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The growth and biochemical responses of *Aspergillus niger* to treatment of malathion

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ABSTRACT

Organophosphates are widely used pesticide in various agricultural fields causing soil pollution and has health hazards. The persistence nature of these pesticides in environment and its toxic effect on biodiversity make it necessary for rapid removal from the environment. A study was undertaken to determine the effect of organophosphate malathion on growth and biochemical activities of *Aspergillus niger* with an aim to examine the potential of the fungus for decontamination of malathion from soil. The fungus was treated for five days at an initial inoculums of 10^3 spores/ml on exposure to graded concentrations of malathion (10-1000 mM). The species was affected differentially by malathion, with concentrations up to 50 mM causing growth acceleration and toxicity observed thereafter. Quite a significant growth of organism was obtained at 1000 µM on prolongation indicating the high tolerant nature of the fungus towards pesticide. As expected there was biochemical alteration of cellular metabolism resulting in the reduction of cellular protein and carbohydrate and concentration dependent changes of carbohydrate enzymes (cellulase, invertase and amylase) and dehydrogenase. There was also major change in stress enzymes like phosphatases and esterases, both the enzymes being more active as a response to insecticide treatment. Enhanced activities of these enzymes was considered to be the basis for tolerance of the fungus to high concentrations of malathion.

1. Introduction

One of the most widely used agricultural pesticide groups are the organophosphates (OP), which are being exclusively used worldwide for control of the sulking and chewing pests in the orders diptera, lepidoptera, hemiptera, and coleoptera, due to their low effective concentrations. One such OP insecticide is malathion, S-(1,2-dicarbethoxyethyl)-O,O-dimethyldithiophosphate, which is being widely used since 1950 as a non-systemic, wide spectrum, phosphorodithioate organophosphorus compound (Goda *et al.,* 2010; Xie *et al.,* 2013). The pesticide is used in over 100 food crops in agriculture sector and against domestic pests like mosquitoes, head lice, animal parasite, etc. worldwide because of its quick knock down action against target pests by inhibition of acetylcholine esterase and comparatively low persistence in the environment (Barlas, 1996; Singh *et al.,*2012). However, prolonged use of the chemical has caused residual toxicity in crop fields affecting the soil microbiota (Megharaj *et al.*, 1987; 1994; Jena *et al.,* 2012). Residual toxicity of OP insecticides including malathion at field concentrations, has been reported in crop fields and in aquatic conditions leading to the decrease of productivity of the agricultural sectors and freshwater ecosystems (Van Donke *et al*., 1992; Megharaj *et al*., 1994; Mohapatra and Schiewer, 1996; Pandey and Gopal, 2012).

Any pesticide when applied not only affects the target organism but the main sufferers are the non-targeted soil microorganisms which get adversely affected by the application of the compounds. Pesticides get incorporated in soil directly, during plant treatment, and indirectly, via residues of plant or water and animal origin (Johnen, 1977; Anderegg and Madisen, 1983). A study on the effect of Triazophos-AN shows a great reduction in the population of soil bacteria up to 81%. There is also reduction in the population of the soil fungi by field application of

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insecticides but bacteria are more sensitive to the pesticide as compared to fungi (Kalyani *et al*., 2015). However, similarly profenofos, diazinon and malathion caused adverse impact on soil bacteria, nitrogen fixing bacteria and actinomycetes and effects are similar to that of the soil fungi (El-Ghany and Masmali, 2016). The application of organophosphate herbicides affects the size and composition of the soil organisms (Milosevia and Govedarica 2002; Ayansina *et al*., 2003). Liu *et al.* (2001) observed that dimethoate is inhibitory to the growth and biochemical activities of *A. niger* but the fungus also metabolically degrade the insecticides dimethoate and chlorpyriphos. There is no proper study of the effects of malathion on growth of *A*. *niger* in general and on enzymatic activities in particular. This study deals with the effect of malathion on growth and cellular enyme activities of *A. niger.*

2. Material and methods

2.1 The experimental conditions

The fungal strain *Aspergillus niger* was obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh. The pesticide used in the study was commercial grade malathion (Kalyani Industries Ltd., Mumbai). All the chemicals used for the sample analysis were of Hi media. The selected fungus was batch-cultured in 250ml Erlenmeyer flasks, each containing 100ml of liquid CzepakDox culture medium and with an initial inoculum of 10^3 spores/ml. Aqueous solution of malathion, after extraction of the active ingredient in acetone, was added to the medium to achieve the desired concentrations (10 - 1000 µM). The stock and experimental cultures were grown in an incubator shaker (NBiotech, Korea) at 26 ± 1°C in dark under continuous shaking (120 rpm).

2.2 Analytical procedures

Samples were taken at twenty four hours intervals up to five days for the estimation of the growth (fresh and dry weight). However, for protein, carbohydrate, and for other enzyme assays, samples were taken after 5 days of incubation. The mycelium was harvested using Whatman filter paper and the fresh weight was taken after one hr of shade drying. The samples were then kept in hot air oven at 80°C for 24 hours to obtain the dry weight. Total cellular protein content was determined by the methodof Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard. The total carbohydrate content was quantified by method of Roe (1955), using glucose as standard. The activities of carbohydrate enzymes (invertase, amylase and cellulase) were measured after incubating the mycelia, macerated in Sorensen's buffer for 24 hour in the respective substrate (1% starch for amylase, 1% carboxymethyl cellulose for cellulase and 1% sucrose for invertase). The amount of glucose produced was measured using 3,5 – dinitrosalicilic acid (DSA) and the standard was prepared using glucose (Chhotaray *et al.*, 2014). Dehydogenase activity was measured following the method of Casida *et al.* (1964), using triphenylformazan as standard. Phosphatase activity was determined spectrofluorimetrically by the breakdown of 4-Methylumbelliferyl phosphate to methyl umbelliferone using two buffers- citrate buffer for acidic phospatase and Tris HCL buffer for alkaline phosphatase. Quantification of phosphatase activity was done by using the linear regression equation of standard curve of methyl-umbeliferone (Chandrakala, 2016). Esterase activity was determined by breakdown of florescein-diaceate into florescein in Tris HCL buffer at pH 7. Quantification of esterase activity was made using the linear regression equation of standard curve of fluoriscein disodium salt (Chandrakala, 2016). For all biochemical and enzyme assays, a mass of 0.05g of fresh mycelium (after 1 hr of shade drying) was taken and macerated in the respective buffers.

2.3 Statistical analysis

The treatments were made in triplicates and the experiments were done twice. The data presented as figures and tables are the means of the replicates pooled together from two experiments. Comparisons among the concentrations as well as among days of treatment were made using least significant difference test (LSD). The trend of change was made by regression analysis among the replicates of the treatments. As and when needed the regression coefficients have been given in the text. The data analysis was done in Excelstat following the standard statistical procedures (Gomej and Gomej, 1984).

3. Results and discussion

From the pattern of change in the hyphal biomass during the period of observation showed that both fresh weight and dry weight of *A. niger* increased with increase in the days of incubation irrespective of the applied concentration of the malathion indicating linear growth increment of the fungus (Table 1). However, significant concentration dependent change (enhancement/reduction) in the growth could be seen on any day of the observation. The insecticide, up to concentration of 50µM, caused increase in growth of the fungus resulting in increase in biomass as well as cellular protein and carbohydrate content. The enhancement of growth was found to be high in the beginning as compared to prolongation for five days. The fresh weight after one day of treatment was 2.85 times higher at 50µM concentration as compared to control. Corresponding increase in the dry weight was also observed

at this concentration. On the other hand, after five days of incubation the growth enhancement at 50µM malathion was 35% higher than that observed in control (Table 1). The protein and carbohydrate content at this concentration were also higher than of control but such difference was found insignificant (LSD = 1.837% and 1.002% for protein and carbohydrate, respectively). However, with further increase in the concentration of the insecticide, protein and carbohydrate content significantly decreased but reduction of carbohydrate content was only significant at 1000µM (Fig 1).

The activity of invertase was found to be the highest followed by amylase and cellulase in the fungus. The activity of these enzymes were high in culture treated with the insecticide up to 50µM but further increase of concentration caused a continuous concentration dependent decrease of the enzyme activity (Fig 2). As compared to control, the highest rate of reduction in activity (at 1000µM compared to control) was 29% for cellulase followed by amylase (45%) and invertase (80%). However, at 50µM of the insecticide the enhancement of the enzyme activity was highest for cellulase (105%) followed by amylase (26%) and invertase (23%). The difference in the enhancement rate of invertase and amylase was found insignificant (t=0.48; n=12). This indicated that at non-toxic concentrations of the insecticide, the activities of the carbohydrate enzymes and effectively enhanced to encourage the growth of the fungus.

The activities of phosphatases and esterases were measured in order to estimate the insecticide metabolizing efficiency of the fungus. As expected, alkaline and acidic phosphatase activity increased significantly at each of the applied concentrations of the insecticide up to 50µM (LSD = 1.36 and 1.85 for alkaline and acid phosphatase, respectively). The alkaline phosphatase activity drastically decreased at 100µM concentration but further increase of the insecticide concentration did not cause significant change in the enzyme activity (Fig 3). The activity at these concentrations (100-1000µM) also did not significantly differ from that of untreated control. The acid phosphatase on the other hand continuously decreased with increase in malathion concentration beyond 50µM but at 100µM the activity was significantly higher than of untreated control. At other higher concentration the acid phosphatase activity was insignificantly different from the untreated control.

Similarly esterase activity increased continuously up to 50µM concentration of the insecticide but showed a concentration dependent decrease thereafter (Fig. 3). The activity enhancement at 50µM concentration was about 2.7 times of the untreated control. At other higher concentration also there was significantly high activity of the esterase, when compared to the untreated control (LSD = 2.113).The activity of the dehydrogenase was estimated as a measure of metabolic efficiency of the fungus. Dehydrogenase activity showed enhancement up to 50µM of the insecticide and reduction thereafter (Fig. 4). The rate of enhancement were found insignificant (LSD = 0.039) but reduction beyond 50µM insecticide concentration was found significant.

Table 1

The fresh weight and dry weight (g/100 ml) after different days of treatment with different concentrations of malathion

Conc. (mM)	Fresh wt (g/100 ml) Days after treatment					Dry wt (g/100 ml) Days after treatment				
	1	2	3	4	5	1	2	3	4	5
0	0.024 ±0.002	0.391 ±0.022	1.743 ±0.078	2.663 ±0.139	2.887 ±0.149	0.002 ±0.001	0.057 ±0.003	0.233 ±0.012	0.341 ±0.014	0.384 ±0.021
10	0.053 ±0.003	0.392 ±0.015	1.798 ±0.094	2.679 ±0.148	3.145 ±0.166	0.004 ±0.001	0.062 ±0.003	0.245 ±0.011	0.359 ±0.019	0.424 ±0.022
50	0.061 ±0.003	0.581 ±0.035	1.913 ±0.086	2.757 ±0.153	3.875 ±0.191	0.006 ±0.001	0.066 ±0.003	0.273 ±0.014	0.368 ±0.018	0.508 ±0.021
100	0.023 ±0.001	0.355 ±0.017	1.577 ±0.082	2.318 ±0.126	2.685 ±0.142	0.003 ±0.001	0.051 ±0.002	0.208 ±0.011	0.344 ±0.013	0.359 ±0.015
500	0.009 ±0.001	0.183 ±0.009	0.932 ±0.054	2.117 ±0.101	2.258 ±0.113	0.001 ±0.001	0.021 ±0.001	0.143 ±0.009	0.222 ±0.015	0.282 ±0.016
1000	0.004 ±0.001	0.011 ±0.001	0.448 ±0.026	0.475 ±0.029	1.073 ±0.055	0.001 ±0.001	0.002 ±0.001	0.071 ±0.003	0.065 ±0.003	0.133 ±0.007

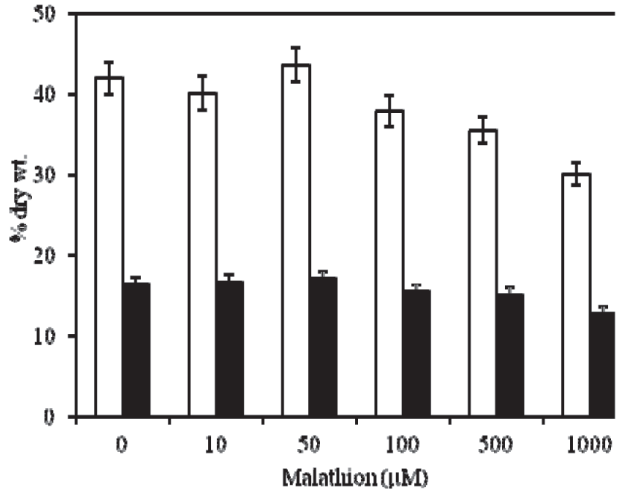

Fig. 1. The percentage of protein (empty columns) and carbohydrate (solid columns) contents of *A. niger* in response to 5 days of treatment with graded concentrations of malathion.

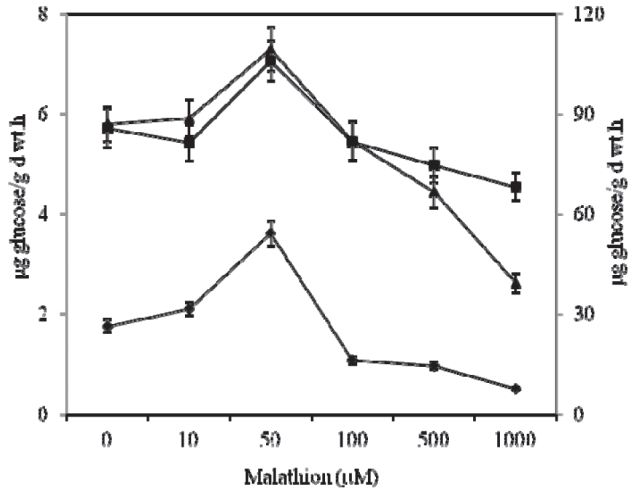

Fig. 2. The cellulase (diamond), amylase (triangle) and invertase (square) activities of *A. niger* in response to 5 days of treatment with different concentrations of malathion. The activity of invertase has been presented in secondary Y axis.

The fungus was found to have significantly high tolerance to malathion, being encouraged to grow up to 50 mM of the insecticide (Table 1). Enhanced growth and biochemical performances (Fig. 1 and 2) indicated that the fungus probably metabolised the insecticide and used it as a carbon source. The day dependent increase in growth and metabolic activities in the treated cultures supports this assumption. There are a number of cyanobacteria, algae, bacteria and fungi, which are known to metabolically degrade insecticides and other organochemicals causing decrease in their environmental toxicity (Ratna Kumari *et al.,* 2012; Ibrahim *et al.,* 2014; Kadhim *et al.,* 2015; Azmy *et al.,* 2015; Khan *et al.,* 2016; Nadalian *et al.,* 2016). Many fungal species belonging to phycomycetes, ascomycetes and white-rot fungi are found to be very useful for biodegradation of pesticides (Liu *et al.,* 2001; Jauregui *et al.,* 2003, Mohapatra *et al.,* 2018). Fungal species like *Aspergillus glaucus* (Anderegg and Madisen, 1983), *A. flavus* and *A. sydowii*

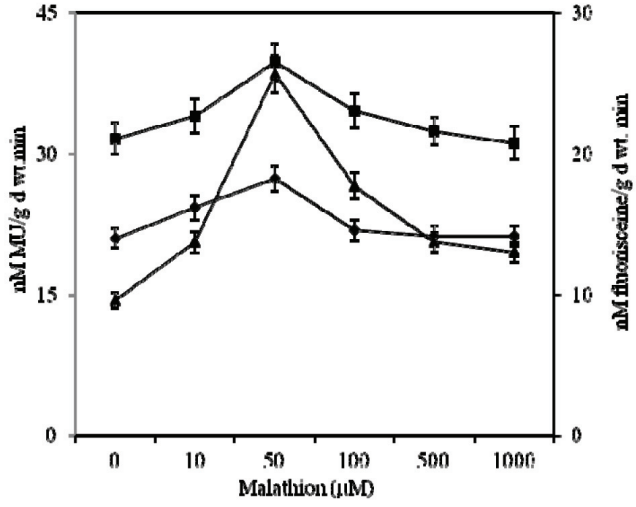

Fig. 3. The alkaline (diamond) and acid (triangle) phosphatase and esterase (square) activities of *A. niger* in response to 5 days of treatment with graded concentrations of malathion. The activity of estarase has been presented in secondary Y axis.

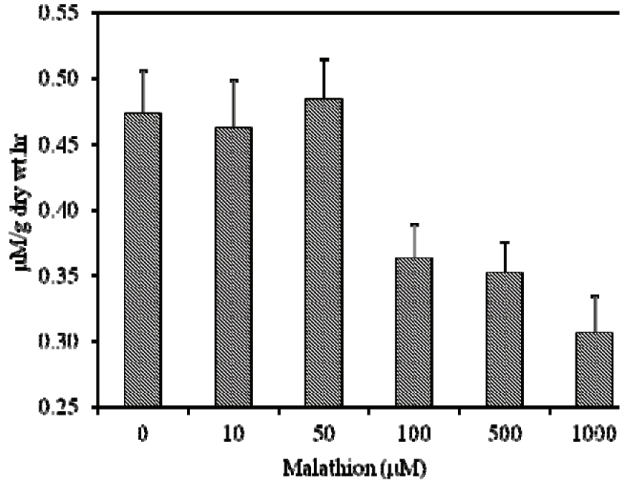

Fig. 4. The dehydrogenase activity of *A. niger* in response to 5 days of treatment with malathion.

(Hasan, 1999), *A. niger* (Liu *et al*., 2001) and *Trichoderma viride* (Matsumura and Boush, 1966) are known to degrade the pesticides under ambient environmental set up, even when exposed concentrations are much higher than of the residual level. Ramadevi *et al.,* (2012) reported that *Aspergillus niger* isolated from soil samples from malathion contaminated cotton cultivated field soils of Guntur district showed degradation ability of the chemical under laboratory conditions.

Significantly high level of esterases and phosphatases was noticed in the cultures treated with 50 mM of malathion. Liu *et al.* (2001) have reported that *A. niger* expressed a special enzymatic protein, comparable to bacterial phosphotriesterase on prolonged exposure to the OP insecticide dimethoate. They also observed the degradation of chlorpyrifos by the dimethoate tolerant *A. niger* strain indicating the broad substrate range of the induced enzyme. The high esterase activity in the present case proved that in *A. niger*, malathion treatment enhanced esterase level but the type of esterase so formed needs to be characterized. Further it was also observed that malathion treatment enhanced phosphatase activity which has not been reported earlier with OP treatment in fungal systems.

