%	9.73	1.67
9	PCP-12	5'-GACAGACAGACA GACAGACAGACAG ACAGACAG-3'	480-1600	8	7	1	0	87.5%	5.37	1.53
10	Oligo 1(b)	5'-AGAGAGAGAG AGAGAGG-3'	480-1200	6	4	2	0	66.66%	11.02	0.68
11	Oligo 2(b)	5'-GAGAGAGAG AGAGAGAG-3'	500-1050	4	2	2	0	50%	6.13	0.55
12	Oligo 3(b)	5'-GACAGACAG ACAGACA-3'	480-2600	7	3	4	0	42.85%	9.68	0.53
13	Oligo 4(a)	5'-GACAGACAG ACAGACAT-3'	480-2000	9	6	3	0	66.66%	12.9	1.01
14	Oligo 4(b)	5'-TGACAGACAG ACAGACA-3'	600-1900	7	4	3	0	57.14%	10.84	1.43
15	Oligo 5(a)	5'-GGACAGACAG ACAGACA-3'	600-1600	5	2	3	0	40%	9.6	0.34
16	Oligo 5(b)	5'-GACAGACAG ACAGACAG-3'	700-1815	4	0	4	0	0%	8	0
17	Oligo 8(a)	5'-CTCTCTCTC TCTCTCTG-3'	400-1900	8	5	3	0	62.5%	12.13	1.71
18	Oligo 9(a)	5'-GCTCTCTCT CTCTCTCT-3'	700-2000	6	4	2	0	66.66%	7.91	1.44
19	Oligo 11(a)	5'-GCTGTCTG TCTGTCTGT-3'	900-3200	5	4	1	0	80%	4.93	1.65
20	Oligo 11(b)	5'-CTGTCTGTC TGTCTGTC-3'	800-3000	6	4	2	0	66.66%	8.8	1.06

It could be derived from the similarity matrix (Jaccard, 1908) that accessions CG43 and CG44 of *Calamus guruba* collected from Harishankar hills of Bolangir district had maximum genetic similarity of 99%; lowest similarity (65%) was observed between accessions CG1 (Khallikote, Berhampur) and CG45 (Harishankar, Bolangir) and between CG30 (Chandaka WL sanctuary) and CG39 (Nuapada, Athgarh district). All the accessions of the nine populations of *Calamus guruba* had an average similarity of 0.82.

The dendrogram constructed on the basis of ISSR data divided the 45 accessions from 9 individual population of *Calamus guruba* divided them into two clusters; one with CG29 and CG30 both collected from Chandaka sanctuary having a similarity of 74% with the rest 43 accessions (Fig. 5). Further, out of the second major clade, 5 accessions of Harishankar (Bolangir) population (CG41- CG45) got segregated sharing a node at 75% level of similarity. In many cases, accessions of a particular population formed compact and clear groups in the dendrogram demonstrating their genetic relatedness as can be seen among the individuals of populations from Khallikote, Ganjam (CG1-CG5), Anandapur, Keonjhar (CG31-CG35), Nuapada, Athgarh (CG36-CG40) and Harishankar, Bolangir (CG41-CG45).

3.4. Combination of RAPD and ISSR analysis

Using pooled data of both ISSR and RAPD analysis, a total of 259 bands were observed in the 45 accessions of *Calamus guruba* collected from 9 different localities of Odisha. Of these, 169 bands were polymorphic and 90 were monomorphic in nature. However, no unique band was detected. The accession CG17 (Odapada, Cuttack district) produced the highest number of bands (229) taking all primers in to consideration and lowest number of bands (155) in the accession CG 42 (Harishankar, Bolangir).

The Jaccard's similarity coefficient (Jaccard, 1908) was maximum (0.94) between accessions CG1 and CG2 (Khalikote, Ganjam) and CG11 and CG12 (Jasabantpur, Jagatsinghpur) and the lowest (0.62) between CG23-CG42, CG29-CG39 and CG29-CG40 representing different locations. All the accessions of *Calamus guruba* had an average similarity of 0.79. The dendrogram constructed using the combination of RAPD and ISSR data (Fig. 6) placed five accessions of many populations together in single cluster as in case of CG1-CG5 (Khallikore, Berhampur), CG21-

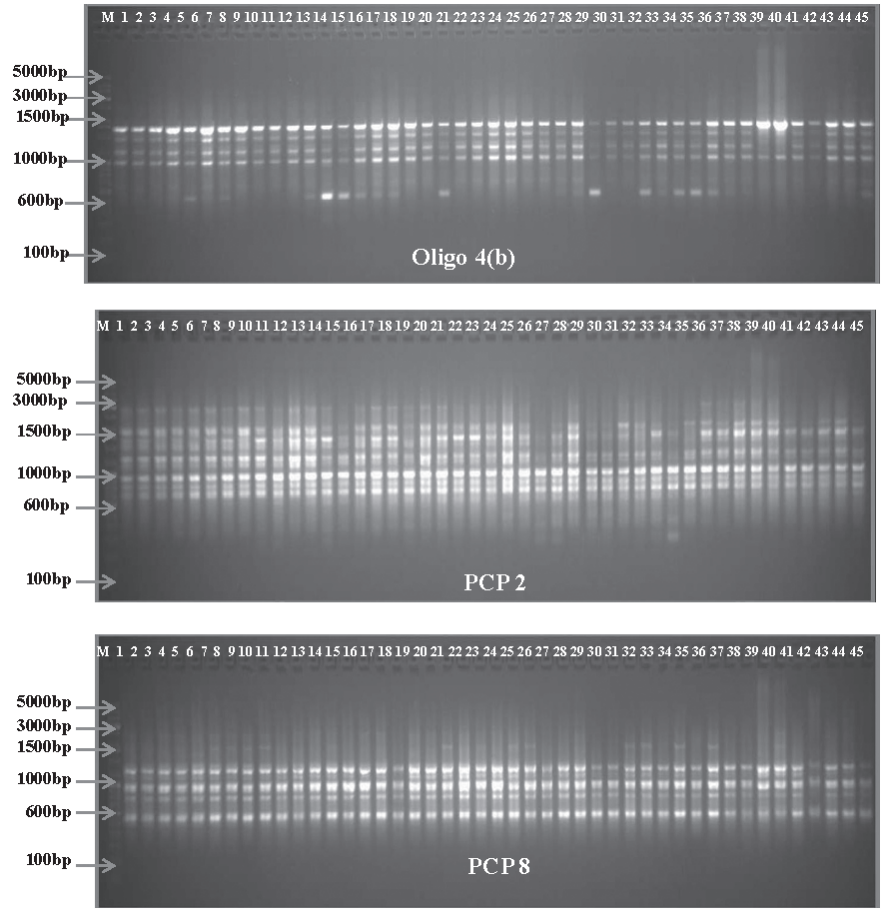

Fig. 3. ISSR banding patterns of 45 accessions of *Calamus guruba* with primers Oligo 4(b), PCP2 and PCP 8; M-Genei ruler (Medium range) (100bp-5kb); Lanes 1 to 45, (CG1-CG45).

CG25 (Bhatapada, Balugaon), CG31-CG35 (Anandapur, Keonjhar) and CG41-CG45 (Harishankar, Bolangir). Interestingly, the five accessions (CG41-CG45) collected from Harishankar (Bolangir) formed a segregated cluster from the rest 40 accessions at 70% level of similarity in the dendrogram.