The result showed that *A. niger* has quite a high degree of tolerance to malathion showing improved growth and metabolic performance under treated condition up to 50 mM of dimethoate. This indicates that the fungus has the potential to degrade the organophosphate pesticide malathion under in vitro condition and can be a very good organism for the degradation of this compound. Moreover the organism also showed potential to grow in high concentration, though slowly, and also showed good sign to have the ability to survive in natural condition.

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Qualitative and quantitative assessment of plant diversity of Chandaka-Damapara Sanctuary with special emphasis on identification of useful, keystone and threatened species

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ABSTRACT

An exhaustive survey of flora and vegetation of Chandaka-Dampada Sanctuary revealed the occurrence of 655 species of angiospermic plants belonging to 454 genera under 119 families. Of these, *Uvaria lurida, Uvaria hamiltonii, Cycas sphaerica, Pterocarpus marsupium, Alphonsea maderaspatana, Litsea glutinosa, Salacia chinensis* and *Oroxylum indicum* were classified as threatened species needing immediate attention for conservation. Besides, several wild relatives of crop plants, parasites, epiphytes, lithophytes, insectivorous plants and botanically interesting species were also identified during the study. Quantitative assessment of 113 species of trees (GBH e"30cm) was made by laying 79 sample quadrates in the sanctuary and stand density of 222.21 stems/ ha and stand basal area of 11.2 m^2/ ha were recorded. The values of diversity indices such as Shannon-Weiner Index, Simpson Index and Evenness Index were calculated as 3.962, 0.03 and 0.83 respectively. The sanctuary was found to be rich in terms of biological diversity of plants.

1. Introduction

Lying between 20°12' 30"-20°26' 03" N latitudes and 85°49'35"-85°34'42" E longitudes, Chandaka-Damapara Sanctuary occupies part of Khurda and part of Cuttack District of Odisha state (Figs.1). The district boundary runs SW-NE almost in the middle of the sanctuary. The sanctuary is situated in a close proximity of the state capital, Bhubaneswar and spreads over an area of 193.39 sq.km. The legal boundary has been duly described by forest block boundaries in the State Govt. Notification No. 13482/FFAH, dated 10.06.1988 comprising of Reserved Forests, Demarcated Protected Forests, revenue lands, private lands and human settlements (Table-1).

1.1. General physiography, soil, geology and climate

The oldest-rock exposed in the area is Athagarh sand stones belonging to Gondwana group and found in western and northern sectors. The sand stone is medium grained, grey to pink in colour. This is overlaid with laterite caps. On the top, lies lateritic red loamy soil or sand-silt-clay deposits. Bulk of the sanctuary urea is covered with the latter with lateritic soil dominating in Bharatpur-Jagannaihprasad and Kodanmnda blocks. Soil depth is generally good and sub soil moisture is sufficient to promote good forest cover but soil humus is very poor due to forest fire and over-grazing.

The land is generally undulating, broken by low hills of moderate slope exceeding 35^0 with abrupt sleep slopes at places. The valleys are narrow and spread in linger like projections in the centre. Hills lying to the west and North-west are moderate to sleep while those on the East and South-east side are of gentle to moderate slope (Figs. 3-4). The altitude varies from 35m in Jagannathprasad block (near Baraman) and Chudanga block (near Ranipokhari enclave)

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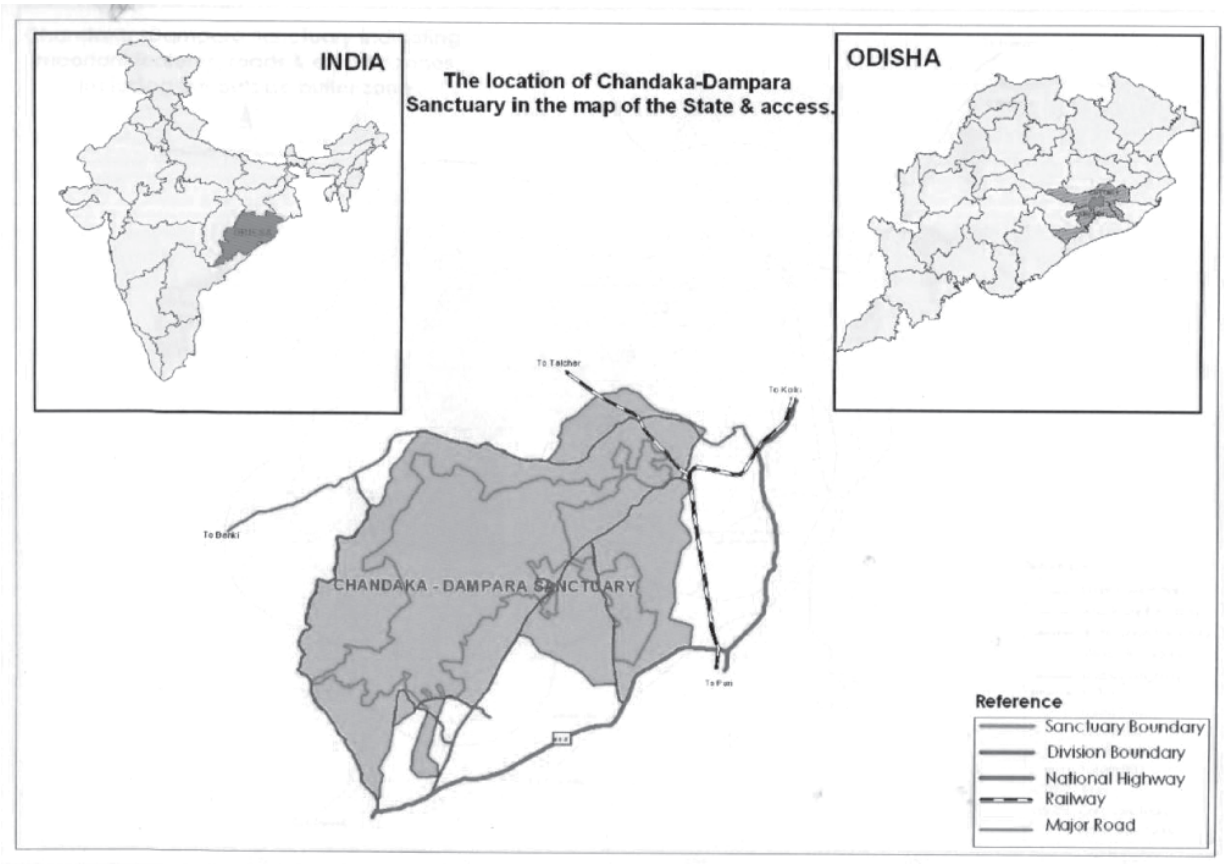

Fig. 1: Location map of Chandaka-Dampara Sanctuary in the map of Odisha and India

Table 1

Details of area coverage and classification of forests

Type/ classification of land	Area
Reserved Forests (7 nos. of RFs)	9,140.81 ha
Demarcated P.F. (11 nos. of DPFs)	9,219.19 ha
Private land acquired	53.87 ha
Bhalunka corridor	19.00 ha
Garjit village	195.52 ha
Other land area connecting Ratanpur and Koduamunda DPFs, Bhola & Daspur RFs, Jagannathprasad & Dalua DPFs	710.61 ha
Total area	19,339.00 ha

to 219m at Pandari mundia in Sunakhani-II D.P.F. Some other prominent hills (mundias) are Kansal (204m), Tarkei (203m), Barapita (194m) and Bathua (191m). In all, there are 32 small hillocks.

The climate of the area is tropical climate with three distinct and well marked seasons i.e. summer, rainy season and winter. The monsoon normally commence from mid June and lasts till mid October. Winter is short lived from mid November to and January. Hot and humid weather continues till the onset of monsoons. Average annual rainfall at Bhubaneswar is about 1560 mm.

Though there are no natural wetlands and marshes within the protected area, some seasonal marshes are found near Haridamada, Kalajhar, Saurabila and Kankadajhar. However, there are man-made reservoirs like Kumarkhunti and Deras within the area and Jhumuka, Guptapada, Kajalaganda (Manpur) Maclua (Bhjiput), Dulanapur, Baghua, Ashok Bandh and Jaria near the periphery of the sanctuary. Kushpangi pata, Jaripata, Chudanga pata (Chandiprasad) and Kanjia pata (lake) are natural marshes on the northern periphery of the sanctuary.

2. Study of flora and vegetation: Past and present

One can find occasional reference to the plants collected from Chandaka in "The Botany of Bihar and Orissa" (Haines, 1921-25) and nothing at all in its "Supplement" (Mooney, 1950). Biswal *et al*. (2005) made an inventory of plants of Chandaka Wildlife Sanctuary and listed 542 angiosperms, 16 pteridophytes and 1 gymnosperm species from the area. Behera *et al.* (2008) conducted an

ethnobotanical study of the plant resources of the area. However, absolutely no information is yet available on the population size and structure, diversity, distribution pattern, abundance, frequency and rarity of any species. Quite a good number of exotic species have been planted as avenue trees and forestry plantations. Several alien weeds like *Mikania micrantha, Parthenium hysterophorus, Cleome rutidosperma, Euphorbia heterophylla*, have invaded a wide range of habitats in Chandaka especially along the roads adversely affecting the indigenous flora. It is essential that that the present status of flora need be documented in quantitative terms with thrust on identification of critical habitats and conservation-worthy species before further deterioration takes place.

In the present study, the trees, shrubs, climbers and herbs occurring in the entire area were collected, identified and listed. For quantitative ecological study, the area was divided into smaller sampling grids and transects of 1000 m X 5 m (0.5 ha) were laid in representative vegetation types to capture maximum diversity. For each transect, data on geographical coordinates, soil types, forest types and general information about the sites were collected. All species occurring in each transect were enumerated and field level data on density, abundance, frequency, regeneration potential, use value of each of them were recorded. Each species was photographed and GPS data of place of occurrence were recorded in case of rare and economically important species so that the species can be relocated. Emphasis was laid on plant biodiversity documentation in special habitats and recording data on special groups of plants like weeds, epiphytes, lithophytes, grasses, wetland plants etc.

3. Methodology

3.1. Study site

The floristic inventory of the entire 193 sq. km area under the jurisdiction of Chandaka-Damapara Wildlife Sanctuary was made which included 9140.81 ha of Reserved Forests (7 RFs), 9219.19 ha of DPFs (11 DPFs) distributed in four forest ranges.

3.2. Plant collection, identification and preservation:

The entire area coming under Chandaka-Damapara Wildlife Sanctuary was divided into smaller units for floristic inventory. Multi-seasonal survey (summer, rainy and winter) will be conducted in these floristic survey units and all trees, shrubs, climbers and herbs were collected, preserved as herbarium specimens and identified. As far as possible, the flowering and fruiting specimens were collected for study of detailed morphology and botanical description and also for preservation as herbarium specimens. Standard field and herbarium methods (Jain & Rao, 1977 and Bridson & Forman, 1998) were followed for plant collection and preservation of voucher specimens. Landscape level photographs of habitats and plant association were taken in the field besides photograph of each plant species encountered during the survey. The plantation crops, avenue plants and introduced species were also listed in the enumeration of taxa.

The specimens were pressed in between blotters of field press applying light pressure and some representative samples were kept in polythene bags for dissection and diagnosis in the camp or laboratory. On return from a day's trip, the plants pressed in the field press were pruned to convenient sizes, poisoned by dipping them in 2% solution of mercuric chloride in absolute alcohol and plant parts were well-spread to reveal morphological details. Again the specimens were pressed in blotters for drying and the blotters were frequently changed to facilitate quick drying of specimens. After complete drying, the specimens were mounted on to standard mount boards of 42 x 28 cm. size using animal glue. The specimens were often stitched with cotton thread to attach entire or parts of specimens to mounting board, especially thick twigs and large fruits. The detached leaves, flowers, fruits, seeds or other parts were put in specially prepared paper capsules and mounted on the sheet for future study. The data recorded in the field were transferred to herbarium labels, which were then pasted on the lower right corner of the herbarium sheets. The field and herbarium methods followed during the present work were as per the guidelines provided by Jain & Rao (1977) and Bridson & Forman (1989).

Regional floras such as Haines (1921-25), Gamble and Fischer (1915-1935), Saxena and Brahmam (1994-1996), Mooney (1950) were used besides expertise available at the institute for identifying plants, and voucher specimens were housed in the Herbarium of Regional Plant Resource Centre (RPRC), Bhubaneswar. Trained research workers and surveyors undertook the field work.

3.3. Quantitative assessment of plant diversity

3.3.1.Sampling protocol

The entire Sanctuary area was divided into smaller grids, which formed the sampling plots for quantitative assessment of plant biodiversity (Fig. 5). In each grid, random quadrates of 20 m X 20 m were laid fixing GPS points for enumeration of all standing trees e"30cm girth at breast height (gbh). Tree seedlings/ saplings, shrubs and climbers/ lianas were enumerated from one 5 m X 5 m plot within the quadrate. Quantitative data in respect of herb

species were collected from one small sample plot measuring 1m X 1 m.

For each species, data on number of individual in each quadrate, associated taxa, phenology, regeneration potential, maturity classes and local uses were also recorded. While enumerating tree species, measurements of height and DBH were noted. In case of herbaceous elements where counting of number of individuals was impracticable, percentage of area covered by each taxon was calculated. The density, frequency, dominance of each species was derived using standard ecological methods. Quantitative data was collected in data sheets designed depending on the requirement of the project.

3.3.2.Data analysis

For tree species in the wild, phytosociological characteristics of plant communities like; a) Frequency (percent of all transects in which a species was present), b) density (ratio of total number of trees to total number of transects) and c) abundance (ratio of total number of trees to total number of transects of occurrence) were recorded. Importance Value Index (IVI) was calculated following Curtis and Macintosh (1950). The dominance was determined by Simpson's index (Cd =Σ(ni/ N)2, and diversity as Shannon's Index (H = -Σ(ni/N) log (ni/N), where n*i* = importance value index of species i, N= sum of importance value index for the community. Evenness Index was calculated by Pielou's index (D= -Σ pi2 / In S), where *S* is the species richness of the community (Magurran, 1988). The girth (GBH) was converted into basal area (BA) as BA= GBH2/4ð for deriving biodiversity indices.

4. Results and discussion

4.1. Study of flora and vegetation

4.1.1.Natural vegetation

The flora of Chandaka-Dampada Sanctuary was observed to be moderately diverse with intimate mixture of evergreen and deciduous elements. The area comes under semi-evergreen forest zone but the interplay of biotic factors has changed the original character of the vegetation. The resultant secondary growth is stunted in nature, which seldom goes beyond 10m in height, There is preponderance of thorny bushes and weed growth *(Chromolaena odorata)* as under-storey in the forest, wherever there are permanent gaps. Species composition is heterogeneous. Thorny bamboo *(Bambusa bambos)* occurs as pure formations in valleys and mixed with tree growth on hill slopes. Based on the species composition, the natural forests can be broadly classified under "Moist Bamboo brakes", "Secondary moist bamboo brakes", "Lateritic semi evergreen forests", "Peninsular (coastal) Sal forests", "Northern Secondary moist mixed deciduous forests" and "Tropical Dry evergreen forests" as per the forest type classification of Champion & Seth (1968). However, (i) Lateritic semi-evergreen forests dominated by *Xylia xylocarpa* (ii) Moist bamboo brakes and (iii) Secondary sal forests are the three dominant types in the sanctuary.

Most part of the sanctuary is covered with lateritic semi-evergreen forests characterised by the presence of *Xylia xylocarpa*, which does not form homogenous crop and all the trees are found in pole stage due to lot of biotic pressure on the forest over past years. The chief associates of *Xylia* are *Lagerstroemia parviflora, Strychnos nux-vomica, Aegle marmelos, Bridelia retusa, Pterocarpus marsupium, Madhuca latifolia, Alangium salvifolium, Terminalia bellirica, Careya arborea, Cassia fistula* etc. The second storey is composed of a number of small trees and shrubs, of which *Suregada multiflora, Polyalthia cerasoides, Cipadessa baccifera, Helicteres isora, Tarenna asiatica* and *Holarrhena pubescens* deserve special mention. Usually, climbers are plenty and are represented by *Combretum roxburghii, Gouania leptostachya, Ichnocarpus frutescens, Spatholobus parviflorus, Hemidesmus indicus, Ampelocissus latifolia, Argyreia nervosa, Atylosia scarabaeoides, Lygodium flexuosum* and many such others. Scattered trees of mango, figs (*Ficus* spp.) and clumps of spiny bamboo *Bambusa bambos* are also not uncommon. The only gymnospermous member *Cycas sphaerica* is sparsely distributed throughout the drier regions. Several herbaceous taxa like *Desrnodium gangeticum, Dicliptera bupleuroides var. roxburghiana, Eranthemum capense, Phaulopsis imbricata* and *Peristrophe paniculata* in association with some grasses and sedges compose the vegetation of forest floor. Along shady streams, *Terminalia arjuna, Barringtonia acutangula* and *Diospyros malabarica* occur as a narrow fringe. In disturbed areas, however, alien weeds like *Chromolaena odorata*, *Lantana camara* var. *aculeata, Cassia tora* and *Parthenium hysterophorus* etc. occupy the place of other herbs and adversely affect the indigenous flora.

On exposed sand-stone hills, *Xylia xylocarpa, Vitex pinnata, Lepisanthes tetraphylla, Strychnos nux-vomica, Pterospermum xylocarpum* and *Diospyros montana* in association with shrubs like *Eugenia rothii, Hugonia mystax, Tarenna asiatica* and *Diospyros ferrea* form an interesting plant community.

Few patches of mixed sal forests of secondary nature are scattered in the sanctuary area on drier habitats where the soil is clayey and with grits and pebbles. The Sal (*Shorea robusta*) occurring here is of coppiced origin and plants hardly reach more than 10 m. in height. *Soymida febrifuga,*

Table 2

Comparative list of ten dominant angiosperm plant families in Chandaka Dampada Sanctuary, erstwhile Puri district, Odisha and British-India

Chandaka Dampada Sanctuary (Present work)	Puri district (Undivided) (Panda & Patnaik, 1997)	Orissa (Saxena & Brahmam, 1994-96)	British India (Hooker, 1904)
Fabaceae (86 spp.)	Fabaceae	Fabaceae	Orchidaceae
Poaceae (59 spp.)	Poaceae	Poaceae	Fabaceae
Euphorbiaceae (34 spp.)	Cyperaceae	Cyperaceae	Poaceae
Acanthaceae (28 spp.)	Euphorbiaceae	Orchidaceae	Rubiaceae
Rubiaceae (29 spp.)	Acanthaceae	Asteraceae	Euphorbiaceae
Asteraceae (21 spp.)	Rubiaceae	Euphorbiaceae	Acanthaceae
Cyperaceae (21spp.)	Asteraceae	Rubiaceae	Asteraceae
Scrophulariaceae (17 spp.)	Scrophulariaceae	Acanthaceae	Cyperaceae
Convolvulaceae (16 spp.)	Convolvulaceae	Scrophulariaceae	Lamiaceae
Amaranthaceae (14 sp.)	Lamiaceae	Lamiaceae	Urticaceae & Verbenaceae

Table 3

Ten dominant angiosperm plant families in Chandaka-Dampada Sanctuary with number of genera, species and habit category.