4. Discussion

Molecular characterization of 45 individuals belonging to 9 populations of *Calamus guruba* collected from 7 districts of Odisha was made with a view to establish genetic relatedness with in individuals of a particular population and among populations and also identify populations with maximum genetic diversity to serve as source of seeds and other planting materials. Genetic richness can be assessed by estimating the genetic diversity parameters (viz. percentage of polymorphic loci and gene diversity index (Yeh, 2000) and in rattans and palms, high levels of genetic polymorphism have been documented (Bon *et al*., 1996; Cardoso *et al*., 2000). In the present study, two molecular markers (RAPD and ISSR) and their combination were used to study the inter- and intra-population genetic variability in *Calamus guruba* Buch.-Ham., a species with dwindling populations and genetic bottle-necks requiring conservation intervention and genetic improvement. The RAPD and ISSR technique has been successfully used in a variety of taxonomic and genetic relatedness studies on palms (Witono *et al*., 2006; Loo *et al*., 1999; Thawaro and Te-chato, 2009; Sathish and Mohankumar, 2007, Karim *et al*., 2015; Adawy, 2002) and the same is also applicable to assess genetic diversity of *Calamus guruba* as it has the ability to generate reproducible polymorphic markers. The results of the present study demonstrated the utility of RAPD and ISSR markers to characterize genetic relatedness and diversity among population of the studies species.

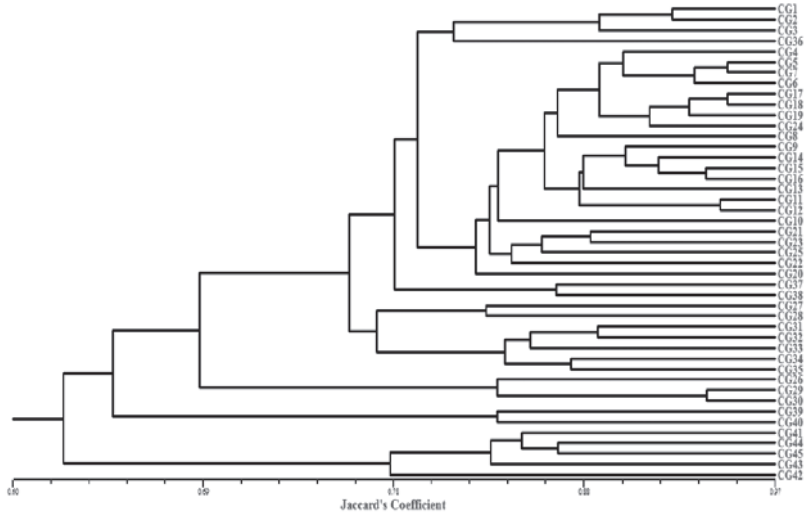

Fig. 4. Dendrogram showing genetic relationship among 45 accessions of *Calamus guruba* (CG1-CG45) using RAPD markers

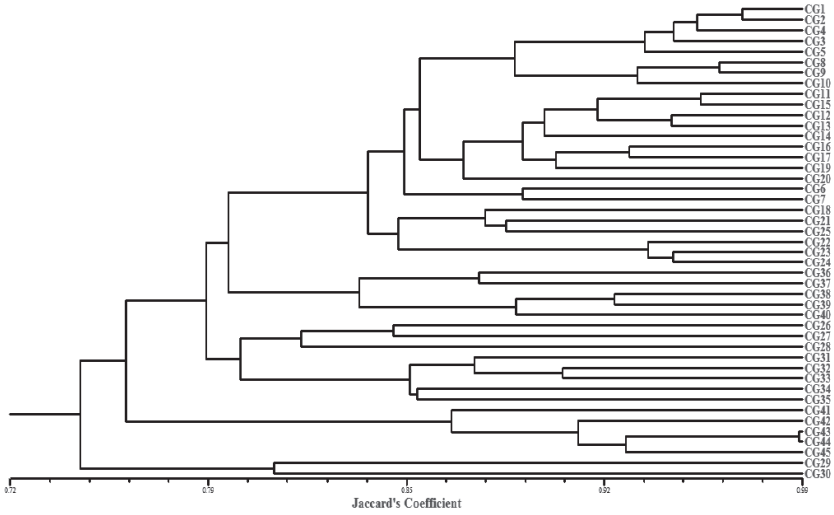

Fig. 5. Dendrogram showing genomic relationship among accessions of *Calamus guruba* (CG1-CG45) using ISSR markers

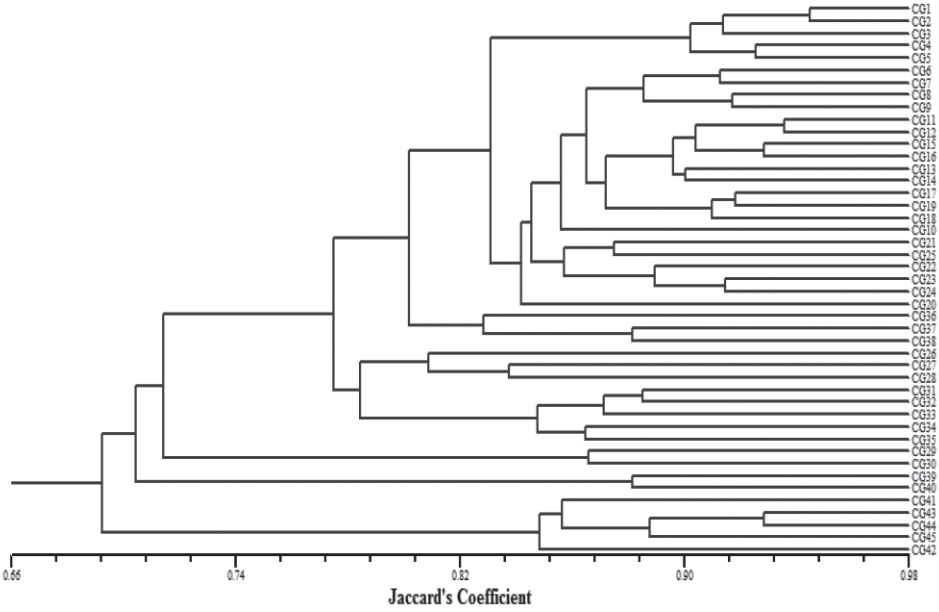

Fig. 6. Dendrogram showing genetic relationship among accessions of *Calamus guruba* (CG1-CG45) using combination of RAPD and ISSR markers

A total 121 bands were amplified using RAPD primers which include 82 polymorphic bands and the average percentage of polymorphism was 66%. Similar range of polymorphism has been obtained in different Indian and Sri Lankan populations of *Calamus rivalis* and *C. metzianus* using RAPD markers (Shreekumar *et al*., 2006). High level of polymorphism was also detected in India rattan genotypes by Sarmah *et al*. (2006) as in case of the present study. The genetic diversity of *Calamus thwaitesii* occurring in Western Ghats of India and Sri Lanka was estimated by using RAPD markers (Shreekumar and Renuka, 2006) and high percentage of polymorphism (40.00 to 60.83) was detected. They observed that majority of genetic diversity resides within population (70.79%) and only 29.21% among populations.

With ISSR primers, a total number of 138 bands got amplified. Out of these, 87 loci were polymorphic, 51 monomorphic and no unique band could be found. Highest level of polymorphism (88.88%) was observed with primers PCP1 and PCP9; the average percentage of polymorphism being 60.62%. Karim *et al*. (2015) reported as high as 90% polymorphism in *Phoenix dactylifera* in South Tunisia with the application of ISSR molecular markers. The finding of the present work corroborates the above data. Similar observations were also made by Zehdi *et al*. (2004) in Tunisian date palm.

Using RAPD and ISSR markers in combination, a total of 259 bands were produced in the present investigation, of which 169 bands (65.25%) were polymorphic. Adawy (2002) reported high polymorphism among and within the populations of Egyptian date palm (*Phoenix dactylifera*) applying RAPD and ISSR marker combination. Similar observations were made by Khierallah *et al*. (2014) in 17 date palm cultivars of Iraq. Our results are in conformity with the above observations on genetic diversity of palms.

The 45 accessions selected for genetic diversity assessment using molecular markers belonged to nine wild populations of Odisha with 5 individuals per population. Out of these, the population from Chandaka Wildlife Sanctuary, Bhubaneswar was found to possess maximum genetic diversity in terms of percent polymorphism (37.5%) and minimum (18.98%) in case of Khallikote, Ganjam population. In view of this, it is suggested that *Calamus guruba* population from Chandaka possessing higher genetic variability be used as source of seed and other planting materials for enrichment of genetic stock and raising quality planting materials for raising plantations in suitable forest habitats.

Acknowledgements

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A new record of *Graphis* (Lichenized Ascomycota) from India

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ABSTRACT

A collection from Darjeeling district in West Bengal state was identified as *Graphis ruiziana* (Fée) A. Massal. This lichen species is recorded for the first time from India. Detailed description, illustration and relevant notes are provided.

1. Introduction

Graphis Adans., which is the largest genus in the family *Graphidaceae*, comprises over 370 species worldwide; the members of the genus are found mostly in tropical regions, with few members also being found in the temperate parts. The genus *Graphis* is characterized by mostly lirellate ascomata; partially to completely carbonised excipulum; hyaline, amyloid, transversely septate to muriform ascospores (Staiger, 2002; Lücking, 2009).

Singh & Sinha (2010) reported the occurrence of 111 species of *Graphis* in India. Later, few more species were added to the genus by Jagadeesh Ram and Sinha (2009b), Singh and Swarnalatha (2011b), Singh *et al.* (2011), Chitale *et al.* (2011), Sharma and Khadilkar (2011), Gupta and Sinha (2012), Sethy *et al.* (2012), Singh and Singh (2014, 2015 & 2016), Singh *et al*. (2015). While studying the specimens of *Graphidaceae* the author came across an unidentified collection from Darjeeling hills, which on critical examination was found to be *Graphis ruiziana*. The species *Graphis ruiziana* has not been recorded from India so far in any revisionary/floristic work. Therefore, this is a new distributional record for the species in India.

2. Materials and Methods

The morphological examinations were carried out under a stereo zoom microscope (Nikon SMZ 1500). Thin hand-cut sections of thalli and ascomata were mounted in water, 10% KOH and Lugol's solution; all anatomical measurements were made in water mounts and anatomical characters were examined under a compound microscope (Magnüs MLX-Tr). The lichen chemicals were investigated with Thin Layer Chromatography (TLC) in solvent system A, following White & James (1985). The spot tests were performed with the usual chemical reagents (K, C and P). The specimen was also examined under UV light (365 nm). The specimen was taken on loan from the herbarium of Central National Herbarium, Botanical Survey of India, Howrah (CAL) and studied by the author at Lichen Laboratory, Botanical Survey of India, Central Regional Centre, Allahabad, in December 2011. Identification of the specimen followed the key by Lücking *et al.* (2009).

3. New Record

Graphis ruiziana (Fée) A. Massal., Mem. Lichenogr.: 111. 1853. *Opegrapha ruiziana* Fée, Essai Crypt. Écorc.: 27. 1824. *Graphina ruiziana* (Fée) Müll. Arg., Mém. Soc. Phys. Genève 29(8): 38. 1887.

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Fig. 1. *Graphis ruiziana* (Fée) A. Massal.

Thallus corticolous, epiphloeodal, thallus 22–32 µm thick above the bark, 4–5 cm diam., finely cracked, irregular in outline, prothallus indistinct; surface white to creamy to ashy-grey, smooth. Thallus in section lacks upper cortex, clusters of small calcium-oxalate crystals present in the thallus.

Ascomata lirellate, simple, jet-black, numerous, scattered all over the thallus, straight to occasionally curved, ellipsoid or oblong to elongate, sessile, 0.4–1.7 mm long, 0.3–0.4 mm broad, obtuse at the ends; disc closed to slit-like, ashy-white pruinose along the slit; excipulum completely carbonized; not covered by thalline margin; labia convergent, entire; hymenium hyaline, not inspersed, I–, 176–208 µm high; epihymenium indistinct; subhymenium hyaline to brown to black, becoming carbonised with age; paraphyses simple, tips without brown walls. Asci clavate, 128–150 × 19–29 µm. Ascospores 8 per ascus, sub-biseriate to biseriate, obovatus or broadly ellipsoid, muriform, 25–58 × 14–19 µm, 8–11 × 3–4 locular, hyaline, I+ blue-violet.

Chemistry: Thallus K– red, C–, KC–, P–, UV–; No lichen substances detected by TLC.

Remarks: This species is characterised by its simple, jet-black, short, sessile ascomata; completely carbonized excipulum; convergent, entire labia; 8-spored asci; obovatus or broadly ellipsoid, muriform, 25–58 × 14–19 µm, 8–11 × 3–4 locular, hyaline, I+ blue-violet ascospores and lack of lichen substances. In morphology, anatomy and chemistry *Graphis ruiziana* is very similar to *G. nuda* and *G. subruiziana,* but *G. nuda* has shorter (25–40 × 14–18 µm) ascospores and *G. subruiziana* has larger (55–90 × 20–35 µm) ascospores and non-pruinose disc.

Distribution: Brazil, Brittany, Costa Rica, France, Ireland, Portugal, Scotland, Venezuela; now recorded here from India also.

Specimen examined: INDIA: West Bengal, Darjeeling District, Manebhanjan, May 1985, K. N. Roy Chowdhary 4803 (CAL).

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New distributional record of the endemic plant *Dipcadi montanum* var. *madrasicum* (E. Barnes & C.E.C. Fisch.) Deb & S. Dasgupta (Asparagaceae) from Andhra Pradesh

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ABSTRACT

The endemic taxon, *Dipcadi montanum* var. *madrasicum* (Asparagaceae) is reported for the first time from Narasimha Konda Sacred Grove in the Eastern Ghats of Andhra Pradesh. As most of the species of *Dipcadi* occurring in India are found in the Western Ghats hotspot area, this collection from a sacred grove in the Eastern Ghats is interesting from the phytogeographical and conservation point of view. Detailed description, photo plate, distribution map and relevant notes are provided.

1. Introduction

Sacred groves often harbour diverse and sometimes unique assemblages of plant and animal species and, therefore, are important for biodiversity conservation. This is evident by the fact that surveys of these groves often yield species that are significant from the conservation point of view. While on a field exploration trip to assess the conservation status of the sacred groves of Nellore District, Andhra Pradesh, a collection of *Dipcadi* species from Narasimha Konda Sacred Grove (14^{0}27'24.2"N, 079^{0}52'51.8"E; alt. 111 m.s.l) was made, which on critical examination were identified as *Dipcadi montanum* (Dalz.) Baker var. *madrasicum* (Barnes & Fischer) Deb & Dasgupta. The plant *Dipcadi montanum* var. *madrasicum* is known so far from Madhya Pradesh, being found in Balaghat plateau, the northern most extension of its distribution in peninsular India, Eastern Ghats/coast of Tamil Nadu and Eastern Ghats of Odisha (Saxena & Brahmam, 1995). Perusal of relevant literature and consultation of major herbaria reveals that the genus *Dipcadi* was not reported from Andhra Pradesh (Pullaiah, 1997; Sudhakar Reddy *et al*. 2008) and hence is now reported as a new distributional record for the state of Andhra Pradesh. Detailed description, distribution and photographs are provided to facilitate its easy identification.