Family	No. of species	No. of genera	Trees	Herbs	Shrubs	Climbers
Fabaceae (including Caesalpiniaceae and Mimosaceae)	86	47	28	26	17	15
Poaceae	59	43	2	57	0	0
Euphorbiaceae	34	17	11	16	7	0
Acanthaceae	28	20		22	5	1
Rubiaceae	29	20	12	14	3	0
Asteraceae	21	21	0	20	1	0
Cyperaceae	21	9	0	21	0	0
Scrophulariaceae	17	9	0	17	0	0
Convolvulaceae	16	6	0	7	1	8
Amaranthaceae	14	8	0	14	0	0

Table 4

Habit-wise classification of plant species occurring in Chandaka-Damapada Sanctuary

Life forms	No. of species	No. of genera	No. of families
Trees	161	123	47
Shrubs	93	70	42
Climbers	69	57	27
Herbs	332	236	66
Total	655	454	119

Table 5

Diversity of different plant groups representing the flora of Chandaka Dampada Sanctuary

Plant groups	Genera	Species	Families
Angiosperms			
Monocot	93	129	20
Dicototyledons	357	522	95
Gymnosperms	1	1	1
Pteridophytes	3	3	3
Total	454	655	119

Table 6

Biodiversity parameters of tree species enumerated from Chandaka Wildlife Sanctuary

No. of tree species	113
No. of genera	83
No. of families	36
Number of individuals	2108
Stand Density (No. of stems ha-1)	222.21
Total Basal Area (m^2)	34.52
Stand Basal Area (m^2 ha-1)	11.2
Maximum tree GBH (cm)	120
Mean tree GBH (cm)	43.7
Shannon-Weiner Index	3.96
Simpson Index	0.03
Evenness Index	0.83

Acacia leucophloea, Cleistanthus collinus, Diospyros melanoxylon, Chloroxylon swietenia, Phyllanthus emblica, Woodfordia fruticosa, Albizia odoratissima, Acacia pennata etc. are found in association with sal. The under growth has bushes of *Phoenix acaulis, Flacourtia indica, Diospyros chloroxylon, Carissa spinarum* and the ground flora is composed of *Pentanema indicum, Blumeopsis flava, Isleima prostratum, Cynodon dactylon, Digitaria granularis* and *Atylosis scarabaeoides.* The two leafless total parasites found in profusion here are *Cassytha filiformis* and *Cuscuta reflexa*. Plantations of cashew (*Anacardium occidentale*) exist at several locations.

Moist bamboo brakes dominated by a single species of thorny bamboo Bambusa bambos are found in the western portion of the sanctuary on slopes and sheltered localities. The bamboos form dense clumps and hardly allow any other plant to come up underneath except few bulbous perennials, shrubs and grasses. There is apparently no weed growth on the forest floor due to low light and sufficient litter deposition. However, *Chromolaena odorata, Cassia tora, Cassia occidentalis,* and *Lantana camara* var. *aculeata* are the weeds invading the area where the canopy is considerably open. Bulbous or cormous perennials like Curcuma sp., *Geodorum densiflorum, Costos speciosus, Amorphophallus paeoniifolius* and *Asparagus racemosus* inhabit the floor and send off shoots with the first conventional shower of rain. They are very often associated with shade tolerant species viz. *Antidesma ghaesernbilla, Clerodendrum viscosum, Oplismenus compositus* and *Oplismenus burmanii*. Straggling amongst the clumps of bamboo are the climbers *Dioscorea wallichii, Smilax zeylanica, Abrus precatorius, Calamus viminalis, Cayratia trifolia* and few others.

Several groves of mango are seen near human settlements, a little away from core area of the sanctuary. *Scindapsus officinalis*, *Vanda* spp., *Acampe praemorsa* and *Dendrophthoe falcata* are the epiphytes on mango trees. Several acres of land have been brought under plantation in recent years besides the existing old plantation.

4.1.2.Statistical analysis of the flora

An exhaustive floristic survey of Chandaka Dampada Sanctuary has revealed the occurrence of 655 species of angiosperms belonging to 454 genera under 119 families. Table-2 & 3 give the comparative account of 10 dominant angiospermic plant families in the British-India, Bihar-Orissa, and undivided Puri District and Chandaka Dampada Sanctuary and their classification according to habit. It is observed that Fabaceae (including Papilionaceae, Caesalpiniaceae and Mimosaceae) and Poaceae occupy first two positions in order of species diversity in the flora of Chandaka, Puri District and Orissa. However, in British-India, the first position is taken by Orchidaceae, which does not figure in the list of 10 dominant plant families either in the present work but occupy fourth position in the State flora for Odisha. Asteraceae (Compositae), the largest family in the world adorns 6th position in Chandaka and 7th in Puri district. The Convolvulaceae, which does not find a place in the list for British India occupies 9th position in the flora of the region under reference. In general, the dominance of angiospermic plant families in Chandaka Sanctuary is very much similar to that recorded for the flora of Orissa except for the family Orchidaceae (only 5 species in Chandaka), the members of which are predominantly forest dwellers. The habit-wise distribution of species, genera and families are presented in Table-4.

The ratio of monocot families to dicot families; monocot genera to dicot genera and monocot species to dicot species are 1:4.75, 1:3.839 and 1:4.046 respectively. A statistical analysis of the flora shows the percentage of dicotyledonous and monocotyledonous plants as 75.29: 24.71 as against 81.30: 18.70 in the World flora. This shows relatively less number of monocotyledonous plant species in the flora of Chandaka as compared to flora of Odisha where this ratio is 70.75: 29.25. This is mainly due to the occurrence of large number of grasses and sedges in the flora. Of the 129 species of monocotyledonous plants collected during the present work, 80 species belong to two families namely Poaceae and Cyperaceae and the remaining 18 monocot families are represented by only 49 species. The genus-species ratio is 1: 1.443 as against 1: 1.7 for undivided Puri District, 1: 2.59 for Orissa and 1:7 for British-India. Evidently, therefore, in Chandaka region less number of species occurs under a particular genus in comparison to

Table 7

No. of individuals, GBH, basal area, density, frequency, dominance and IVI of tree species in Chandaka Wildlife Sanctuary

Name of the Taxon	Family	No. of Individuals	Total GBH (cm)	Total Basal area in m2	Frequency	Density	Relative Frequency	Relative Density	Relative Dominance	IVI
Xylia xylocarpa	Mimosaceae	279	12045	4.370	58.442	90.584	5.085	13.235	12.659	30.979
Diospyros sylvatica	Ebenaceae	104	4340	1.548	54.545	33.766	4.746	4.934	4.485	14.164
Aegle marmelos	Rutaceae	107	4214	1.388	54.545	34.740	4.746	5.076	4.021	13.842
Careya arborea	Barringtoniaceae	70	3653	1.651	40.260	22.727	3.503	3.321	4.783	11.606
Strychnos potatorum	Strychnaceae	82	3579	1.327	41.558	26.623	3.616	3.890	3.845	11.351
Tectona grandis	Verbenaceae	92	4335	1.756	18.182	29.870	1.582	4.364	5.086	11.033
Strychnos nux-vomica	Strychnaceae	85	3749	1.391	33.766	27.597	2.938	4.032	4.030	11.000
Lagerstroemia parviflora	Lythraceae	83	3328	1.158	38.961	26.948	3.390	3.937	3.354	10.681
Holarrhena pubescens	Apocynaceae	56	2320	0.793	45.455	18.182	3.955	2.657	2.296	8.908
Cassia fistula	Caesalpiniaceae	49	2033	0.714	36.364	15.909	3.164	2.324	2.068	7.556
Terminalia bellirica	Combretaceae	49	2191	0.826	32.468	15.909	2.825	2.324	2.394	7.543
Terminalia alata	Combretaceae	48	2227	0.901	16.883	15.584	1.469	2.277	2.611	6.357
Mangifera indica	Anacardiaceae	37	2236	1.104	15.584	12.013	1.356	1.755	3.199	6.310
Alangium salvifolium	Alangiaceae	35	1618	0.673	23.377	11.364	2.034	1.660	1.949	5.643
Azadirachta indica	Meliaceae	32	1548	0.634	25.974	10.390	2.260	1.518	1.837	5.614
Polyalthia cerasoides	Annonaceae	44	1749	0.584	20.779	14.286	1.808	2.087	1.692	5.587
Syzygium cumini	Moraceae	29	1424	0.601	24.675	9.416	2.147	1.376	1.741	5.263
Pterospermum xylocarpum	Sterculiaceae	33	1446	0.539	23.377	10.714	2.034	1.565	1.560	5.160
Madhuca indica	Sapotaceae	38	1835	0.764	12.987	12.338	1.130	1.803	2.214	5.147
Lannea coromandelica	Anacardiaceae	24	1143	0.490	24.675	7.792	2.147	1.139	1.420	4.705
Streblus asper	Moraceae	31	1376	0.513	19.481	10.065	1.695	1.471	1.487	4.653
Lagerstroemia reginae	Lythraceae	27	1247	0.479	20.779	8.766	1.808	1.281	1.389	4.477
Bridelia retusa	Euphorbiaceae	25	1168	0.475	16.883	8.117	1.469	1.186	1.376	4.031
Zizyphus xylopyrus	Rhamnaceae	25	1052	0.374	19.481	8.117	1.695	1.186	1.084	3.964
Vitex pinnata	Verbenaceae	40	1514	0.478	6.494	12.987	0.565	1.898	1.384	3.847
Diospyros montana	Ebenaceae	25	1001	0.338	18.182	8.117	1.582	1.186	0.978	3.746
Naringi crenulata	Rutaceae	25	934	0.287	19.481	8.117	1.695	1.186	0.832	3.712
Cleistanthus collinus	Euphorbiaceae	32	1221	0.379	9.091	10.390	0.791	1.518	1.099	3.408
Pongamia pinnata	Fabaceae	21	1126	0.510	10.390	6.818	0.904	0.996	1.477	3.377
Acacia leucophloea	Mimosaceae	21	900	0.353	14.286	6.818	1.243	0.996	1.023	3.262
Suregada multiflora	Euphorbiaceae	20	763	0.248	18.182	6.494	1.582	0.949	0.718	3.249
Buchanania lanzan	Anacardiaceae	24	1112	0.440	9.091	7.792	0.791	1.139	1.276	3.205
Terminalia chebula	Combretaceae	18	803	0.302	16.883	5.844	1.469	0.854	0.876	3.198
Diospyros melanoxylon	Ebenaceae	15	624	0.214	16.883	4.870	1.469	0.712	0.621	2.802
Antidesma ghaesembilla	Euphorbiaceae	18	805	0.312	11.688	5.844	1.017	0.854	0.903	2.774
Catunaregam spinosa	Rubiaceae	20	699	0.200	14.286	6.494	1.243	0.949	0.579	2.771
Shorea robusta	Dipterocarpaceae	17	1061	0.542	2.597	5.519	0.226	0.806	1.571	2.604
Casearia elliptica	Flacourtiaceae	15	554	0.170	15.584	4.870	1.356	0.712	0.492	2.559
Xantolis tomentosa	Sapotaceae	18	773	0.273	9.091	5.844	0.791	0.854	0.792	2.437
Lepisanthes tetraphylla	Sapindaceae	13	507	0.163	14.286	4.221	1.243	0.617	0.472	2.332

Mimusops elengi	Sapotaceae	17	886	0.380	3.896	5.519	0.339	0.806	1.100	2.245
Dalbergia paniculata	Fabaceae	14	719	0.326	6.494	4.545	0.565	0.664	0.945	2.174
Memecylon umbellatum	Melastomataceae	16	581	0.172	10.390	5.195	0.904	0.759	0.499	2.162
Canthium dicoccum	Rubiaceae	14	484	0.136	7.792	4.545	0.678	0.664	0.394	1.736
Semecarpus anacardium	Anacardiaceae	11	508	0.195	6.494	3.571	0.565	0.522	0.564	1.651
Gmelina arborea	Verbenaceae	10	495	0.204	5.195	3.247	0.452	0.474	0.591	1.517
Albizia lebbeck	Mimosaceae	8	376	0.154	7.792	2.597	0.678	0.380	0.445	1.503
Morinda pubescens	Rubiaceae	9	367	0.122	6.494	2.922	0.565	0.427	0.353	1.345
Hymenodictyon orixense	Rubiaceae	6	330	0.156	5.195	1.948	0.452	0.285	0.451	1.188
Samanea saman	Mimosaceae	9	438	0.182	2.597	2.922	0.226	0.427	0.528	1.181
Ochna obtusata	Ochnaceae	6	211	0.060	7.792	1.948	0.678	0.285	0.173	1.135
Cipadessa baccifera	Meliaceae	8	241	0.058	6.494	2.597	0.565	0.380	0.167	1.112
Phyllanthus emblica	Euphorbiaceae	7	248	0.072	6.494	2.273	0.565	0.332	0.209	1.106
Vitex leucoxylon	Verbenaceae	6	255	0.087	6.494	1.948	0.565	0.285	0.252	1.101
Chloroxylon swietiana	Rutaceae	10	395	0.137	2.597	3.247	0.226	0.474	0.396	1.096
Cassia siamea	Caesalpiniaceae	6	295	0.117	3.896	1.948	0.339	0.285	0.338	0.962
Anogeissus acuminata	Combretaceae	5	230	0.090	3.896	1.623	0.339	0.237	0.262	0.838
Gliricidia sepium	Fabaceae	3	213	0.120	3.896	0.974	0.339	0.142	0.349	0.830
Olax psittacorum	Olacaceae	4	149	0.045	5.195	1.299	0.452	0.190	0.130	0.772
Wrightia arborea	Apocyanaceae	4	128	0.033	5.195	1.299	0.452	0.190	0.095	0.736
Guazuma ulmifolia	Sterculiaceae	4	174	0.064	3.896	1.299	0.339	0.190	0.184	0.713
Limonia acidissima	Rutaceae	5	166	0.044	3.896	1.623	0.339	0.237	0.128	0.704
Tamarindus indica	Caesalpiniaceae	4	215	0.094	2.597	1.299	0.226	0.190	0.271	0.687
Diospyros ferrea	Ebenaceae	3	143	0.055	3.896	0.974	0.339	0.142	0.158	0.640
Anacardium occidentale	Anacardiaceae	3	133	0.048	3.896	0.974	0.339	0.142	0.139	0.621
Fagerlindia fasciculata	Rubiaceae	4	124	0.031	3.896	1.299	0.339	0.190	0.089	0.617
Simarouba glauca	Simmaroubaceae	4	173	0.062	2.597	1.299	0.226	0.190	0.179	0.594
Dalbergia sissoo	Fabaceae	3	115	0.036	3.896	0.974	0.339	0.142	0.104	0.586
Gardenia latifolia	Rubiaceae	3	109	0.033	3.896	0.974	0.339	0.142	0.096	0.577
Mitragyna parvifolia	Rubiaceae	3	108	0.031	3.896	0.974	0.339	0.142	0.091	0.572
Ixora pavetta	Rubiaceae	3	93	0.023	3.896	0.974	0.339	0.142	0.066	0.548
Polyalthia suberosa	Annonaceae	3	133	0.048	2.597	0.974	0.226	0.142	0.139	0.508
Haldinia cordifolia	Rubiaceae	3	128	0.044	2.597	0.974	0.226	0.142	0.127	0.495
Anacardium occidentale	Anacardiaceae	4	169	0.057	1.299	1.299	0.113	0.190	0.166	0.468
Pterocarpus marsupium	Fabaceae	3	113	0.034	2.597	0.974	0.226	0.142	0.099	0.467
Neolamarckia cadamba	Rubiaceae	2	107	0.046	2.597	0.649	0.226	0.095	0.132	0.453
Bridelia monoica	Euphorbiaceae	2	99	0.042	2.597	0.649	0.226	0.095	0.123	0.443
Terminalia arjuna	Combretaceae	3	96	0.025	2.597	0.974	0.226	0.142	0.071	0.439
Cleistanthus patulus	Euphorbiaceae	3	152	0.062	1.299	0.974	0.113	0.142	0.180	0.435
Stereospermum chelonoides	Bignoniaceae	2	83	0.028	2.597	0.649	0.226	0.095	0.081	0.402
Salacia chinensis	Hippocrateaceae	2	82	0.027	2.597	0.649	0.226	0.095	0.078	0.399
Miliusa velutina	Annonaceae	3	120	0.039	1.299	0.974	0.113	0.142	0.114	0.369
Ehretia laevis	Ehretiaceae	2	62	0.015	2.597	0.649	0.226	0.095	0.044	0.365
Vitex peduncularis	Verbenaceae	2	62	0.015	2.597	0.649	0.226	0.095	0.044	0.365
Diospyros malabarica	Ebenaceae	2	60	0.014	2.597	0.649	0.226	0.095	0.041	0.362

Manilkara hexandra	Sapotaceae	2	60	0.014	2.597	0.649	0.226	0.095	0.041	0.362
Grewia tiliifolia	Tiliaceae	2	100	0.040	1.299	0.649	0.113	0.095	0.115	0.323
Litsea glutinosa	Lauraceae	2	91	0.033	1.299	0.649	0.113	0.095	0.096	0.304
Discospermum abnorme	Rubiaceae	4	125	0.031	0.000	1.299	0.000	0.190	0.090	0.280
Litsea monopetala	Lauraceae	1	61	0.030	1.299	0.325	0.113	0.047	0.086	0.246
Ficus glomerata	Moraceae	1	57	0.026	1.299	0.325	0.113	0.047	0.075	0.235
Cassine albens	Celastraceae	1	47	0.018	1.299	0.325	0.113	0.047	0.051	0.211
Vitex glabrata	Verbenaceae	1	47	0.018	1.299	0.325	0.113	0.047	0.051	0.211
Aglaia elaeagnoidea	Meliaceae	1	43	0.015	1.299	0.325	0.113	0.047	0.043	0.203
Barringtonia acutangula	Barringtoniaceae	1	42	0.014	1.299	0.325	0.113	0.047	0.041	0.201
Bauhinia purpurea	Caesalpiniaceae	1	42	0.014	1.299	0.325	0.113	0.047	0.041	0.201
Protium serratum	Burseraceae	1	39	0.012	1.299	0.325	0.113	0.047	0.035	0.195
Atalantia monophylla	Rutaceae	1	37	0.011	1.299	0.325	0.113	0.047	0.032	0.192
Diospyros chloroxylon	Ebenaceae	1	37	0.011	1.299	0.325	0.113	0.047	0.032	0.192
Mallotus philippensis	Euphorbiaceae	1	35	0.010	1.299	0.325	0.113	0.047	0.028	0.189
Ficus benghalensis	Moraceae	1	34	0.009	1.299	0.325	0.113	0.047	0.027	0.187
Acacia arabica	Mimosaceae	1	32	0.008	1.299	0.325	0.113	0.047	0.024	0.184
Pavetta indica	Rubiaceae	1	32	0.008	1.299	0.325	0.113	0.047	0.024	0.184
Acacia auriculiformis	Mimosaceae	1	31	0.008	1.299	0.325	0.113	0.047	0.022	0.183
Acacia nilotica	Mimosaceae	1	31	0.008	1.299	0.325	0.113	0.047	0.022	0.183
Crateva magna	Capparaceae	1	31	0.008	1.299	0.325	0.113	0.047	0.022	0.183
Diospyros ovalifolia	Ebenaceae	1	31	0.008	1.299	0.325	0.113	0.047	0.022	0.183
Manilkara zapota	Sapotaceae	1	31	0.008	1.299	0.325	0.113	0.047	0.022	0.183
Alstonia scholaris	Apocyanaceae	1	30	0.007	1.299	0.325	0.113	0.047	0.021	0.181
Antidesma acidum	Euphorbiaceae	1	30	0.007	1.299	0.325	0.113	0.047	0.021	0.181
Erythrina suberosa	Fabaceae	1	30	0.007	1.299	0.325	0.113	0.047	0.021	0.181
Meyna spinosa	Rubiaceae	1	30	0.007	1.299	0.325	0.113	0.047	0.021	0.181
Ziziphus rugosa	Rhamnaceae	1	30	0.007	1.299	0.325	0.113	0.047	0.021	0.181

Table 8

Girth class distribution according to density and basal area of tree species

Girth Class	Density (Stem ha-1)	Basal Area (m^2 ha-1)
30-60 cm	618.83	8.613
61-90 cm	63.63	2.445
91-120 cm	1.94	0.149

Puri District or Orissa. A summary of representative plant groups in the flora of the region is shown in Table-5.