Dipcadi montanum (Dalz.) Baker var. ***madrasicum*** (E. Barnes & C.E.C Fisch.) Deb & S. Dasgupta in J. Bomb. Nat. Hist. Soc. 75: 59. 1978; Saxena, H. O. & Brahmam, Fl. Orissa 3: 1963. 1995. *Dipcadi madrasicum* Barnes & Fischer in Kew Bull. 1940: 301. 1941; Matthew, Mat. Fl. Tamilnadu Carnatic 360. 1981; Mathew, Fl. Tamilnadu Carnatic 1642. 1983.

Ornithogalum turbinatum var. *madrasicum* (E. Barnes & C.E.C. Fisch.) J.C. Manning & Goldblatt, Edinburgh J. Bot. 60: 552. 2003 (publ. 2004).

Type: Chingleput Dist., Tambaram, 70m, Nov. 1937, *E. Barnes* 1801 (Lecto - K); ibid. Jan 1939, *E. Barnes* 2085 (Para - K)

Small bulbous herbs, *c*. 46 cm high; bulbs ovoid, white, glabrous, 2.5-3 x 2-2.5 cm. Leaves 2 per bulb, linear, 10-18 x 0.2 cm long, green, slightly broader and white at base,

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entire, acute at apex, glabrous. Scapes *c*. 38 cm long, terete, glabrous. Inflorescence *c*. 8 cm long, racemose, loose 7-flowered. Flowers *c*. 12 x 3.5 mm, pedicellate, green or greenish white, glabrous; pedicel 3-4 mm long, filiform, glabrous; bracts deltoid, 5-8 mm long, acuminate at apex, 6-nerved. Perianth 3+3, unequal, green, mildly perfumed, glaucous or glabrous, slightly hooded, sparsely glandular pubescent at sub-apex; outer perianth united up to one-third of its length, campanulate; lobes oblong-obovate, *c*. 8 mm long, 2-2.5 mm wide, rounded or acute at apex, reflexed below the middle; inner perianth united up to two-thirds of their length, coherent to form a flask-shaped structure with apical parts spreading; lobes 3-4 mm long, 2-2.2 mm wide, obtuse at apex. Stamens 6, *c*. 4.1 mm long; filaments arising from the base of the perianth, adherent throughout the tube, slightly free above, *c*. 1 x 0.4 mm; anthers linear, *c*. 3.1 x 0.5 mm, dorsifixed, versatile, introrse, green-yellow. Gynoecium *c*. 9 mm long, glabrous; ovary sub-sessile, obovoid, narrowed at base, 4-4.5 mm long, trilobed; stipe ca. 0.4 mm long; style 4.5-5 mm long; stigma trifid, glandular pubescent. Fruit not seen. Fig.1.

Flowering /Fruiting period: April- September/July-November

Distribution: Endemic to peninsular India (Ahmedullah & Nayar, 1987), Madhya Pradesh, Tamilnadu (Deb & Dasgupta, 1981), Odisha (Saxena & Brahmam, 1995) and now from Andhra Pradesh.

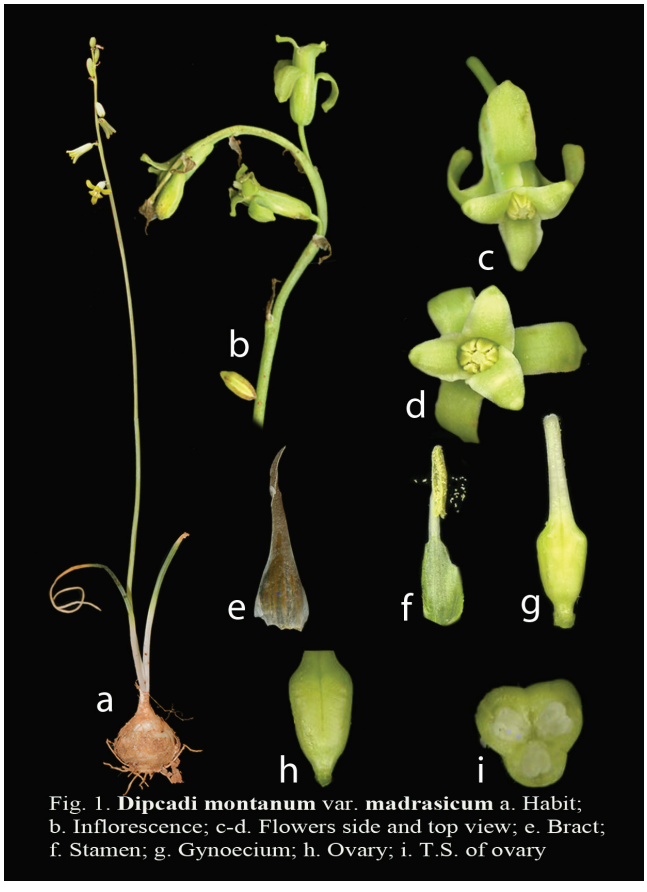


Fig. 1. **Dipcadi montanum** var. **madrasicum** a. Habit; b. Inflorescence; c-d. Flowers side and top view; e. Bract; f. Stamen; g. Gynoecium; h. Ovary; i. T.S. of ovary

Habitat: The plant is found in sandy/marshy places/rock crevices along dry stream beds in scrub forests. The plant is found from the coastal plains to elevations of about 1500 m altitude.

Uses: The bulb serves as an alternate food source for local communities during drought conditions (Deb & Dasgupta, 1981).

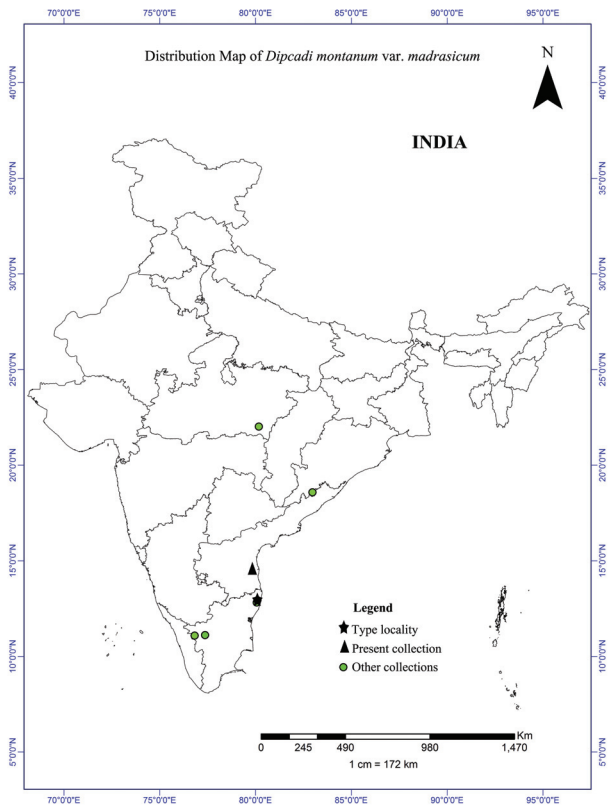
Specimen examined: India: Andhra Pradesh, Nellore District, Narasimha Konda, 14^{0}27'24.2"N, 079^{0}52'51.8"E, 111 m.s.l., 17.07.2016, *J. Swamy & S. Nagaraju* 007902 (BSID).

Additional specimen images seen: Coimbatore district, Perumal mudi shola, 5000 ft (1524. m.s.l.), 29.7.1930, *V. Narayanasamy* 3961 (MH, Acc. No. 80012); Coimbatore District, Mangarai area, 500 m.s.l., 5.4.1980, *R. Maruthan* 46083 (MH, Acc. Nos. 127483, 127484).

Conservation status: The taxon has so far been recorded from about six far-flung localities in the peninsular Indian region. However, not much information/data is available on recent collections, except the present one, to assess the status of the plant. The population observed at Narasimha Konda sacred grove is very small with barely 3-4 individuals. There is a strong possibility of the taxon located in its area of occurrence, if extensively searched. Considering this, the taxon is categorised as Data Deficient for the present.

Notes

The genus *Dipcadi* comprises *c*. 41 species (The Plant List, 2013), distributed in the Mediterranean region, Madagascar, Africa and South West Asia (Mabberley, 1997; 2008 rev.ed). In India, the genus is represented by 11 species (including four varieties), distributed from the Western Himalayas to Peninsular India (Deb and Dasgupta, 1978; Deshpande *et al.,* 2015). *Dipcadi erythraeum* is a native of Arabia-Egypt-Baluchistan region having its eastern extremity of distribution in Sind, Pakistan, and NW Rajasthan (Jodhpur, Barmer), India. *Dipcadi serotinum* is found to occur in the Western Himalaya (including Pakistan & Nepal) extending southwards to Central India (Madhya Pradesh). *Dipcadi reidii* is endemic to the W. Himalaya (Uttarakhand). Ahmedullah & Nayar (1987) reported 7 taxa (4 species and 3 varieties) of *Dipcadi* as endemic to peninsular India; these are *Dipcadi concanense*, *D. maharashtrense*, *D. minor*, *D. montanum* var. *madrasicum*, *D. saxorum*, *D. ursulae* var. *ursulae* and *D. ursulae* var. *longiracemosum*. Subsequently, another species, *Dicpadi goaense*, was described from Goa (Prabhugaonkar, Yadav & Janarthanam, 2009). More



recently, the species *Dipcadi krishnadevarayae* was described from Anantapuram, Andhra Pradesh (Rao, *et al*., 2016). The typical *Dipcadi montanum,* which is endemic to India, is distributed from peninsular India to W. Himalaya in the north. As such, there are now 8 taxa of *Dipcadi* that are known to be strictly endemic to peninsular India; most of these, except for *D. montanum* var. *madrasicum,* are confined to W. Ghats. *D. montanum* var. *madrasicum* is endemically restricted to the Deccan region, including Balaghat plateau (fig.2); it is reported here for the first from the Eastern Ghats of Andhra Pradesh. The report of *D. montanum* var. *madrasicum* (Saxena & Brahmam, 1963) from Odisha (Koraput Dist., Pottangi-Sunki) could not be confirmed through field/herbarium studies. However, this taxon may well be found in other localities in the eastern region of peninsular India.

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Diversity of *Ceratium* along Astaranga coastal water, Bay of Bengal, Odisha

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ABSTRACT

Ceratium spp. are important marine phytoplankton of phylum Myzozoa. They are the primary producers in sea ecosystem and also act as biological indicators of the water. The present study was carried out in Astaranga coastal water of Bay of Bengal during winter season to study the diversity of *Ceratium* spp. found in the study area. Physicochemistry of the water was also studied to relate with *Ceratium* diversity. In all nine different *Ceratium* spp. namely *C. azoricum*, *C. contrarium*, *C. declinatum*, *C. furca*, *C. fusus*, *C. longirostrum*, *C. lunula*, *C. macroceros*, and *C. trichoceros* were recorded. Their diversity in the water during the season could be related to high nutrient and salt concentration and also could be the reason of clear water condition in the locality.

The Bay of Bengal (BoB) connects a number of rivers along Odisha coast and receives about 6.6% of total global river fluxes. Nutrients are injected to the mouth of the Bay of Bengal through river discharge, which significantly affects the growth of phytoplankton. In marine ecosystem physical processes such as upwelling, down welling and terrestrial run off play significant role in nutrient composition in continental shelf, which affects the phytoplankton distribution and abundance (Platt *et al.,* 2005). Since it is known that the coastal marine environments are experiencing dinoflagellate blooms causing red tides (Reynolds, 2006), study on entire phytoplankton distribution and hydrology in the ecosystem is essential and interesting.

The phytoplankton *Ceratium* (dinoflagellate) comes under the microplankton group (20-200µm). It is a common and widespread mixotrophic genus among marine plankton. *Ceratium* spp. have significant role in marine environment as they are the primary producer community and also act as the biological indicator of water masses (Ibrahim, 2014). High population density of the genus causes red tides, which in turn cause nutrient and oxygen depletion. The photosynthetic cells contain chromatophores (yellow, brown yellow or green), which is distributed in cell cytoplasm. Body contains two valves i.e. epi-theca and hypo-theca bearing the horns. Most species have three horns but some have two or single horn also. The horns help the organism to float in the water column. *Ceratium* can produce resting cysts that may remain dormant within the sediments during unfavourable condition (Moreira *et al.,* 2015).

Significant work has been done on phytoplankton diversity and distribution at different areas of the coastal environment. Some of the noteworthy studies include reports on Northern Arabian Sea (Sarangi *et al.,* 2005), Gulf of Aquaba (Al Qutob *et al.,* 2002), coastal waters of Pakistan (Naz *et al.,* 2012), Bay of Bengal water (Gomes *et al.,* 2000; Paul *et al.,* 2007), Palk Bay, South east coast of India (Sridhar *et al.,* 2006), Eastern Indian Coast (Choudhury and Pal, 2010) and coastal water of Chennai (Subramanian and Mahadevan, 1999). Study on *Ceratium* diversity and its relation to water quality is scantily available, though some reports are available from the Red sea (Ibrahim, 2014), Southeast Brazil (Moreira *et al.,* 2015), South Africa (Hart

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and Wragg, 2009) and Central Europe (Padisak, 1985). However *Ceratium* records of BoB are not found. This work presents the *Ceratium* diversity along coastal water of Astaranga providing a check list of species recorded.

The present study was carried out in coastal waters of Astaranga during winter season (December 2015-February 2016). Sampling was done from the coast (19°56'55.40''N and 86°23'36.70''E) to 10 km (19°51'35.34''N and 86°22'51.93"E) in the sea at 9.00-10.00 AM covering eleven different stations in a transect with about 1 km between the stations. Surface water samples were collected for analysing various water quality parameters like sea surface temperature (SST), pH, dissolved oxygen (DO; modified Winkler's method), salinity (argentometric), silicate (silico-molybdic method), total phosphate (perchloric acid digestion) and nitrate (Azo-dye method) (Strickland and Parsons, 1972). Plankton samples were collected by filtering water through a plankton net (50µm pore size) and preserved immediately in 5% formaldehyde (Fig. 1). Measurement of *Ceratium* cell size was carried out by using ocular micrometer. For taxonomic identification photographs of the organisms were taken by the help of light microscope (Magnus MLX with camera at 10x40 zoom). Onsite measurement of SST was done by using a digital thermometer and pH by Systronic water analyser. The water samples for DO were fixed on site after sampling. The reported taxa are those recorded from all the 11 sampling site of the transect.

The water quality of Astaranga coastal water showed significant variation during the study period. SST varied from 24.27-25.52°C with an average of 24.65°C. The surface water was moderately alkaline (pH 7.58) with a narrow pH range. This indicated efficient utilization of dissolved CO_2 for phytoplankton production. On the other hand, DO ranged from 2.88-4.32mg/l with an average of 3.73 mg/l showing sub-optimal aerobic condition. This may be attributed to a good amount of dissolved organic carbon (DOC) drifted from the terrestrial system. As the water had a high transparency the DOC is probably the primary driving force for high *Ceratium* density. Salinity varied from 32.86-35.02psu with an average of 34.06psu showing high saline condition. Nutrients like phosphate, silicate and nitrate concentrations of the study site were 77-138µg/l with an average of 112.82µg/l, 8.6-22.3µg/l with an average of 13.8µg/l and 1866.7-2746.9µg/l with an average of 2298.9µg/l, respectively (Table 1).