4.1.3.Special groups of plants

(a) Threatened plants

Chandaka-Damapada Sanctuary harbours a number of endangered species of Odisha which includes species such as *Uvaria lurida, Uvaria hamiltonii, Cycas sphaerica, Pterocarpus marsupium, Alphonsea maderaspatana, Litsea glutinosa, Salacia chinensis* and *Oroxylum indicum.*

(b) Parasites

Although a few species of parasitic angiosperms are found to occur in and around the sanctuary, they are an interesting group of plants from ecological stand-point. The parasites occurring in the region belong to two distinct types viz. stem parasites and root parasites and may be total or partial parasites. The stem parasites are represented by *Cuscuta reflexa, Cassytha filiformis, Dendrophthoe falcata* and *Viscum articulatum*, the first two being leafless total parasites. Besides, *Striga angustifolia* is the only inconspicuous root parasite found in the region.

(c) Insectivorous plants

Drosera burmanii and *Utricularia aurea* are the two

common insectivorous plants found in the area. While the first one is found in sandy soils close to rice fields, the later is common in ponds, ditches and shallow water bodies.

(d) Wild relatives of crop plants:

Several wild progenitors of cultivated plants also occur within Chandaka sanctuary which are important as germplasm materials for conservation and use in crop improvement programmes. Some important wild relatives of crop plants known to occur in the egion are *Oryza rufipogon*, *Atylosia scarabaeoide*, *Dolichos trilobus*, *Vigna sublobata, Vigna trilobata*, *Abelmoschus manihot* ssp. *tetraphyllum, Solanum virginianum*, *Solanum nigrum*, *Corchorus aestuans*, *Saccharum spontaneum* etc.

(e) Epiphytes

Three species of wild orchids such as *Acampe praemorsa, Cymbidium aloifolium* and *Vanda tessellata* were found to occur in the sanctuary area. While most of these orchids were observed on mango (*Mangifera indica*) trees, *Scindapsus officinalis* grows as an epiphyte on a number of host plants and on rock surfaces. *Schefflera venulosa* was a robust epiphyte growing along streams and in damp places.

(f) Lithophytes

Sarcostemma acidum and *Anisochilus carnosus* grow as lithophytes on barren rock surfaces in selected habitats of the sanctuary and sustain themselves with whatever little amount of organic matter available the form of deposition in the crevices of rocks. *Tephrosia maxima* occur on exposed rocks and apparently behave as a lithophyte.

(g) Wild plants of horticultural importance

A large number of wild plants with beautiful flowers, foliage or interesting crown characters have great potentiality to be used as garden ornamentals. Some of them which occur in Chandaka and can be introduced as garden ornamentals are: *Crotalaria verrucosa*, *Crotalaria laburnifolia, Crotalaria spectabilis*, *Carissa spinarum*, *Pavetta indica, Gloriosa superba, Calycopteris floribunda, Dalbergia rubiginosa, Ichnocarpus frutescens, Sarcostemma acidum* etc.

4.2. Quantitative assessment of tree species

4.2.1.Species diversity and richness

A total number of 2108 individuals of tree species with e" 30 cm GBH were recorded from 79 sample quadrates (39.5 ha) of Chandaka-Domapada Sanctuary. They represent 113 species belonging to 83 genera under 36 families (Table-6). The family Fabaceae (including Papilionaceae, Mimosaceae and Caesalpiniaceae) with 28 species was the most dominant taxon in terms of species content followed by Euphorbiaceae (10 species), Rubiaceae (10 species), Verbenaceae (8 species) and Moraceae (8 species). Eighteen (18) families were represented by single species only. The values of diversity indices such as Shannon-Weiner Index, Simpson Index and Evenness Index were calculated as 3.962, 0.03 and 0.83 respectively.

4.2.2.Importance Value Index (IVI)

The IVI depicts the sociological structure of a species in its totality in the community. Though the Tropical deciduous forests of Odisha state are dominated by the *Shorea robusta* (Sal), for Chandaka Damapada Sanctuary *Xylia xylocarpa* scored the highest IVI of 30.979 pointing at the dominance of the species in terms of density, basal area and frequency of occurrence (Table-7). The top 10 species and their contribution to density, basal area and IVI in studied area were *Xylia xylocarpa* (IVI=30.979), *Diospyros sylvatica* (IVI=14.164), *Aegle marmelos* (IVI=13.842), *Careya arborea* (IVI=11.606), *Strychnos potatorum* (IVI=11.351), *Tectona grandis* (IVI=11.033), *Strychnos nux-vomica* (IVI=11.000), *Lagestroemia parviflora* (IVI=10.681), *Holarrhena pubescens* (IVI=8.908) and *Cassia fistula* (IVI=7.556). Twenty-four species were represented by single individuals and are considered rare in the area.

4.2.3.Family composition

A total number of 2108 standing trees with e" 30 cm GBH were recorded from 79 sample quadrates (39.5 ha) of Chandaka- Domapada Sanctuary, which come under 113 species under 83 genera and 36 families. In terms of tree density, Mimosaceae with 7 species and maximum of 320 trees dominated the area followed by Strychnaceae (167 stems), Verbenaceae (151 stems), Ebenaceae (151 stems), Rutaceae (148 stems), Combretaceae (123 stems) and Lythraceae (110 stems). Mimosaceae scored the maximum FIV of 30.099 followed by Verbenaceae (FIV=19.881), Ebenaceae (FIV=19.695), Euphorbiaceae (FIV=17.791) and Strychnaceae (FIV=17.567).

4.2.4.Stand density, basal area and girth class distribution

A total of 2108 trees were enumerated from on the study sites of Chandaka-Dampara sanctuary and the total basal area (BA) was found to be 34.517 sq. m. The stand density was calculated as 222.211 stems/ ha. The stand density and basal area showed decreasing trend with increasing girth class in Chandaka (Table-8). The highest stem density of 618.83 stems/ ha and basal area of 8.613 sq.

m/ ha was recorded for lowest girth class of 30-60 cm GBH. The lowest stem density of 1.94 stems/ ha and basal area of 0.149 sq. m/ ha was calculated for trees of lowest girth class (91-120 cm).

The density, abundance and distribution of individual species are measurable indicators of plant diversity (Wattenberg and Breckle, 1995). The species richness of 113 tree species over 39.50 ha sampled area in three forest ranges of Chandaka- Damapada Sanctuary reflects a moderate level of diversity. The result of the study compared well with other large-scale inventories conducted in tropical forests both in India and elsewhere. For example, 63 species were recorded for 50 ha plot at Mudumalai Forest Reserve, India, to 272 species in the 60 ha area in southern Eastern Ghats (Arul Pragasan and Parthasarathy, 2010).

The stand density of 222.211 stems/ ha now reported for Chandaka is much lower than reported for other tropical forests of Indian subcontinent such as 516.23 stems ha^{-1} for Nayagarh Fortest Division (Sahoo & Panda, 2015; Sahoo et al., 2017), 352 stems ha^{-1} in northern Eastern Ghats (Panda *et al*., 2013); 443 stems ha-1 in Malyagiri hills of Odisha (Sahu *et al*., 2012); 298 stems ha”1 at Mudumalai Forest Reserve, India and 689 stems ha”1 at Sinharaja, Sri Lanka (Condit, 2000). Density of trees (30 cm GBH and above) in tropical forests ranges between 245 and 859 (Ashton 1964; Campbell *et al*., 1992; Richards, 1996) but the mean stand density of trees now reported for Chandaka Damapada Sanctuary (222.211 stems ha^{-1}) is less than the reported range for tropical forests of India.

The species diversity depends on the adaptation of species which increases with the stability of community and Shannon’s Index (H’) is generally higher for tropical forests (Knight, 1975). In Indian forests, the value is reported to vary in the range of 0.83 to 4.0 (Singh *et al*., 1984). In the present study, Shannon’s Index of diversity of tree species was found to be 3.962, which is within the reported range for the forests of Indian sub-continent (Ayyapan and Parthasarathy, 1999; Pandey, 2003; Panda *et al*., 2013). Comparison of diversity indices is very difficult because of the difference in the area sampled and lack of uniform plot dimensions. However, the index now determined is lower than the value reported for Northern Andhra Pradesh (Reddy *et al*., 2011), Niyamgiri hills, Odisha (Dash *et al*., 2009).

In most of the studies relating to vegetation composition and site quality of forests, basal area acts as an important attribute (Mani and Parthasarathy, 2005; Parthasarathy and Karthikeyan, 1997; Srinivas and Parthasarathy, 2000). The stand basal area recorded in the present study was calculated as 11.20 m^2 ha^{-1}, which is comparable to the value of 10.47 m2 m^2 ha^{-1} reported for southern Eastern GHat region (Panda et al., 2013) but lower that that known for Nayaharh Forest Division, which is 16.90 m2 m^2 ha^{-1} (Sahoo et al., 2017).

The diameter distribution reflects the disturbance effect within the forests (Denslow, 1995; Hett and Loucks, 1976) and helpful in detecting trends in regeneration patterns (Poorter *et al*., 1996). The low basal area values in all the sample plots in the present study revealed the extent of forest disturbance with poor representation of trees in higher girth class. Tree density decreased with increasing size class of trees indicates how well the growing forest is utilizing site resources. A few small-to-medium sized trees per hectare may imply that land is not being fully utilized by the tree crop (Hitimana et al., 2004). Quantitative floristic data from the present study will provide base-line information on distribution, richness and relative abundance of taxa for formulating management and conservation actions for Chandaka Damapada Sanctuary in general and other protected areas, in particular.

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Studies on for genetic polymorphism of 16 Odisha landraces of *Vigna mungo* (L.) Hepper for YMV resistance as revealed by RAPD marker

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ABSTRACT

Genetic variability of 16 blackgram (*Vigna mungo*) genotypes of the family Leguminaceae, one of the important pulses of Odisha, was was studied through RAPD markers. DNA marker analysis revealed an average variation of 42.61% polymorphism in banding patterns of the total score of 176 RAPD amplicons. Cluster analysis of RAPD markers showed that all the genotypes of blackgram studied from Odisha belongs to two distinct clusters i.e. cluster I and II. All the landraces showed narrow genetic diversity except cluster-II (BG-3 of Mahimunda and BG-4 of Berhampur). Among the 10 genotypes of cluster I, BG9, BG10 and BG11 showed close affinity collected from Panthnagar having highest divergence in BG9 which could be used as a potential source of genetically diverse germplasm of blackgram for improved crop breeding for developing YMV resistance.

1. Introduction

Vigna mungo commonly known as black gram of the family Leguminaceae is a bean grown in the Indian subcontinent. Blackgram is very nutritious and play an important role in human diet. However, crop loss in this crop is high due to Mungbean yellow mosaic virus (MYMV) caused by geminivirus transmitted through whitefly (*Bemicia tabaci*). The blackgram, mungbean and soybean are most seriously affected leguminous crops as reported by Verma and Singh (1986). MYMV disease symptoms shows irregular patches of yellow green spots on older leaves and young leaves of susceptible infected plants shows complete yellowish leaves.

Blackgram commonly known as Urd bean in India is a self pollinating diploid (2n=2x=22) annual crop having a genome size of approx. 574 Mbp. Improvement of blackgram against MYMV through breeding is quite difficult as MYMV is transmitted through white fly without s uniform procedure. Mostly, resistance is controlled by recessive genes. The limited success has made with development of disease free and high yielding varieties through conventional breeding. The cytogenetic and genetic mechanisms controlling the organization and evolution of genomes of *Vigna* species are not clearly understood. Hence, selection of land races and local genotypes using molecular markers linked to resistant genes should be an alternative and effective approach to overcome the inaccuracy in only field evaluation of morphological traits (Tanksley *et al*., 1989). Studies on disease resistant gene have indicated a high level of polymorphism and presence of SSRs at certain loci (Yu *et al*., 1996). SSR are highly polymorphic which are abundant as well as dispersed throughout the genome. Present study deals with SSR marker analysis in 15 land races of *Vigna mungo* collected from different localities of Odisha to estimate an preliminary study for YMV resistance genotypes if any suitable for future use in breeding purpose and MYMV marker development.

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2. Materials and Methods

2.1 Plant materials

Sixteen landraces of blackgram including local and established varieties were collected from different agro-ecological parts of Odisha (Table 1). Information regarding the varieties was collected using rural appraisal. The landraces were maintained in green house and experimental nursery of Department of Botany, Utkal University using standard nursery practices.

2.2. DNA isolation, purification and quantification

Nuclear DNA was isolated from young and juvenile leaves (2 g) of one month old seedlings using CTAB method (Saghai-Maroof *et al.*, 1984). The leaf collected from each landraces was ground to a fine powder form using liquid nitrogen with the help of motor and pestle and suspended in six volume of CTAB extraction buffer [2% CTAB,100mM Tris HCl (pH 8.0), 20 mM EDTA, 1.5M NaCl and 2% â-mercaptoethanol (v/v)]. The suspension was incubated in water bath at 60°C for 1-1.5h. An equal volume of chloroform:isomayl alcohol (24:1) was added to the suspension bringing down to room temperature and gently emulsify for 15 min and finally centrifused at 12000 rpm for 20 min at 4°C. The aqueous phase was transferred into new sterile centrifuge tube and DNA was precipitated with two third of its volume of chilled isopropanol. DNA was spooled or the pellet was transferred to 70% ethanol and washed and dried in room temperature. Dried DNA was dissolved in $T_{10}E_1$ (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) and treated with RNase A (Sigma 10 µm ml^{-1}) at 37°C for 1h. The DNA was purified by phenol: chloroform (1:1) extraction and precipitated in ethanol (2.5 volumes) in the presence of 0.3 M sodium acetate (pH 5.2). The DNA was spooled out, washed in 70 % ethanol, air-dried, dissolved in $T_{10}E_1$ buffer and its concentration was determined and diluted to 25 ng μl^{-1} using $T_{10}E_1$ buffer for use as a template for RAPD analysis with different primers (Tables 2).

2.3 PCR analysis and RAPD profile

RAPD profiles were generated by using primers (Table 2) in Polymerase Chain Reaction (PCR) following the standard protocol of Williams *et al.* (1990). A total 20 RAPD primers were used to generate amplified bands in PCR. Each amplification reaction mixture contained 25 ng of template DNA, 200 µM of each dNTPs, 25 ng of primer, 0.5 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 10 × Taq buffer (50 mM KCl, 10 mM Tris–HCl, 1.5 mM MgCl2, pH 9.0) in a final reaction volume of 25 µl. The reaction was carried out in a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer, USA). The first cycle consisted of denaturation (94°C) for 5 min, primer

Table 1

List of blackgram landraces collected from different districts of Odisha for genetic analysis.

Sl No.	Accession No.	Local Name	Place	District	Latitude/Longitude
1.	BG1	Bhawanipatna local	Bhawanipatna	Kalahandi	19.91°N 83.12°E
2.	BG2	Badamba local	Badamba	Cuttack	20.31°N 85.47°E
3.	BG3	Mahimunda local	Mahimunda	Balangir	20.72°N 83.48°E
4.	BG4	B-3-8-8 (Prasad)	OUAT, Berhampur	Berhampur	19.32°N 84.78°E
5.	BG5	Ujjala	OUAT, Berhampur	Berhampur	19.32°N 84.78°E
6.	BG6	Nayagarh local	Nayagarh	Nayagarh	20.12°N 85.10°E
7.	BG7	Berhampur local	Berhampur	Berhampur	19.32°N 84.78°E
8.	BG8	Kothagarh local	Kothagarh	Kandhamal	20.47°N 84.23°E
9.	BG9	PU-31	Pantnagar	Udham Singh Nagar	28.98°N 79.40°E
10.	BG10	PU-40	Pantnagar	Udham Singh Nagar	28.98°N 79.40°E
11.	BG11	PU-19	Pantnagar	Udham Singh Nagar	28.98°N 79.40°E
12.	BG12	Badachana local	Badachana	Jajpur	20.85°N 86.33°E
13.	BG13	Ranipeta local	Ranipeta	Gajapati	18.88°N 84.20°E
14.	BG14	Keonjhar Pejua	Keonjhar	Keonjhar	21.63°N 85.60°E
15.	BG15	Similiguda local	Similiguda	Koraput	18.85°N 82.73°E
16.	BG16	Soroda local	Soroda	Mayurbhanj	21.93°N 86.73°E

Table 2
RAPD profile and DNA polymorphism of 16 blackgram landraces.