Table 1

The physicochemistry of surface water of Astaranga coast of Bay of Bengal

	SST(°C)	PH	Salinity (psu)	DO (mg/l)	Phosphate (µg/l)	Silicate (µg/l)	Nitrate (ig/l)
Max	25.52	7.64	35.02	4.32	138	22.3	2746.9
Min	24.27	7.48	32.86	2.88	77	8.6	1866.7
Aver	24.65	7.58	34.06	3.73	112.82	13.80	2298.9
Stdev	0.26	0.05	0.65	0.33	16.78	3.04	208.46

Note: The data points are the means ±standard deviation of 33 replicates of 11 stations located at a transect of 10km.

A total of 9 *Ceratium* spp. viz., *C. azoricum*, *C. contrarium*, *C. declinatum*, *C. furca*, *C. fusus*, *C. longirostrum*, *C. lunula*, *C. macroceros*, and *C. trichoceros,* as detailed below, were reported during the study (Fig. 2).

1) *Ceratium azoricum* Cleve : Apical horn is very short and centrally placed. Antapical horn is relatively short, continuously curved and directed anteriorly. Right antapical horn is closely positioned to the cell body. Widest point of the cell is adjacent to the antapical horns. Chromatophore small, numerous, disc shaped and distributed in periphery. Cell length is about 140-146µm and width about 142-148µm.

2) *Ceratium contrarium* (Gourret) Pavillard: Cell body is subtrapezoidal with nearly flat and oblique posterior margin. Apical horn is very long and straight. Proximal parts of the antapical horn forms a shallow notch between them and are generally parallel to each other. Antapical horns are often undulating. Widest point is adjacent to the antapical horns. Parietal dark brown chromatophores present in the body as well as in the horns. Cell length is about 340-350µm and width about 265-270µm.

3) *Ceratium declinatum* (Karsten) Jørgensen: Cell body is subtriangular and longer than the width. Apical horn is moderately long. Proximal part of the antapical horns are directed laterally, distally they bend continuously and are directed anteriorly. Widest point is adjacent to the cingulum or to the antapical horns. One or two parietal chromatophores containing carotenoides and xanthophylls as accessory pigments. Cell length is about 190-210µm and width about 125-135µm.

4) *Ceratium furca* (Ehrenberg) Claparède & Lachmann: Cells contain two robust antapical horns, which are unequal

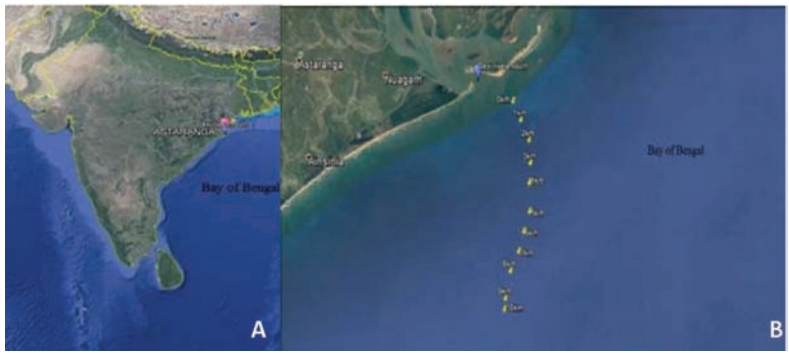

Fig. 1. Satellite imagery of (A) BoB coast and the (B) the sampling sites of the study area in a transect up to 10 km.

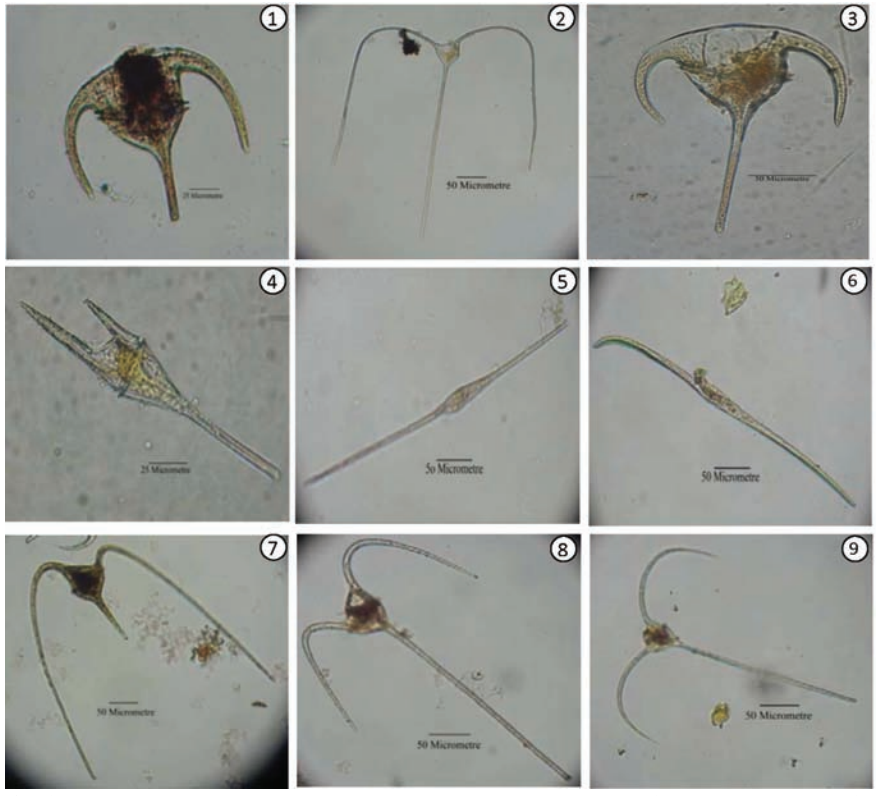

Figure 2. Light micrograph of 1) *Ceratium azoricum,* 2) *Ceratium contrarium,* 3) *Ceratium declinatum,* 4) *Ceratium furca,* 5) *Ceratium fusus,* 6) *Ceratium longirostrum,* 7) *Ceratium lunula,* 8) *Ceratium macroceros,* and 9) *Ceratium trichoceros* distributed in the Astaranga coast of BoB.

and slightly divergent from each other. Left antapical horn is longer than the right one. Epitheca tapers in to a long apical horn. Hypotheca between the horns is prominently inclined towards the cingulum. Chromatophores numerous, yellow brown, peripherally distributed in the cell. Measured cell length is about 185-198 µm and width about 32-37 µm.

5) *Ceratium fusus* (Ehrenberg) Dujardin : Cell is spindle shaped and long in size. Epitheca tapers to form a long apical horn and hypotheca tapers to form the long left antapical horn. Apical and left antapical horns are equal or subequal in length and slightly curved. A reduced right antapical horn may be present. Chromatophores are numerous, yellow brown chloroplast performing photosynthesis. Cell length is about 400-410 µm and width about 20-25 µm.

6) *Ceratium longirostrum* Gourret: Cell is needle shaped with epitheca tapering to form a long apical horn and hypotheca a left antapical horn. Horns are equal or subequal; apical horn is slightly curved and left antapical horn curved prominently. Thin and crowded chromatophores present in the body. Cell length is about 400-410 µm and width about 18-22 µm.

7) *Ceratium lunula* Schimper ex Karsten: Cell body is robust almost triangular in shape with prominently long and curved horns. Posterior margin of the cell is slightly flat and oblique. Apical horn is straight and positioned centrally, slightly inclined to the right and significantly shorter than the antapical horns. Small, angular, yellowish chromatophores are scattered about the amyloid body. Cell length is about 370-400 µm and width about 195-205 µm.

8) *Ceratium macroceros* (Ehrenberg) Vanhöffen: Cell body is subquadrangular with nearly flat and oblique posterior margin. Apical horn is very long and almost straight. The antapical horns are divergent after bending downward. Proximal parts of the antapical horns are directed posteriorly, forming a deep notch between them. Chromatophores are yellowish brown, numerous and discoidal. Cell length is about 400-420 µm and width about 260-270 µm.

9) *Ceratium trichoceros* (Ehrenberg) Kofoid: The cell body is subtrapezoidal with nearly flat and oblique posterior margin. Apical horn is very long and straight. Antapical horns are parallel to each other and are often undulating. Chromatophores are golden brown in colour. Cell length is about 300-310 µm and width about 245-253 µm.

In the locality diversity of *Ceratium* indicated nutrient rich condition during winter. Ibrahim (2014) reported that *Ceratium* diversity increased in nutrient rich waters elsewhere. Light availability was maximum in the photic zone and salinity concentration was also higher in the study site reaching up to 35.02 psu. Abundance of *Ceratium* spp. in the study area indicated clear water condition. Similar results were reported by Padisak (1985) and Buck and Zurek (1994). Species diversity and abundance of *Ceratium* at SST i.e., 24.65°C and DO i.e., 3.73mg/l revealed that they can survive in low temperature and low oxygen level. On the other hand, oxygen depletion in the water could be related to oxygen utilisation through metabolic activity that was driven by DOC as reported in Albert Falls (Hart and Wragg, 2009).

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A note on the occurrence of *Talinum portulacifolium* (Forssk.) Asch. & Schweinf. (Taliniaceae) in Odisha

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ABSTRACT

Wild occurrence of *Talinum portulacifolium* (Forssk.) Asch. & Schweinf. (Taliniaceae), an exotic of Tropical African origin, from different forest areas and urban habitats of Odisha has been reported in this paper. Though the species has got thoroughly naturalized in a wide range of habitats, it has not so far been reported in Floras and literature on flora and vegetation of Odisha. The probable cause of escape from cultivation and establishment in nature has been discussed. Nomenclature, botanical description, phenology, pattern of distribution of the species have been provided with ecological notes on its occurrence and spread in the state of Odisha.

Talinaceae is a small dicotyledonous plant family within the order of the Cariofilales. It includes two genera, *Talinella* and *Talinum* and about 27 species distributed in America and Africa, including Madagascar (Christenhusz & Byng, 2016). This family has been recognized by modern classification systems such as the system APG III (Bremer *et al*., 2009). Previously, the genera that constitute this family had been arranged in Portulacaceae, but phylogenetic analyses on molecular data indicate that the two genera that constitute the family forms a monophyletic clade to be considered as a separate family.

The members of the family are succulent shrubs, lianas or herbaceous in habit and are native to the Americas, Africa and Madagascar. They have tubers and underground parts. Some species exhibit Crassulacean acid metabolism (CAM). Leaves alternate, sometimes clustered on short shoots, flat, mucilaginous, slightly succulent. Flowers bisexual, actinomorphic, in terminal paniculate inflorescence. The fruit is a berry, mucilaginous and indehiscent.

The genus *Talinum,* commonly known as "fame flower" and "flame flower" is represented by about 15 species (Nyffeler & Eggli, 2010), which are native of America and South Africa. Species of *Talinum* are mostly herbs and have succulent stems and leaves. The stems are decumbent, ascending or erect, 30 – 100 cm high. The basal leaves usually form a small rosette of up to 6 leaves from which arise the stem and a paniculate inflorescence. Two species namely, *Talinum portulacifolium* (Forssk.) Asch. ex Schweinf. and *T. triangulare* (Jacq.) Willd. are introduced and naturalized in several parts of India. They are grown as ornamental plants, leafy vegetables or as a medicinal herb.

During the course of botanical studies in Odisha, the authors collected plant specimens from as many as ten districts, which were later identified as *Talinum portulacifolium* (Forssk.) Aschers & Schweinf. (Talinaceae). The nomenclature, botanical description, notes on ecology, distribution, factors responsible for invasion of the species and uses have been provided.

Talinum portulacifolium (Forssk.) Aschers & Schweinf, Weinf., Bull. Herb. Boiss. 4. App. 172. 1896; Manilal & Sivar., Fl. Calicut 40. 1982; M.K.V. Rao in B.D. Sharma & Sanjappa, Fl. India 3: 9. 1993; Sasidh.,Fl. Chinnar WLS 29.

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1999; Anil Kumar *et al*., Fl. Pathanamthitta 68. 2005; N.P. Balakr. in P. Daniel, Fl. Kerala 1: 319. 2005; Sunil & Sivadasan, Fl. Alappuzha Dist. 104. 2009. *Orygia portulacifolia* Forssk., Fl. Aeg.-Arab. 103. 1775. *Portulaca cuneifolia* Vahl, Symb. Bot. 1: 33. 1790. *Talinum cuneifolium* (Vahl) Willd., Sp. Pl. 2: 864.1799; Hook. f., Fl. Brit. India 1: 247. 1874; Gamble, Fl. Pres. Madras 66(48). 1915. *Talinum indicum* Wight & Arn., Prodr. 356. 1834.

Herbs or sub-shrubs, robust, glabrous with rootstock. Leaves subsessile, 6 - 8 x 2 - 3 cm, obovate or oblanceolate, obtuse or rotund and mucronate at apex, entire, fleshy, glossy above, obscurely nerved. Inflorescences terminal, racemose or paniculate. Flowers 1.5 - 2 cm across; bracts 1- 6 mm long, linear; pedicels 0.7 - 1.5 cm long. Sepals 2, 4 - 6 x 3 mm, ovate-lanceolate, acuminate, 3-nerved. Petals 5, pink, purple, or white, 9 - 12 x 5 - 6 mm, obovate to ovate-rotund. Stamens many; filaments 2 - 3.5 mm long, unequal, basally connate; anthers ca 1 mm long, oblong. Ovary superior, ca 2 mm long, 1-loculed; ovules many on free central placenta; styles 3-armed. Capsules 5 - 7 mm in diam., globose, 3-valved. Seeds ca 35 in each capsule, 1 mm long, ovoid or sub-reniform, black, shining, with concentric striations (Fig. 1).

Flowering & Fruiting: February-August

Distribution: Tropical Africa, Arabian Peninsula, East Asia, Introduced and cultivated in several tropical countries; India (Tamilnadu, Kerala, Karnataka, Andhra Pradesh, Odisha).

Uses: The plant is gathered from the wild for use as a food and medicine. It is sometimes cultivated in tropical Africa and India as a vegetable, and is also grown as an ornamental plant.

Specimens examined: North Orissa University campus, Takatpur, Baripada, N. C. Rout & A. K. Biswal, NOU 2394, Dt. 22.09.2015, IMMT Campus, Bhubaneswar, N. C. Rout & A. K. Biswal, NOU 2708 Dt. 14.9.16; Joranda, Dhenkanal District, A. K. Biswal, NOU 2624, Dt. 20.10.2016.

The species has been naturalised in several states of India, especially in the western peninsula of the country. It might have been introduced as a medicinal herb or as a leafy vegetable. *Talinum portulacifolium* is found to occur sporadically in more than 10 districts of Odisha. The species is mostly found in marshy wastelands, as weed in gardens and along forest fringes. However, it is likely that because of its exotic origin, It does not find place in regional floras (Haines, 1921-25; Mooney, 1950 and Saxena & Brahmam, 1994-96) and other floristic literature. Even, there is no mention of its occurrence in the flora of the neighbouring states like West Bengal, Jharkhand, Bihar, Chhatisgarh and Madhya Pradesh. Therefore, the species turned out to be new plant record for Odisha state and also for Eastern India. The species does not appear to be an invasive one and rapid colonizer.