Primer name	Sequence (5'- 3')	No. of bands amplified	No. of polymorphic bands	Polymorphic %	Size range of amplified bands
OPA08	GTCACGTAGG	12	5	41.66	200-800
OPA11	CAATCGCCGT	10	6	60.00	300-1200
OPA14	TCTGTGCTGG	8	5	62.50	200-1100
OPD-08	GTGTCCCCCA	7	4	57.14	300-800
OPD-12	CACCGTATCC	10	4	40.00	200-900
OPE03	CCAGATGCAC	6	2	33.33	200–1000
OPF08	GGGATATCGG	7	2	28.57	200–1200
OPF10	GGAAGCTTGG	4	2	50.00	200–900
OPF12	ACGGTACCAG	12	5	41.66	300–1300
OPF13	GGCTGCAGAA	10	3	30.00	250–1100
OPF16	GGAGTACTGG	5	2	40.00	300–800
OPW02	ACCCCGCCAA	11	3	27.27	150–1300
OPW03	GTCCGGAGTG	10	4	40.00	200–1350
OPW05	GGCGGATAAG	9	5	55.55	200–1100
OPW06	AGGCCCGATG	5	2	40.00	250–900
OPW08	GACTGCCTCT	11	4	36.36	250–1050
OPW11	CTGATGCGTG	10	4	57.14	300–900
OPN-04	GACCGACCCA	14	6	42.85	200-800
OPN-11	TCGCCGCAAA	8	5	62.50	100-1200
OPN-15	CAGCGACTGT	7	2	28.57	400-1300
		176	75	42.61	

annealing (42°C) for 1 min and DNA polymerization (72°C) for 2 min. In the next 40 cycles the period of denaturation was maintained at 92 °C for 1 min while the primer annealing and DNA polymerization was same as in the first cycle. The last cycle consisted of only primer extension (72°C) for 8 min. The amplified products were stored at 4°C and separated by electrophoresis on 1.5 % agarose gel in 1×TAE buffer for 2 h at 60 V. Gene Ruler 100 bp DNA ladder plus (MBI Fermantas, Lithuania) was used as the size standard to determine the size of the polymorphic fragments. DNA fragments were visualized by staining the gel with ethidium bromide and images were documented using Gel Doc G700 (BioRad, USA). Only those amplification products that appeared consistently in three replications were scored for further analysis.

2.4 Data analysis

The visualization of presence or absence of the bands was taken into consideration in RAPD analysis, but the differences in their intensity were ignored. Amplified bands of RAPD primers were scored as present (1) or absent (0) in each landraces for each set of primers and only clear and reproducible bands were used in this study. A binary matrix was obtained from the RAPD profile using NTSYS-pc programme (Rohlf, 2008). Binary matrix was transformed into a similarity matrix using Jaccard's coefficient. From this matrix a phylogenetic dendrogram was obtained by cluster analysis following the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method (Sneath and Sokal, 1973).

2.5 Principle component analysis (PCA)

The Jaccard's similarity matrix was subjected to principle component analysis. This coordination method makes use of multidimensional solution of the observed relationship. PCA resolves complex relationships into a function of fewer and simpler factor. Principal components were derived for each landraces using eigen vectors and

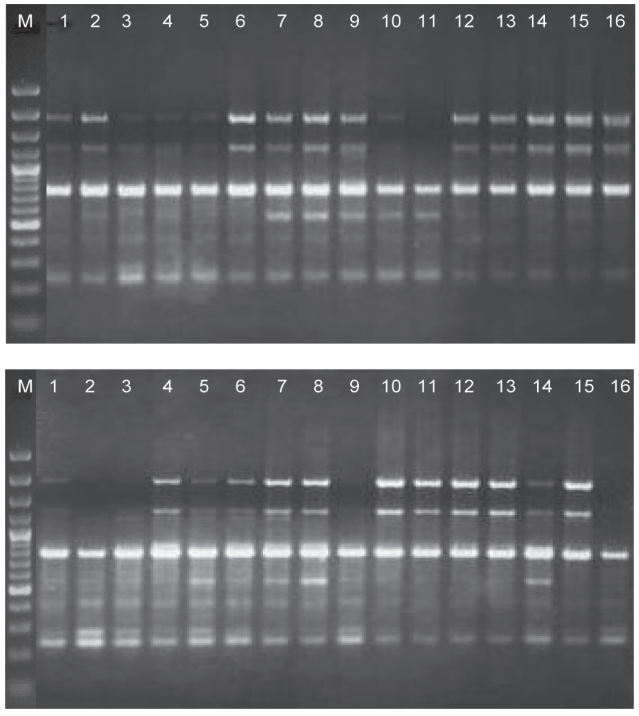

Fig. 1. RAPD profile of 16 land races of blackgram amplified with OPA-08 primer.

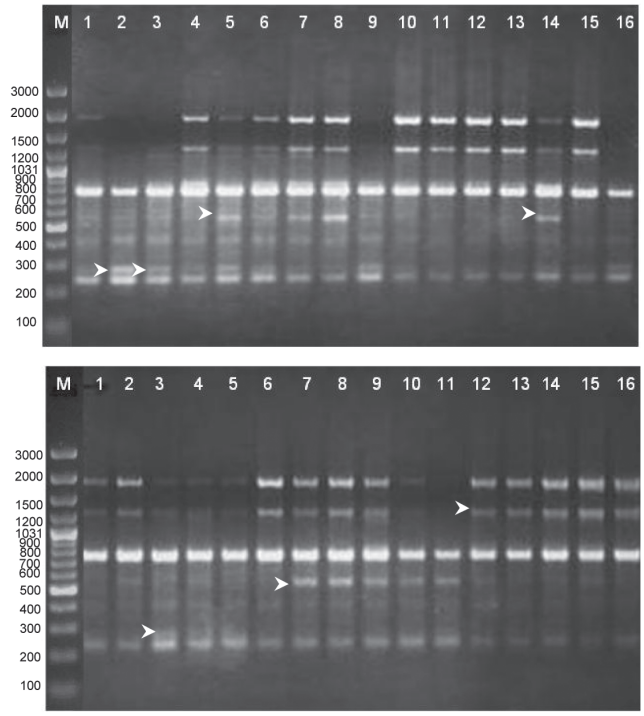

Fig. 2. RAPD profile of 16 land races of blackgram amplified with OPN-04 primer.

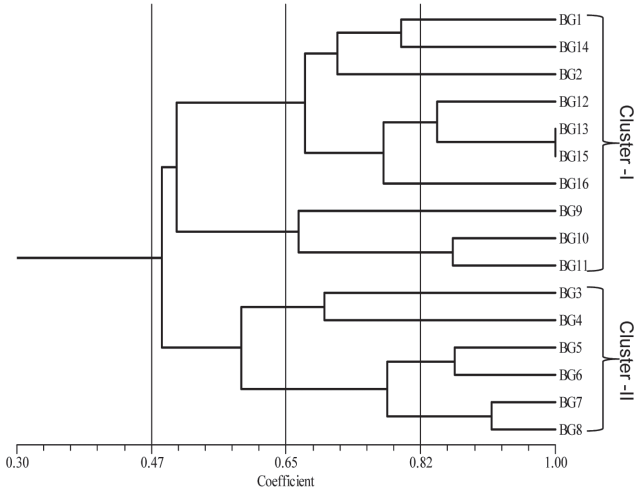

Fig. 3. Dendrogram of 16 land race of blackgram constructed using UPGMA based on Jacard's similarity coefficients for the RAPD data set.

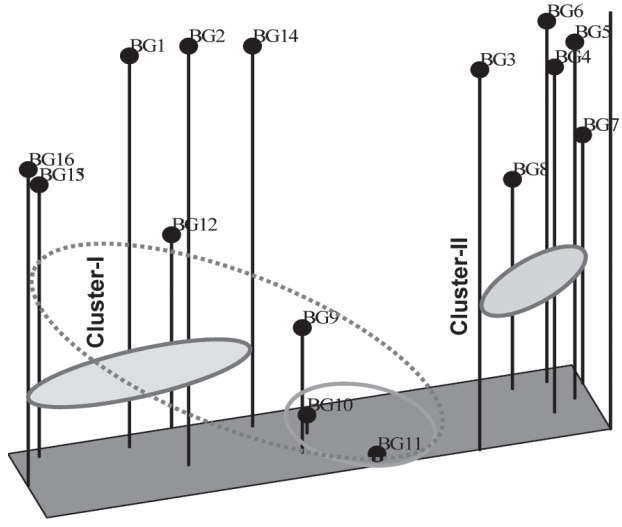

Fig. 4. Three-dimensional plot of principal component analysis of 16 genotypes of blackgram with different genotypes showing genetic diversity.

eigen values extracted from a correlation matrix among the markers that was obtained from a standardized data matrix. All the above analyses were carried out using NTSYS-pc (Version 2.02e, Applied Biostatistics) program. Bootstrap analysis (1000 iterations) of the binary data was performed using the WINBOOT programme (Yap and Nilsson, 1996) to determine the confidence limits of the UPGMA based dendrograms and boot-strap of 50 % majority rule consensus tree was constructed. Average discrimination coefficients (D) of each RAPD primers were estimated for all the 48 landraces with band differences ranging from one to five using the Power Marker (Liu and Muse, 2005) software tool. In this, data matrix was derived from the distance or similarities between the operational taxonomic units.

3. Results and discussion

Genetic variability was found to be associated with morphological traits. Morphological markers are offen

influenced by environmental factors. So, RAPD markers have been used in many instances to assess the genetic variability in genetic resource utilization (Panigrahi *et al.,* 2015; Shelke and Das, 2015; Sahu *et al.*, 2016). Total 176 RAPD amplicons were generated by 20 primers, of which 75 (42.61%) were polymorphic (Tables 2, Figs. 1 and 2). Each primer gave 4 to 14 amplified bands by OPF-10 and OPN-04 primers respectively having a size range from 100 bp to 2000 bp (Table 2). Genetic similarity on the basis of RAPD analysis for 16 varieties of blackgram ranged from 2% 65%. The results indicate that most divergent group were BG-5 to BG-8 from the rest of the varieties. The unique markers obtained from different varieties could be used to develop variety specific SCAR markers for identification of genotypes. In this study numbers of unique bands were discovered by RAPD marker (Figs. 1 and 2) which could be cloned and utilized for the preparation of variety specific marker (SCAR) and physical localization on chromosomes through FISH. Present study of RAPD marker throws light on phylogeny and classification of local landraces of blackgram germplasms for further study, breeding practices and management of germplasms. $RAPD_{800}$ was the common monomorphic band in OPA8 and OPN4 primer as well as some other primers. Some of the polymorphic bands like $RAPD_{600}$ in BG5, BG6, BG7, BG8 and BG-14 of 'Ujala', 'Nayagarh local', 'Berhampur local', 'Kothagarh local' and 'Keonjhar Pejua' are special marker bands amplified in OPA8 primer. OPN14 similarly produced some marker band like $RAPD_{2000}$ and $RAPD_{1400}$ in some of the land races (Fig. 4, arrow heads). $RAPD_{600}$ produced by BG7, BG8, BG9, BG10, BG11 in OPN4 was also found to be landrace specific. A unique band of $RAPD_{250}$ in 'Mahimunda local' of Balangir district was landrace specific in OPN4 primer. Prasanthi *et al.* (2011) reported RAPD marker based SCAR marker development in blackgram for large-scale application in marker-assisted breeding for YMV resistance. This involves the characterization of the linked maker and the design of locus-specific primers. The conversion of a linked marker to SCAR has been reported in common bean (Melotto *et al.,* 1996), rice (Naqvi and Chattoo, 1996), blackgram (Prasanthi *et al.,* 2011) and tomato (Zhag and Stommet, 2001). Gupta *et al.* (2013) also used F_2 population to tag and map the MYMIV resistance gene using SSR markers in blackgram.

The results presented here showed that 16 land races are considerably different genetically in cluster distribution that depends mainly on the genomic constituents of the genotypes. The close similarities of BG13 (Ranipeta local) and BG15 (Simliguda local) might be of common origin as these two land races are found in nearby districts. Single largest Cluster-I was produced with 10 land races (Fig. 3) out of which BG13 and BG15 (Gajapati and Similiguda) were having very high genetic similarity. Cluster-II formed with rest 6 land races out of which BG3 and BG4 showed more genetic distance from the other 4 land races (Fig. 3). PCA analysis showed that in the Cluster-IBG9, BG10 and Bg11 of Panthnagar are of same genetic background with a high genetic variability (Fig. 4). As such Cluster-I showed high genetic variability than Cluster-II. Saraswathi *et al.* (2011) and Shelke and Das (2015) reported also intra-group diversity among banana using IRAP and RAPD marker systems. The amplified products could be tested at different chromosomal sites among divergent species to check the common ancestor (Cuadrado, 2002; Achrem, 2006). This RAPD polymorphism could be due to deletion and/or amplification from the pre-existing sequences during varietal evolution. Very similar conclusions can be drawn from SSR-based studies (Shang, 2006) and ISSR-based studies (Ren, 2011). A recombinant inbred line (RIL) mapping population (F8) was generated by crossing *Vigna mungo* (cv. TU 94-2) with *Vigna mungo* var. *silvestris* and screened for mungbean yellow mosaic virus (MYMV) resistance. The inter simple sequence repeat (ISSR) marker technique was employed to identify markers linked to the MYMV resistance gene and one ISSR marker was identified as tightly linked to the MYMV resistant gene at 6.8 cM by Anjum *et al*. (2010). The ISSR technique has been used in tagging disease resistant genes in a number of crops (Reddy, *et al.,* 2002). ISSRs have better capacity to reveal polymorphism and offer great potential to determine intra- and inter-genomic diversity when compared with other arbitrary primers like RAPDs (Zietkiewicz, *et al.,* 1994; Souframanien and Gopalakrishna, 2006). A major obstacle is the lack of high resolution genetic markers for blackgram for development of YMV markers. The linkage mapping in black gram can be made possible using genetic markers developed from other related legumes. BAC library for *Vigna* species, except only one on mungbean (Miyagi *et al*., 2004), is one of the constrains in developing markers for disease resistance. Efforts are needed to develop expressed sequence tag (EST) libraries which offer important information for species that have not been sequenced and are a central source of gene based markers and single nucleotide polymorphism (SNP) or indel polymorphisms (Galeano *et al*., 2009). EST–based markers are valuable because they represent sequences that are transcribed and therefore, can potentially be associated with phenotypic differences. Generation of expressed sequence tag (EST) databases for the development of genic microsatellite markers might overcome the paucity of the polymorphic markers in black gram for developing MYMIV resistant genes. A single dominant gene controls the MYMIV resistance in blackgram genotype DPU 88-31 was reported for MYMIV resistance

gene using SSR markers (Gupta *et al.,* 2013). Morphologically distantly related and DNA based resistant varieties obtained from the cluster analysis could be used for plant breeding in black gram to develop high yielding disease resistant varieties.

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Effect of nutrient subsidization on growth performance and soil microbial activity in rice field

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ABSTRACT

The microbial activities of soil in rice agroecosystem are influenced by agro-practices and nutrient regime. The levels of various macronutrients and their ratios determine the plant production. At the same time the soil microbial activities determine the nutrient mobilization and their availability to the rhizosphere. The current work was done at different combinations of nitrogen (N), phosphorus (P), potassium (K) to study the growth and production rate of rice (*Oryza sativa* L.) and the associated changes in soil microbial activity. During 120 days of observation (at 30 days interval), the shoot length showed continuous and significant increase up to 90 days and that of root showed significant increase up to 60 days. The biomass, on the other hand, showed continuous and significant increase with prolongation of cultivation. Each growth parameter showed the highest performance in the plot supplemented with NPK while the lowest was with the plot supplemented with K only. P supplementation was also found to be the most effective in enhancing the growth performance of the crop in the plots. Corresponding values of F_0 and F_M were also observed. As expected the maximum fluorescence yield was recorded in P amended plots, with or without other nutrients. The activities of invertase and amylase were the highest in the plot with manure (3 kg) and the lowest was observed in the unamended plots. No significant difference in the activities of these enzymes was observed among other nutrient amended plots during the period of observation.

1. Introduction

Soil is a fundamental segment of the environment (Kennedy and Smith, 1995) and it is the aftereffect of the mineral, synthetic, physical and biological entity present in the soil (Rolf, 2005). The knowledge of the biological processes that take place within the soil is important for proper soil use and to preserve soil standard (Lavelle et al., 2006). The importance of soil nutrient level in sustaining rice production in tropical paddy fields has been long recognized. The tropical paddy ecosystem has high population of microbes comprising of both cyanobacteria, bacteria and algae, which are responsible for the mobilization of nutrients (Matsuguchi, 1979; Prosser, 2007). Soil microbial activity and nutrient amendments have a direct bearing on the rice plant growth and production potential.

The assurance and preservation of soil biodiversity is critical for a reasonable agro-biological community, particularly under expanding agrarian strengthening (Vandermeer *et al.,* 1998) and has strong financial and environmental ramifications (Gardi *et al.,* 2009). Microbial activity can be influenced by abiotic factors, like moisture, temperature and soil supplements (Singh *et al.,* 2009).

Most of the enzymatic transformations in soil are accomplished by microbial biomass due to which a part of the organic materials is stabilized as humus and the remaining carbon and other nutrients are utilized by microorganisms for their own growth (Anderson and Domsch, 1980). Moreover, there is reduction in microbial biomass and enzyme activities due to excessive cultivation practices (Gupta and Germida, 1988). Nevertheless it is interesting to

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see the relationship between the microbial activity and soil nutrient management and its impact on plant production. This work presents the influence of soil nutrient management on the soil enzyme activity and the dependent changes in the growth and production of rice (*Oryza sativa L*).

2. Materials and methods

2.1. Preparation of experimental plot

The study area is Nimapada block of Puri district in Odisha. It is a coastal area and here rice cultivation is the major agricultural crop. The experimental plot is designed for five different treatments having 15 plots, 1 m^2 of each, and each treatment with three replicates (Table 1).

Table 1

Treatment of different fertilizers in the experimental plots

Treatment	Soil with different fertilizers in the plot
T1	Soil without fertilizer and manure
T2	Manure (3.0 kg) + K (35 g/m^2)
T3	Manure (500g) + K (35 g/m^2) + N (45 g/m^2)
T4	Manure (500g) + K (35 g/m^2) + P (125 g/m^2)
T5	Manure + K (35 g/m^2) + N (45 g/m^2) + P (125 g/m^2)

Note: The quantity of N, P and K given in the table are the weight of urea, super phosphate and Muriate of Potash.