Fig. 1. *Talinum portulacifolium* (Forssk.) Aschers & Schweinf: Habit and Habitat

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Ruellia elegans Poir. (Acanthaceae): A new plant record for Eastern India

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ABSTRACT

Ruellia elegans Poir. (Acanthaceae), a tropical Brazilian species, is added here to the flora of Odisha and Eastern India from Bhubaneswar. Nomenclature, brief botanical description, phenology, distribution and photograph of the species have been provided.

The genus *Ruellia* L. (Acanthaceae) is represented by 150 species and distributed in tropical and sub-tropical parts of the world (Mabbeley, 1997). Out of this, 9 species are recorded to occur in India (Naithani, 1990; Kumar, & Murugan, 2009; Brintha *et al.,* 2015) and so far only one species from Odisha state (Saxena & Brahmam, 1995). While carrying out botanical exploration in Bhubaneswar, Odisha, some specimens of the family Acanthaceae were collected, which on critical study were identified as *Ruellia elegans* Poir. So far, it is known to occur in Andaman Nicobar Islands, Kerala and Tamil Nadu. Hence, it is reported here as a new distributional record for Odisha states and also for eastern India. For an easy identification, a brief description, updated nomenclature, phenology and photographs have been provided. The specimens have been deposited in the Herbarium at Regional Resource Centre (RPRC), Bhubaneswar, Odisha.

Ruellia elegans Poir., Encyl. Suppl. 4:727. 1816; Kumar, & Murugan, J. Indian Bot. Soc. 88 (3&4):102. 2009; Brintha *et al*., Science Research Reporter 5(1): 40. 2015. *Ruellia formosa* Bonpl. Pl. Aequinoct. 1:167. t.48 1843. *Arrhostoxylum formosa* (Bonpl.) Nees Fl. Bras. 9:62. 1847.

Annual or perennial, erect herbs up to 60 cm high; branchlets quadrangular, ascending, multiple branches, pubescent. Leaves simple, opposite, elliptic-oblong or ovate-lanceolate, 5-10 × 2.5-4 cm long, cuneate to attenuate at base, apex at acuminate, margin obscurely crenate or entire, sparsely scabrous adaxially, sub-coriaceous, glabrous abaxilly; lateral nerves 6-8 pairs, prominent; petiole less than 1 cm long, flat, pubescent. Inflorescence axillary, raceme, up to 15 cm long; peduncle up to 10 cm long, pubescent. Flowers 4.5 cm across, red, bisexual, regular, sub-sessile; bracts one pair for each branch, 1.3 × 0.4 cm long, oblong, pubescent. Calyx valvate, 5, less than 1.5 cm long, tube very short, base united; calyx lobes un-equal, linear, each lobe 1-1.3 cm long, green, densely villous. Corolla tubular, 4.5 cm long, slightly bent, red, villous; 5-lobed, lobe obovate to obtuse, 1 cm across, glabrous. Stamens 4, didynamous; filaments connate at middle, unequal, *ca.* 8-15 cm long, glabrous; anthers 5 mm long, oblong, dithecus, versatile, yellowish white. Ovary disc, elliptic, 4 mm long, pubescent; style 4.2 cm in long, linear, glabrous, pinkish white; stigma bifid, dark pink.

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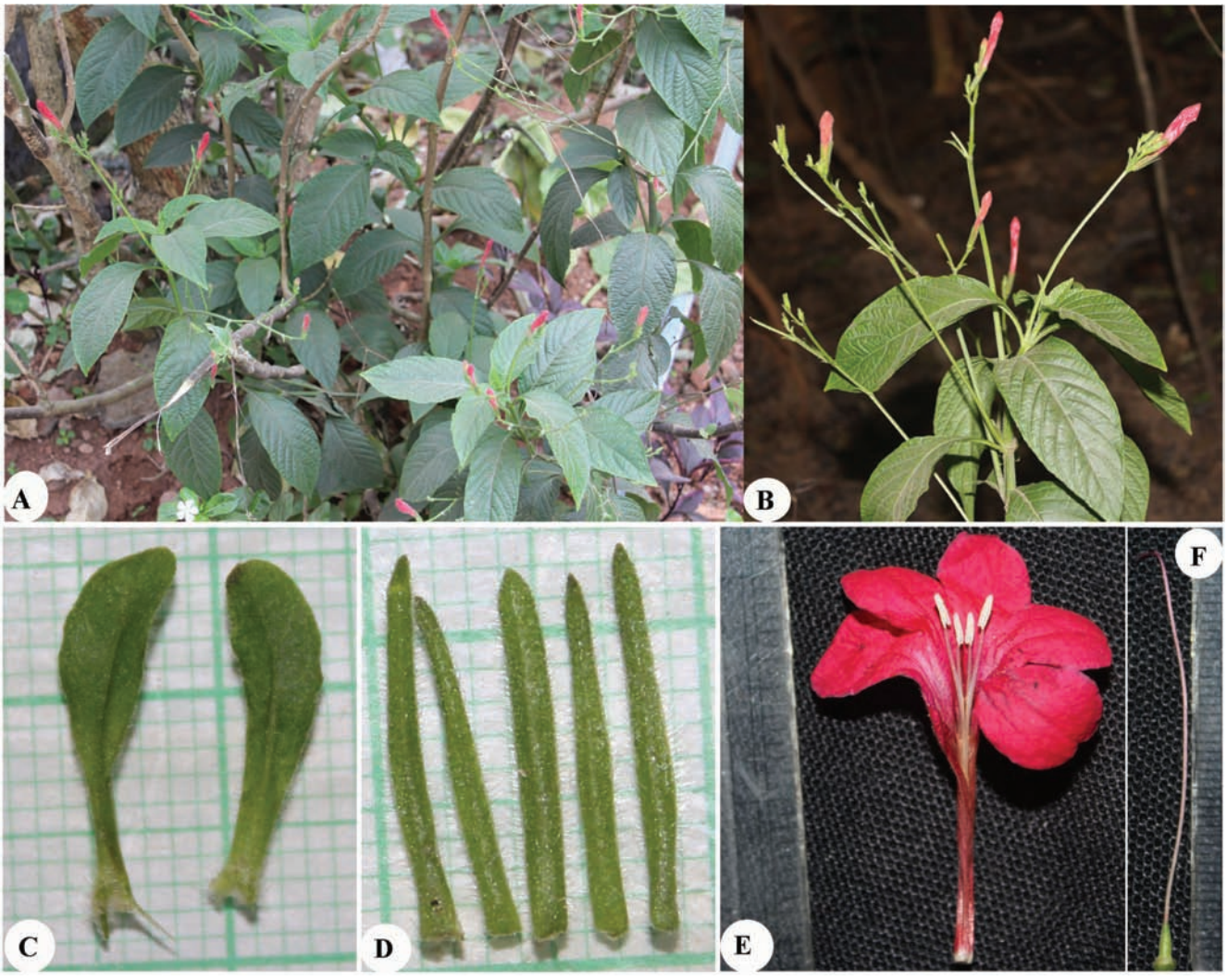

Fig. 1. *Ruellia elegans* Poir. (Acanthaceae): A. habit, B. flower twig, C. bracts, D. calyx, E. split of corolla, F. pistil

Flowering and Fruiting: September - December

Ecology: In the wild, it grows in marshy localities along forest edges and water canals in semi-evergreen forests. Most likely, the species was introduced to Bhubaneswar along with soils and seeds of other garden ornamental plants and got naturalized.

Distribution: INDIA (Andaman & Nicobar Islands, Kerala, Tamil Nadu and Odisha), BRAZIL

Specimens examined: INDIA, Odisha State, Khurda District, Bhubaneswar, IRC village, N20Ú18'356'' E085Ú40'758'', *Kalidass & Murugan* 9497 (RPRC), Dt. 21. 10. 2016.

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