Table 2

The scheme of preparation and amendment of the fields during 120 days of observation

Day	Action
-30	Addition of manure, mechanical dry ploughing
0	Wet ploughing, weeding, irrigation transplantation
30	Full dose of K and P and half dose of N application
60	Half dose N application

Table 3

The physiochemical properties of the field soil before transplantation

Parameter	Value
pH	5.73 ± 0.82
Electrical conductivity (dm/m^2)	0.48 ± 0.03
Mineralization nitrogen (kg/ha)	135.8 ±7.83
Available phosphorus (kg/ha)	18.33 ± 1.42
Available potassium (kg/ha)	118.5 ± 3.85
Organic carbon (g/ha)	4.58 ± 0.21

The plots were prepared through dry ploughing before 30 days of transplantation and through wet ploughing before 7 days of transplantation. The scheme of action and fertilization during 120 days of cultivation has been given in Table 2. Muriate of Potash, urea and super phosphate were used as the source of K, N and P respectively. The physicochemistry of the field soil before manure and fertilizer application was also measured by taking soil sample randomly from five different places in the field (Table 3).

2.2 Analytical parameters

The growth and fluorescence parameters of rice plant and soil enzyme activities were taken as the analytical parameters. The plant performance (shoot length, root length and biomass) were taken at interval of 30 days for a period of 120 days from the day of transplantation. Amount of chlorophyll content was measured spectrophotometrically after extraction with ice cooled absolute methanol following the method of Porra *et al.* (1989). The fluorescence minimum (F_0), maximum (F_M) and yield (F_v/F_M) were determined in the field from the OIP fluorescence transient with the help of a handy PEA (Hansatech, UK) following the method of Chhotaray *et al.* (2014). The activities of carbohydrate enzymes (invertase and amylase) were measured after incubating the soil, in Sorensen's buffer for 24 hours in the respective substrate (1% sucrose for invertase and 1% starch for amylase). The amount of glucose produced was measured using 3,5 – dinitrosalicilic acid (DSA) and the standard was prepared using glucose (Chhotaray *et al.*, 2014).

2.3 Statistical analysis of data

The data were statistically analysed by Excel stat software. The comparisons among the treatments were made through least significant difference test (LSD) and the values of LSD have been given in the text wherever required.

3. Result and discussion

In this study the physiochemical analysis of the soil showed that pH range of the soil condition ranging from 5.73±0.82 (Table 1). The soil pH, organic carbon content and water are the main factors affecting the soil microflora diversity (Zhang *et al.,* 2007). The organic carbon, nitrogen, phosphorus and potassium are important for the development of microflora. Soil enzymes are vital to soil health and fertility management in agroecosystem. These two enzymes amylase and invertase have many significant effects on soil biology, environmental management, growth and nutrient uptake in plant growing in agroecosystem.

The shoot length of the plants constantly increased throughout the observation period but after 90 days the increase was found insignificant irrespective of the treatment (LSD were 4.28 to 6.15 cm; Fig.1A). The root length, on the other hand, showed maximum increase up to 60 days after transplantation and thereafter the increase was insignificant (LSD were 2.19 to 2.73 cm; Fig. 1B). Biomass of the plant increased continuously till 120 days and such increase, when compared among the days of observation, was always significant (Fig. 1C). as expected, the plants in the control plot (without manure and fertilizer) had less number of tillers and lower shoot length as compared to plants in other plots which were supplied with fertilizer and manure. With prolongation of cultivation, the variation in shoot length among the treatments gradually decreased and after 120 days the variation was only 12 % indicating that plant height was not remarkably affected by the cultivation practice. A comparatively higher variation was observed (22%) was observed with respect to root length, when compared to control but among the plots aided with fertilizers, the variation (1%) was insignificant (LSD = 4.88%). This indicated that the addition of fertilizer did not have much effect on the root and shoot length but there is significant impact on the plant biomass. The biomass (Fig 1) shows that there is a gradual increase in the plant growth from 30 days through 120 days of observation.

Significant variations in the activities of amylase and invertase were observed among the treatments which also showed day wise difference (Fig. 2). On each observation day the activity of these enzymes was the highest in in T2 compared to other treatments. The lowest activity was observed in T1 as expected. Irrespective of treatments, there was no significant variation among treatments, except T2, with regard to enzyme activities after 30 days (F = 1.004) but significant variations were recorded thereafter. In each treatment, the activity of invertase and amylase showed insignificant change up to 60 days and significant reduction in the activities were noted thereafter.

From the data presented in Fig. 3 it was found that the chlorophyll content of the plants in the control plot is almost same with the treated plots till 30 days. After 60 days there was a minor change in the chlorophyll content of the control plant whereas significant increase of pigment content was reported in all other treatments. Maximum change was seen in plot treated with manure K and P and manure, K, N, P respectively. After 90 days of observation there was decrease in the chlorophyll content up to 120 days in all treatments but maximum decrease was noticed in T1. This may be due to the rapid utilization of the nutrient and/or the maturation of the crop plant and beginning of senescence of the plants. Nevertheless, the pigment content of T2 remained high, compared to all other treatments throughout observation which could be well corroborated with the soil enzyme activities. Corresponding pattern was also noted for F0, FM and fluorescence yield. While from T2 through T5, the F0 showed insignificant variation with prolongation of cultivation up to 90 days, there was a significant increase in the fluorescence value in T1. This indicated an increased stress on the plant leading to low photosynthetic efficiency. FM did not show significant variation up to 60 days decrease was observed in each treatment thereafter. After 120 days of cultivation the fluorescence and the photosynthetic yield were quite low.

A study on the effect of soil microbes and the enzymes involved on different stage of rice growth showed the increase in microbial biomass and enzyme activities had high rate of release of nutrients for rice crops. Varying soil depth to certain extent also influenced increase in bioactivity (Meena *et al.,* 2014). In another study soil organic carbon, available N, P, K, Zn, Fe and Cu in soil at crop harvest stage significantly increased due to the integrated inoculation of PGPR and Multani mitti based BGA with application of compost and chemical N fertilizer (80 and 120 kg/ha) over N control (Meena *et al.,* 2014). Application of N with inoculation of bacterial and cyanobacterial PGPR along with compost not only improved nutrient availability in soil but also enhanced soil microbial, plant enzymatic activity and crop yield (Meena *et al.,* 2014). An association of zinc solubilizing bacteria with rice plant shows these bacteria facilitate root growth which in terms induces plant growth (Othman *et al.,* 2017). In the present study, the high rate of enzyme activities in T2 may be attributed to the healthy soil microbial population that was supported by the addition of organic manure. It may be noted that the organic manure content of T2 was the highest among treatments. Chhotaray *et al.* (2014) have reported that in organic agroecosystems, a healthy microbial flora and activity is maintained due to high soil organic carbon level. This study showed that wit manure addition, the gain of biomass is not as high as observed with NPK addition, but such practice maintains a healthy microflora, which is necessary to sustain the productivity of the crop field.

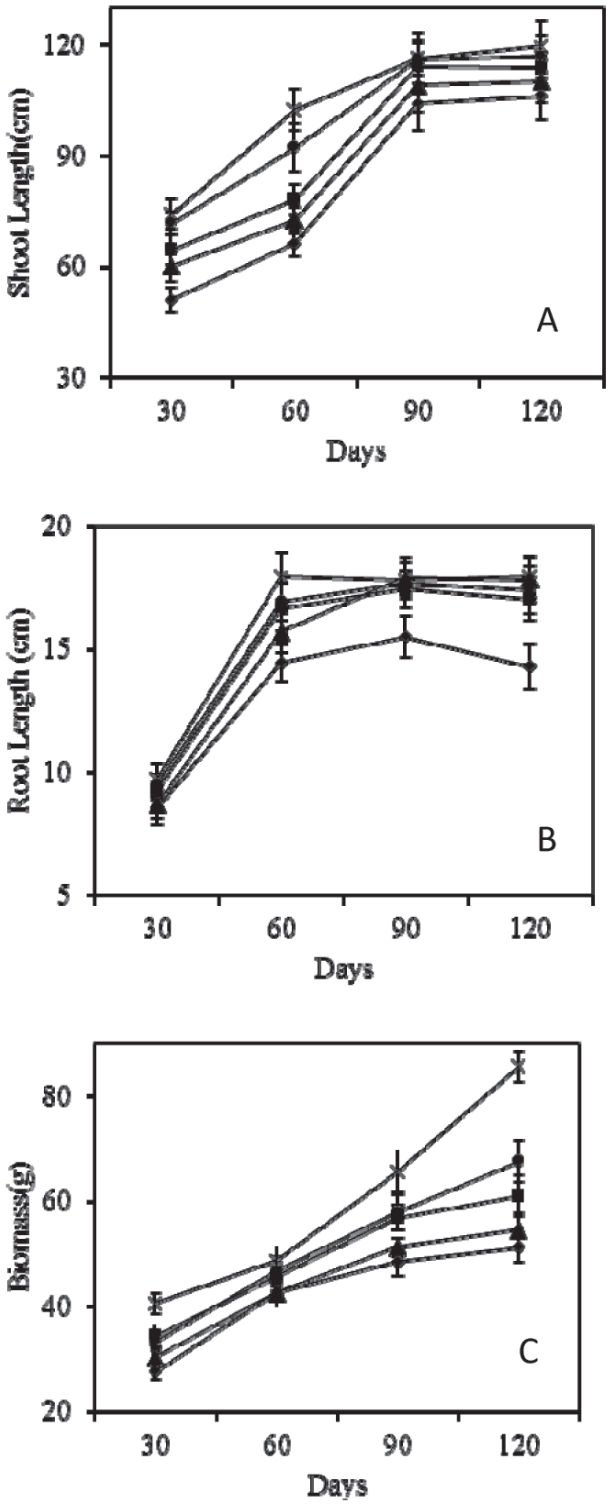

Fig. 1 A,B,C: Control (Diamond), treatment of K (Square), treatment of K&N (triangle), treatment of K&P (circle), treatment of K,P&N (Cross) shows the change of shoot length, root length and biomass of plants at different time period.

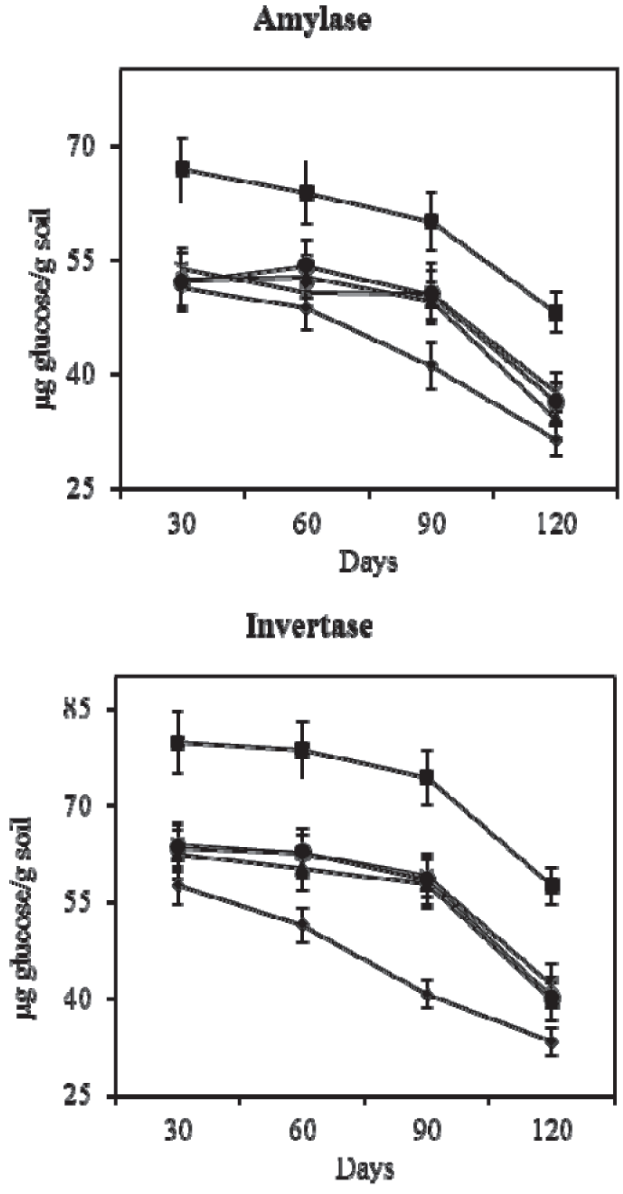

Fig. 2: Control (Diamond), treatment of K (Square), treatment of K&N (triangle), treatment of K&P (circle), treatment of K,P&N (Cross) shows the activities of enzyme amylase and invertase with time period.

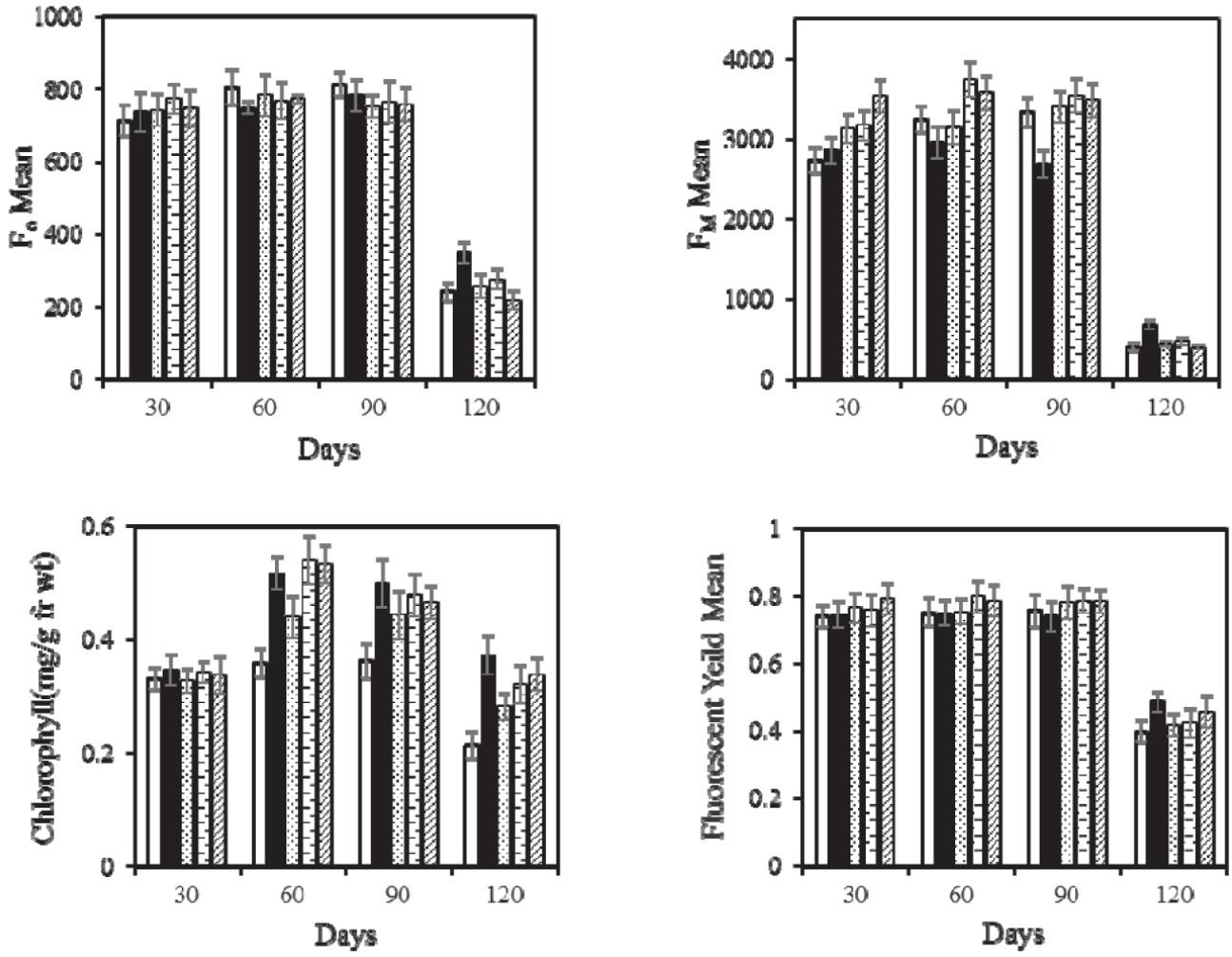

Fig. 3: Control (Empty), treatment of K (Black), treatment of K&N (Dotted), treatment of K&P (Brick), treatment of K,P&N (Oblique) shows the mean value of F_0, F_M, chlorophyll content and Fluorescence yield with different time period.

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Screening for phytochemicals and antioxidant activity of flowers of *Madhuca indica* J.F. Gmel.

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ABSTRACT

Madhuca indica J. F. Gmel. [Syn. *Madhuca longifolia* var. *latifolia* (Roxb.) A.Chev], commonly known as "Mahua" is an economically important plant and known for its medicinal and ethnobotanical uses as well as for its edible flowers having high nutritional values. However, due to lack of scientific knowledge on its food, nutritional properties and industrial applications, the species has not been exploited to the extent possible. Hence, the present study is aimed at screening of phytochemicals, estimation of reducing power and determination of the physicochemical properties of Mahua flowers with various solvent concentrations. The chloroform, acetone, methanol, ethanol and aqueous extracts of the flowers were investigated for its phytochemical activity. Mahua flower shows higher reducing power in aqueous extracts as compared to acetone, methanol and ethanol extracts.

1. Introduction

Since time immemorial, medicinal plants have played a very significant role in curing human diseases especially in traditional system of medicine and pharmaceutical drug formulations (Chaudhary *et al.,* 2015). The large population of the world depends on the medicinal plants for their health care needs in view of their easy availability and having no side effects. The high cost of modern allopathic medicines also compels them to resort to plant-based traditional medicines (Togboto and Towson, 2005). The world health organisation (WHO) estimated that 80% of the population of developing countries relay on traditional medicines, mostly plant drugs, for their primary health care needs (Prabakaran *et al*., 2011). India has one of the oldest, richest and most diverse cultural traditions in the field of utilization of medicinal plants (Mehta *et al*., 2013). Medicinal plant has been providing modern medicine with numerous plant derived therapeutic agent (Evans, 2000).

Madhuca indica (Syn. *Madhuca longifolia* var. *latifolia*), belonging to family Sapotaceae, is one of the multi-purpose wild medicinal plants having great economic importance as almost all parts of the plant are used by human beings for some purpose or other (Banerji and Mitra, 1996) such as producing country liquor from succulent corollas and oil from the seeds (Boral *et al.*, 1999). The plant is known to possess various therapeutic properties and has been one of the noteworthy plants mentioned in various medicinal systems (Chidrewar *et al.,* 2010). *M. indica* is considered as a stimulant, demulcent, emollient, heating and astringent (Awashthi and Mitra, 1967). Thus, the present study aimed to determine the physicochemical parameters, preliminary phytochemical screening and reducing power of flowers.

2. Material and methods

2.1 Collection of plant materials

The fresh flowers of *M. indica* were collected in early

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morning during the month of March, 2017 from the village Patolokota, Keonjhar District, Odisha. The collected flowers were cleaned manually to remove all foreign materials and damaged flowers. The samples were then washed carefully in tap water followed by distilled water to remove remaining unwanted materials. Then the samples were air-dried under shade at room temp to avoid direct loss of phyto-constituents of flowers by sunlight. The anther parts were removed from dried flowers and then the samples were transferred to air tight jars and stored in freeze at 4^0C for further use.

2.2 Preparation of plant extract

The flowers were cut in to small pieces and kept in thimble for extraction. The samples were extracted with different organic solvents (methanol, ethanol and acetone) with different concentrations (≈100%, 75% and 50%) and in aqueous solution by using Soxhlet Apparatus at a temperature not exceeding the boiling points of respective solvents (Pandey and Tripathy, 2014). The liquid extracts were evaporated and stored in a refrigerator at 4^0C in small sterile glass vials for further experimental work.

2.3 Organoleptic characters

The organoleptic character like colour, odour, taste, physical appearance and surface characteristics were studied (Katiyar *et al.,* 2011; Patel *et al.,* 2012; Sanmugarajah *et al.,* 2013).

2.4 Physico-chemical investigation

The flower samples were subjected to physicochemical analyses such as dry biobass, water soluble extractives, alcohol soluble extractives, pH in 1% w/v solution, pH in 10% w/v solution (Sanmugarajah *et al.,* 2013; Katiyar *et al.,* 2011)

2.5 Preliminary phytochemical screening

Phytochemical analysis in chloroform, acetone, methanol, ethanol and aqueous extracts of flowers of *M. longifolia* var. *latifolia* were carried out using standard procedures to identify the possible bioactive compounds (Harborne, 1998; Trease and Evans, 1989).

2.6 Reducing power

The reducing power of different solvent extracts (methanol, ethanol, acetone and aqueous) of Mahua flowers was determined according to the method of Oyaizu (1986). Different concentrations of extracts were mixed with 2.5 ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50^0c for 20 minutes. After incubation, 2.5 ml of 10% TCA was added to the reaction mixture, which was then centrifuged at 3000g for 10 minutes. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ solution. Then absorbency was measured at 700 nm against blank prepared by replacing sample with 1.0 ml distilled water. All the analyses are performed in triplicate and result were averaged. Ascorbic acid was used as standard. Increasing absorbance of the reaction mixture indicated increasing reducing power (Indu and Annika, 2014).

3. Results and discussion

3.1. Organolaptic properties

Organoleptic evaluation can be done by means of sense organs, which provide the simplest as well as quickest means to establish the identity and purity to ensure quality of a particular drug. This is again necessary because once the plant is dried and made into powder, it loses its morphological features and becomes prone to adulteration (Chanda, 2014).

Table 1
Organoleptic properties of Mahua flowers

Parameters	Organoleptic characters
Colour	White creamy (Fresh), Brown (Dry)
Odour	Sweet
Taste	Sweet
Surface	Longitudinal

3.2. Physicochemical characters

The extractive values are useful to evaluate the chemical constituents present in the crude drug and also help in estimation of specific constituents soluble in a particular solvent (Yi Zeng*et al.,* 2004). The water and alcohol soluble extractives of Mahua flowers were 1.28% and 0.53% respectively. A similar result was obtained by Katiyar *et al.* (2011), where they found the percentage of water and alcohol soluble extractives as 0.664% and 0.680% respectively. The percentage of water loss on drying of flowers was 11.62% and it is recommended as the minimum level to discourage the growth of bacteria, yeast or fungi during storage (Soni *et al.,* 2011) (Table-2).

3.3 Phytochemical screening

Alkaloids, flavonoids, glycosides, carbohydrates and lipids were found to be present in aqueous, ethanol, methanol and acetone extracts of Mahua flowers. Quinones, oxalates and amino acids were absent in all these extracts. Tannins and terpenoides were absent in acetone and aqueous extracts,

Table 2
Physico-chemical characteristics of Mahua flowers

Parameters	Values
Water soluble extractives	1.28%
Alcohol soluble extractives	0.53%
Loss on drying	11.62%
pH 1% w/v solution	6.16
pH 10% w/v solution	6.23

whereas they were present in ethanol and methanol extracts. Saponins were reported only in ethanolic extract and absent in others extracts. Terpenoids, lipids and carbohydrates were also found to be present in chloroform extracts of flowers (Table 3).

Flavonoids and tannins are major groups of compounds that act as primary antioxidant free radical scavenger and antioxidant activity (Polterait, 1997). The qualitative analysis of the extracts confirmed the presence of saponins in the flowers of Mahua. The saponins have the property of precipitating and coagulating red blood cells in humans and plants containing this compound are responsible for stimulating activity (Sodipo *et al.,* 2000).

Plants containing alkaloids are used in medicine as anaesthetic agent (Herourat *et al.,* 1998) and are likely to have antibacterial properties (Okwu, 2004). The presence of alkaloids in these extracts indicated that it can be used as an antibacterial agent. The presence of higher terpenoids in flower extracts that have carboxylic acid groups could also be responsible for the activity of organic extracts (Murugesen and Muthysamy, 2011).

Table 3
Phytochemical screening of Mahua flowers

Phytochemicals	Tests	Aqueous extract	Ethanol extract	Methanol extract	Acetone extract	Chloroform extract
Alkaloids	Wagner's test	+	+	+	+	-
Flavonoids	Alkaline reagent test	+	+	+	+	-
Phenols	Ferric chloride test	+	+	+	+	-
Saponins	Foam test	-	+	-	-	-
Tannins	Braymer's test	-	+	+	-	-
Terpenoides	Salkowki's test	-	+	+	-	+
Quinones	Acid test	-	-	-	-	-
Oxalates	Acid test	-	-	-	-	-
Glycosides	Keller-Kiliani's Test	+	+	+	+	-
Carbohydrates	Fehling test	+	+	+	+	+
Proteins	Millon test	+	+	+	-	-
Lipids	Sudan red test	+	+	+	+	+
Amino acids	Ninhydrin test	-	-	-	-	-

3.5. Reducing power

The reducing ability of compound generally depends on the presence of reductants which have been exhibited antioxidant potential by breaking the free radical chains, donating a hydrogen atom.

Presence of reducers causes the conversion of the Fe3+ ferricyanide complex used in this method to the ferrous form. By measuring the formation of Pearl's Prussian blue at 700nm, it is possible to determine the concentration of Fe^{3+} ion. As the concentration of flower extract increases, simultaneously antioxidant power also increases. The reducing powers of extracts were very high and it increases as the quantity of samples increase (Saha *et al*., 2010; Indu and Annika, 2014).

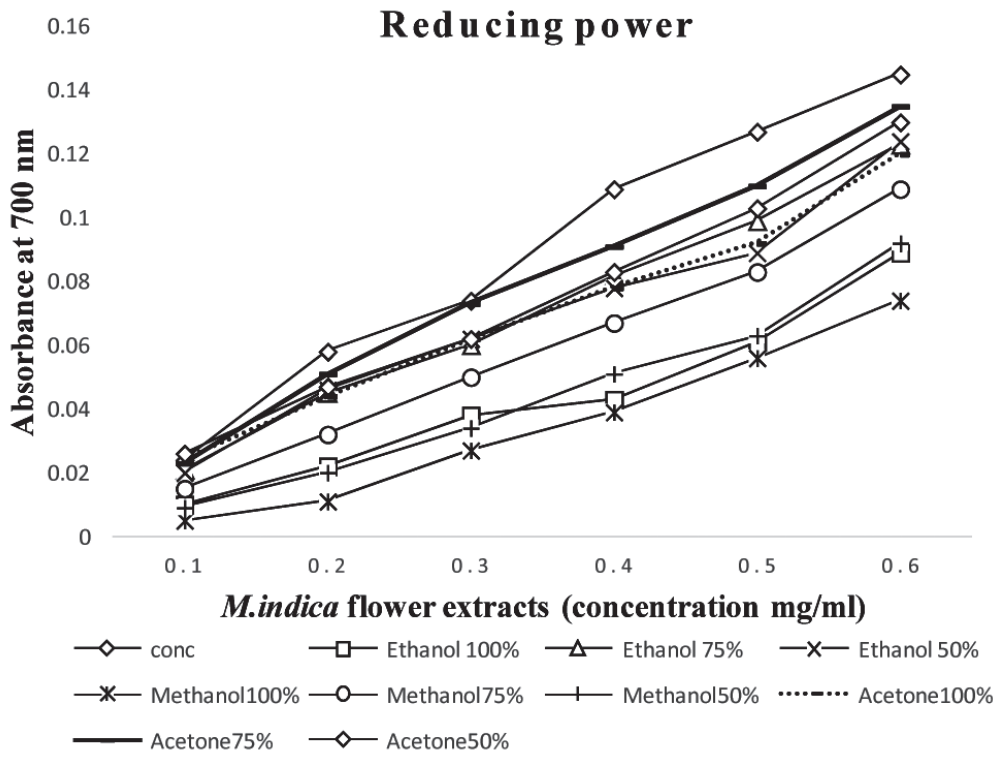

Fig. 1. Graph showing reducing power of *M. longifolia var. latifolia* flowers.

4. Conclusion

Plants are important sources of potentially bioactive constituents for development of new therapeutic agents. *M. indica* - a plant of Indian origin have tremendous medicinal values and wide range of uses but due to lack of knowledge and scientific data, the economic potential of the species has not been fully exploited (Patel *et al.,* 2012).

As the first step towards achieving this goal, flowers of Mahua were analysed for their organoleptic properties, physicochemical characteristics, phytochemical constituents to determine the purity and quality of a crude drug especially in powered form. Plant produces a wide variety of secondary metabolites which are used either directly as precursors or as lead compounds in the pharmaceutical industries. The high reducing power of flower extract of *M. indica (longifolia* var. *latifolia)* implies that it is capable of donating hydrogen atom in a dose dependent manner and can be used as antioxidants. The plant is a rich source of various bioactive compounds useful in curing a number of diseases and the medicinal properties of different plant parts of Mahua need to be studied in detail so that this multi-purpose species could be put to more effective use for human welfare.

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Assessment of genetic diversity and phylogeny of the seagrasses of Odisha coast using molecular markers

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ABSTRACT

Seagrasses are submerged marine flowering plants belonging to four core families Cymodoceaceae, Hydrocharitaceae, Posidoniaceae and Zosteraceae of the monocot order Alismatales, which form critical habitats in the tidal and sub-tidal zones of shallow and sheltered localities of seas, backwaters, lagoons and estuaries. In the present work, the inter and intra-species genetic diversity of six species of seagrass namely, *Halophila ovalis, Halophila ovata, Halophila beccarii* (Hydrocharitaceae)*, Halodule pinifolia, Halodule uninervis* and *Cymodocea serrulata* (Cymodoceaceae) occurring in Chilika lagoon of Odisha coast were assessed using RAPD and ISSR molecular markers. With 10 RAPD primers, 79 loci were amplified, out of which 50 (63%) were polymorphic in nature. Similarly, 54 out of 84 bands (64%) generated with 11 ISSR primers, were found to be polymorphic. The dendrogram constructed using combined RAPD and ISSR data separated members of Cymodoceaceae (*C. serrulata, H. pinifolia* and *H. uninervis*) and Hydrocharitaceae (*H. ovalis, H. ovata* and *H. beccarii*) into two distinct clusters justifying their inclusion in distinct botanical families based on morphological traits. All the accessions of a particular species also formed distinct groups with varying levels of similarities. The present study revealed that RAPD and ISSR markers can be effectively used for species identification of seagrasses even at juvenile and non-flowering stage.

1. Introduction

Seagrasses are submerged marine flowering plants belonging to four core families Cymodoceaceae, Hydrocharitaceae, Posidoniaceae and Zosteraceae of the monocot order Alismatales, which form critical habitats in the tidal and sub-tidal zones of shallow and sheltered localities of seas, backwaters, lagoons and estuaries. Though these marine plants grow in coastal waters of all continents except Antarctica and survive most diverse environmental conditions, the species diversity of seagrasses is relatively low and only 72 species are recognized till date (Short *et al*., 2011). Seagrasses are considered a 'biological group' as they have not evolved from a single lineage, but from four independent evolutionary events between 35 to 65 million years ago and hence form a paraphyletic group including four core angiosperm families and this grouping is based on their shared traits, which allow them to complete their life cycle under submerged conditions in the marine environment (den Hartog, 1970, Les *et al*., 1997, Jannsen & Bremer, 2004).

The seagrasses represent an important component of the seascape's natural history, playing a critical role in sediment accumulation and carbon storage. Seagrass meadows support high rates of secondary productivity; they host algae that support diverse and productive food webs for fishes and birds (Orth *et al*., 1984), and directly provide food for many marine herbivores including the endangered green sea turtle, manatee and dugong (Green and Short, 2003; Larkum *et al*., 2006; Short *et al.,* 2007). These meadows also support coral reef ecosystems by filtering and precipitating pollutants. They serve as nursery ground

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for many fish and invertebrate species too (Beck et al., 2001). Besides, some seagrass species produce highly valuable secondary compounds such as phenolic acids (rosmarinic acid, zosteric acid etc.) used in traditional medicine and biotechnological purposes (Newby *et al*., 2006; Lucas *et al*., 2012). Paradoxically, however, the valuable seagrass resources are declining rapidly throughout the world largely because of eutrophication and high turbidity due to natural and human influences.

Although on a global scale, seagrasses represent less than 0.1% of the angiosperm taxa, the taxonomical ambiguity in species delineation is high and thus, the taxonomy of several genera is unsolved. While seagrasses are capable of performing both, sexual and asexual reproduction, vegetative reproduction is common and sexual progenies are always short lived and epimeral in nature. This makes species differentiation often difficult, since the flower as a distinct morphological trait is missing. Besides, seagrasses, in general, have fewer morphological and anatomical features for species identification than their terrestrial counterparts (Kuo & McComb, 1989). Short living reproductive organs of seagrasses due to high wave and tide action and high seasonal appearance make them not regularly available for identification. This necessitates the development of molecular markers as an alternative tool for identification and to derive phylogeny of seagrass species.

The genetic diversity and phylogenetic relationships among and within genera and species of seagrass have been studied using a variety of biochemical and molecular markers such as isozymes (McMillan & Williams, 1980; McMillan, 1981, 1982; Laushman, 1993; Capiomont *et al*., 1996; Reusch, 2001), RAPD (De Heij & Nienhuis, 1992; Kirsten et al., 1998; Procaccini *et al*., 1999; Angel, 2002; Jover *et al*., 2003; Micheli *et al*., 2005), AFLP (Waycott & Barnes, 2001) and microsatellites (Randall *et al*., 1994; Davis *et al*., 1999; Reusch, 2002; Reynolds *et al*., 2012). However, most of the molecular studies done so far involved species of *Zostera, Thalassia* and *Posidonia* and only few publications are available dealing with some Indian species of *Halophila, Halodule* and *Cymodocea* (Waycott *et al*., 2002; Pharmawati *et al.*, 2016 and Suhardi & Susandarini, 2017). In recent years, many other molecular markers have been used for analysis of genetic variability and phylogenetic studies of Indian seagrasses such as rbcL/matK, trnH/psbA (Lucas *et al*., 2012) and ITS sequence data (Nguyen *et al*., 2015, Dillipan *et al*., 2016) but there is no generally agreed consensus yet on conserved molecular regions useful for seagrass taxonomy and evolutionary history. Considering the non-availability of data on molecular study of seagrasses of Chilika lagoon, Odisha, India, the present investigation was undertaken to assess the intra and inter-species genetic variability of six seagrasses species occurring in Chilika lagoon (Pattnaik *et al.,* 2008) and to identify species of *Halodule* and *Halophila* at juvenile stage using RAPD and ISSR markers, which is difficult to discriminate morphologically.

2. Materials and methods

2.1. Study site

Studded like a zircon on the golden stretch of eastern seacoast of Odisha in India, Chilika lagoon is a unique assemblage of marine, brackish and fresh water eco-system with estuarine characters. The lagoon is situated between 19^0 28' and 19^0 54' "N" latitudes and 85^0 05' and 85^0 38' "E" longitude and the water-spread area varies between 1165^2 km during monsoon to 906^2 km in summer. The lagoon is connected to the sea through a long constricted inlet channel with a comparatively smaller inlet. A 32 km long narrow outer channel connects the main lagoon to the Bay of Bengal near village Arakhakuda. It also receives fresh water from 52 rivers and rivulets, which attribute to its brackish and estuarine character. The lagoon is an avian wonderland and a staging and wintering ground for a large number of bird species. The lagoon has been broadly divided in to four ecological zones, (i) the southern zone, (ii) the central zone, (iii) the northern zone and (iv) the outer channel. Chilika has a typical physiographic feature experiencing the dynamics of the coastal processes along with the riverine interface. Wave, current and tide, along with storms are the coastal processes, responsible for shoreline characteristics and coastal morphology. Chilika can be classified in to different geomorphic units like structural hills, denudated hills and pediments dominated by khondalite, charnockite, gneisses; buried pediment, piedmont zone, deltaic plain, mud flat, coastal plain, barrier spit and coastal sand dunes.

2.2. Plant materials

Six seagrass species namely, *Halophila ovalis, Halophila ovata, Halophila beccarii, Halodule pinifolia, Halodule uninervis* and *Cymodocea serrulata* were collected from 20 identified stations of Chilika lagoon. Plant samples were cleaned by repeated washing in lagoon water, removing clays, sands and other epiphytic organisms growing on leaf blades. The plant samples were brought to the laboratory in zip lock polypack with the lagoon water to prevent dehydration. The samples were then washed thoroughly with tap water to remove sands and debris, if any. The leaves were removed manually and soaked in blotting paper to remove extra water and finally 2 grams of leaves of each species were weighed and kept separate for genomic DNA extraction.

2.3. Genomic DNA isolation and quantification

Tender leaves were collected from fully-grown plants and stored at -80^{0}C prior to use. Total genomic DNA was extracted by using the protocol described by Doyle and Doyle (1990) with required modifications. The quality and concentration of DNA was examined by ethidium bromide-stained agarose gel electrophoresis and spectrophotometric analysis. The quantification was done in comparison with the known standard (ë-DNA). After quantification, the DNA was diluted with $T_{10}E_1$ buffer to a working concentration of 25ng/µl of PCR analysis.

2.4. Random amplified polymorphic DNA (RAPD) analysis

Eighteen random decamer oligonucleotide Operon primers from A, C, D and N series were used for RAPD analysis. Out of these, ten primers responded well and gave very good amplification. 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 step 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 consisted of three temperature steps i.e. one min at 92 ^{0}C for denaturation of template, one min at 37^{0}C for primer annealing followed by two min at 72^{0}C for primer extension. The final step consisted of only one cycle *i.e*. 7 min at 72 ^{0}C for complete polymerization. The soak temperature was 4^{0}C. After the completion of the PCR 2.5 ml of 6X loading dye (MBI Fermentas, Lithuania) was added and amplification products were detected using 1.5% agarose gel stained with 0.5 ìg/ml ethidium bromide at 65V for 2 hours.

2.5. Inter simple sequence repeat (ISSR) analysis

Inter Simple Sequence Repeats were used for PCR amplification. 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). Out of twenty ISSR primers, eleven primers showed reproducibility. 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. 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 (as indicated in the Table 2) for 1 min and primer extension at 72 ^{0}C for 2 min. In the subsequent 42 cycles, the period of denaturation was reduced to 1 min while the primer annealing and primer extension time was the 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 2% agarose gel stained with ethidium bromide.

2.6. Data analysis and construction of phylogenetic tree

The presence/absence of bands in RAPD/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): Rp = ∑IB, 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 modified CTAB protocol yielded good quality of DNA as revealed by Agarose Gel Electrophoresis (Fig.-1). The concentration of whole genomic DNA isolated from 30 samples varied from 60 ng μl^{-1} to 1.5 µg μl^{-1}.

3.2. RAPD analysis

All the 30 samples produced distinct reproducible amplifications with 10 selected RAPD primers out of 18 primers tried. The banding pattern with RAPD primers are represented in Fig. 2 & 3. All the primers amplified wide range of fragments ranging from 100 bp to >3000 bp. With these primers, a total of 79 loci were amplified. The highest numbers of bands (11) were amplified by the primers OPN4 and lowest by the primer OPN6 (3). The highest Resolving Power (RP) was 16.72 for the primer OPN4 and highest PIC (0.37) for OPD20. The details of RAPD analysis are presented in Table-1.

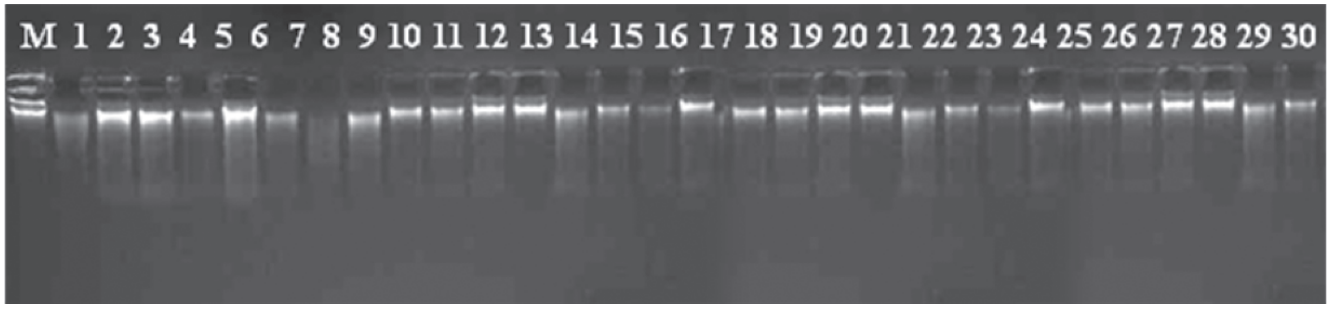

Fig.l: Qualitative and quantitative analysis of total genomic DNA by 0.8* agarose gel. M, uncut phage DNA (600ng); 1 to 5. *H.ovalis.* lanes 6 to 10. *H. ovata.* lanes 11-15, *H.beccarii*. lanes 16-20. *H. uninervis.* lanes 21-25, *H.pinifolia.* and lanes 26-30, C. *serrulata*

Table 1

List of primers used for RAPD amplification, total number of loci, level of polymorphism, resolving power and PIC value

Primer	Primer sequence (5'-3')	Annealing Temperature	Total no. of loci	NPL	(%) PPL	No. of fragments amplified	Rp	PIC
OPA4	AATCGGGCTG	37	9	7	77.7	513	16.52	0.14
OPA11	CAATCGCCGT	37	10	7	70	499	16.08	0.29
OPA18	AGGTGACCGT	37	9	6	66.6	438	14.1	0.32
OPA3	AGTCAGCCAC	37	10	8	80	601	19.4	0.05
OPA20	GTTGCGATCC	37	6	1	16.6	362	11.68	0.05
OPD18	GAGAGCCAAC	37	7	3	42.8	428	13.78	0.04
OPD20	ACCCGGTCAC	37	7	5	71.4	325	10.46	0.37
OPN6	GAGACGCACA	37	3	0	0	186	6	0
OPN16	AAGCGACCTG	37	7	5	71.4	426	13.74	0.04
OPN4	GACCGACCCA	37	11	8	72.7	519	16.72	0.34

NPL: No. of polymorphic loci; PPL: Percentage of polymorphic loci; Rp: Resolving power; PIC: polymorphic loci information content

The dendrogram (Fig. 4) divided the members of two families (Hydrocharitaceae and Cymodoaceaceae) into two distinct clusters, each group with three species. The cluster *Halophila ovata, H. ovalis* and *H. beccarii* shared a common node with *Cymodocea serrulata-Halodule pinifolia-Halodule uninervis* at a similarity level of 23%. Each cluster was further divided into sub-clusters in the dendrogram. While *Haophila ovata* and *Halophila beccarii* came together, *Halophila ovalis* got separated from them. Similarly, *Halodule uninervis* and *Cymodocea serrulata* were segregated from *Halodule pinifolia* at 27% level of similarity. Of all accessions, two genotypes of *Halophila ovalis* (Ho3 and Ho4) were found to exhibit similarity of as high as 99%. In general, all accessions of a particular species formed compact group in the dendrogram.

3.3. ISSR analysis

Out of twenty ISSR primers, ten primers were found to be responsive. A total of 84 bands were amplified, of which 54 bands were polymorphic in nature (Table-2). Therefore, the overall polymorphism was as high as 64.28% (Fig. 5). The maximum numbers of bands (11) were amplified in both $(GA)_9T$ and $(AG)_8T$ and minimum (6) with the primer $(AGG)_6$. The maximum number of polymorphic bands (90.9%), were amplified by the primer $(AG)_9T$. The resolving power for the primer $(AG)_{9T}$ was 22.12 and for the primer $(GACA)_4$ was 9.02. The primer index for the primer $(AG)_8T$ was 0.15 and for the primer $(GAC)_5$ was 0.06.

Two distinct clusters were observed in the dendrogram constructed using ISSR data with 31% similarity between them (Fig. 6). Three species of *Halophila* of Hydrocharticeae family were separated from members of Cymodoceaceae represented by two species of *Halodule* and one species of *Cymodocea*. Two accessions of *Halophila ovata* (Hv3 and Hv4) were found to have maximum genetic similarity (99%) between them followed by two genotypes of *Halophila ovalis* (Ho3 and Ho4) with 97% similarity.

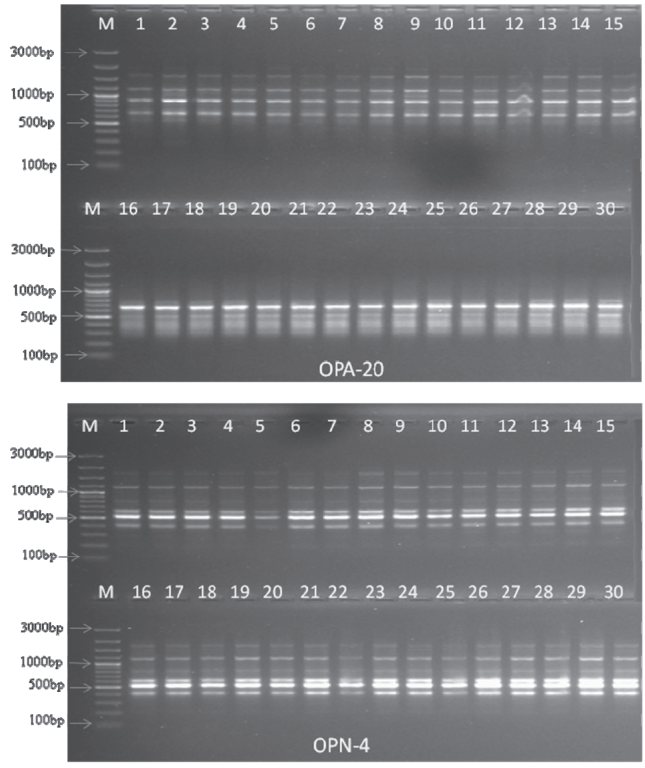

Fig. 2 & 3. RAPD banding patterns of 30 accessions of three genera of sea grasses as revealed by the primer OPA-20. OPN-4; gene ruler (Medium range) 3kb, lanes 1 to 5. *H.ovalis* lanes 6 to 10, *H.ovata*, lanes 11-15, *H.beccarii*, lanes 16-20, *H.uninervis*, lanes 21-25 *H.pinifolia* and lanes 26-30, *C.serrulata*

3.4. Combined markers analysis

Out of 38 primers (18 RAPD + 20 ISSR) used for genetic diversity assessment of 30 accessions of 6 seagrass species, 21 primers were responsible to produce scorable bands. The dendrogram constructed using RAPD and ISSR markers in combination showed an average similarity of 63% among the species (Fig. 7).

3.5. Dendrogram showing clustering pattern

In the dendogram, the six seagrass species studied got separated into two major clusters sharing a node at a 27% similarity. One cluster comprised of three species of the family Hydrocharitaceae (*Halophila ovata, H. ovalis* and *H. beccarii*) and the other with three species of Cymodoceaceae (*Halodule pinifolia, H. uninervis* and *Cymodocea serrulata*). Subsequently, all accessions of a particular species formed clear clusters with varying levels of similarity among them.

4. Discussion

Identification of seagrass species relied on morphological characteristics till date. Seagrasses possess similar morphological and physiological features that facilitate their survival in marine habitats (Les *et al*., 1993; Philbrick and Les, 1996). The possibility of convergent evolution of morphological characters in this group of plants has led to a number of different hypotheses concerning their origins, phylogenetic relationships and evolution (den Hartog, 1970; Larkum and den Hartog, 1989; Cox and Humphries,

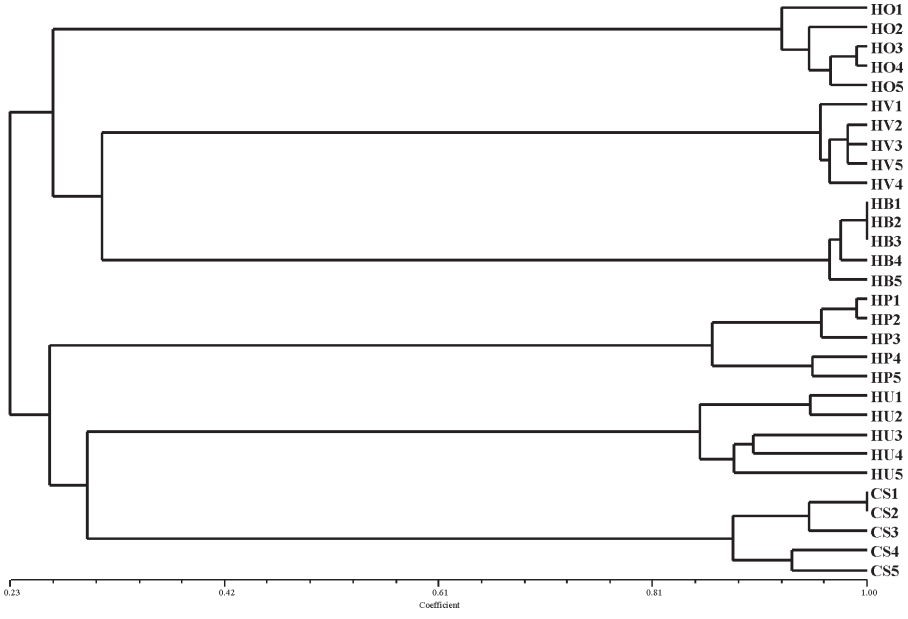

Fig. 4. Dendrogram showing the clustering pattern of 30 accessions of 6 species of seagrass as revealed from RAPD markers

1993; Les and Haynes, 1995; Les *et al.,* 1993, 1997; Philbrick and Les, 1996; Waycott and Les, 1996). At times, separation of different seagrass species becomes challenging, even for a seagrass taxonomist. However, there was the need for a fast, reliable, and cost-efficient system for recognition and identification of seagrasses also by non-experts. In addition, there are a number of questions by ecologists concerning the composition of the seagrass meadows where some unexpected species have been found mingled with known species of the specific habitat. These kinds of questions need to be answered using technically simple molecular data in conjunction with morphological characters. Seagrasses have both sexual and asexual reproduction but the flower, as a distinct morphological trait, is hardly ever found (Papenbrock, 2012). This makes identification of seagrass species difficult. As observed by Short *et al*. (2007), genetic analysis provides a tool to clarify species identity, diversity and distribution.

In the present study, the genetic diversity and phylogeny of three species of Hydrocharitaceae namely, *Halophila ovata, Halophila ovalis* and *Halophila beccarii* and three species of Cymodoceaceae such as *Halodule pinifolia, H. uninervis* and *Cymodocea serrulata* were investigated using RAPD and ISSR markers. Very close genetic similarities (84-95%) were found among accessions of each species justifying their taxonomic identity as species. All the 15 accessions of three species of *Halophila* belonging to the family Hydrocharitaceae and the other 15 accessions of two species of *Halodule* and *Cymodocea serrulata* of family Cymodoceaceae formed distinct phylogenetic clades in the dendrograms constructed using RAPD, ISSR data and the combination of the two sharing nodes sharing nodes at similarity level of 27-31%. Similar segregation of seagrass species from Lombok Island of Indonesia in to two clades as per their family affiliation (Hydrocharitaceae and Cymodoceaceae) in the phylogenetic tree construction based on rbcLgene on has been reported by Suhardi & Susandarini (2017). Lucas *et al*. (2012) on the basis of *rbcL* and *matK* sequence analysis, divided the seagrasses into major clades which represented Hydrocharitaceae, Zosteraceae and Cymodoceaceae families.

Table 2

List of primers used for ISSR amplification, total number of loci, the level of polymorphism, resolving power and PIC value

ISSR Primer	Primer sequence	Annealing Tem. (^{0}C)	Total no. of loci	NPL	(%) PPL	No. of fragments amplified	Rp	PIC
(AGG)6	AGGAGGAGGAGGAGGAGG	55	6	5	83.3	354	11.42	0.09
(GA)9T	GAGAGAGAGAGAGAGAGAT	51	11	10	90.9	686	22.12	0.13
(GAC)5	GACGACGACGACGAC	45	9	5	55.5	541	17.44	0.06
(GACA)4	GACAGACAGACAGACA	43	5	3	60	279	9.02	0.12
(GTG)5	GTGGTGGTGGTGGTG	45	7	5	71.4	414	13.34	0.09
(GTGC)4	GTGCGTGCGTGCGTGC	51	7	4	57.1	417	13.46	0.07
(CAA)5	CAACAACAACAACAA	35	3	2	66.6	176	5.68	0.1
(GGA)4	GGAGGAGGAGGA	35	7	4	57.1	413	13.32	0.09
(AG)8T	AGAGAGAGAGAGAGAGT	45	11	7	63.6	621	20.02	0.15
(GT)8A	GTGTGTGTGTGTGTGTA	45	8	5	62.5	463	14.92	0.12
T(GA)9	TGAGAGAGAGAGAGAGAGA	51	10	4	40	590	19.04	0.09

Among the three species of *Halophila, H. ovata* and *H. beccarii* were found to be genetically close to each other with maximum similarity of 38% and *H. ovalis* was distantly placed. Analysis of ITS sequence data also supported segregation of *H. beccarii* and *H. ovalis* (Waycott *et al*., 2002). Within Cymodoceaceae family, there was no clear grouping of species according to their generic affiliation. The two species of *Halodule* (*H. pinifolia and H. uninervis*) never got together in the cladogram. While *Cymodocea serrulata* formed a cluster with *Halodule uninervis* in the dendrogarm constructed with RAPD data, it came together with *Halodule pinifolia* in the cladogram generated with ISSR data. Using ITS sequences, *Halodule* and *Cymodocea* were grouped in different clades (Nguyen *et al*. 2015), a view supported by Pharmawati *et al*. (2016), who used *matK* sequences for this study. However, using 18S rDNA sequence

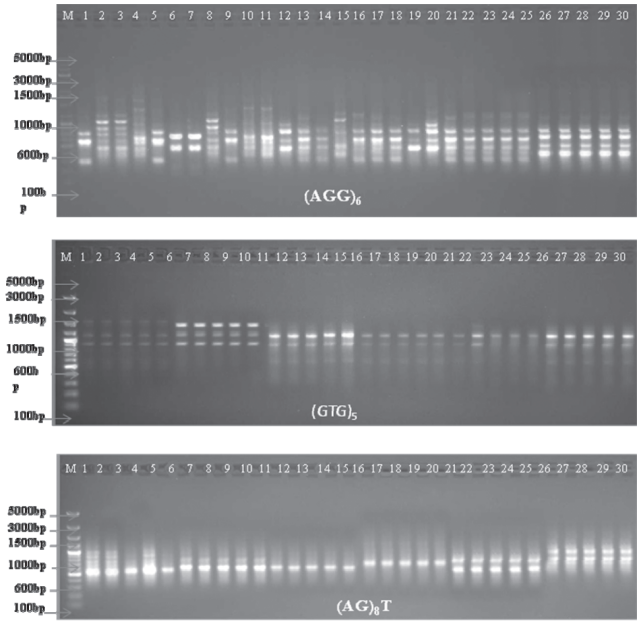

Fig. 5: ISSR banding patterns of 30 Accessions of three genera of sea grasses as revealed by the primar $(AGG)_6$, $(GTG)_5$ and $(AG)_8$ T:gene rules (Medium range) 5kb. lanes 1 to 5. *H.ovalis*, lanes 6 to 10, *H.ovata*, lanes 11-15. *H.beccarii,* lines 16-20, *H.uninervis,* lanes 21- 25. *H.pinifolia* and lanes 26-30, *C.serrulata.*

data, Dilipan et al. (2016) found that *H. pinifolia* is monophyletic and *H. uninervis* might have originated from *H. pinifolia.* However, Nguyen et al. (2015) and Peterson *et al*. (2014) suggested that Cymodoceaceae might be a non-monophyletic group. As suggested by them, sequences from nuclear, chloroplast and mitochondrial DNA need to be carefully combined to further clarify whether Cymodoceaceae is a monophyletic or non-monophyletic group.

The present paper is the first of its kind demonstrating the successful application of RAPD and ISSR markers to characterize the genetic diversity of seagrasses of Eastern Indian coast. These techniques can me fruitfully utilized for identification of species of *Halodule* and *Halophila* occurring in Chilika lake at vegetative or juvenile stage, which are otherwise difficult to identify using morphological characters. However, use of other markers like rbcL, matK and trnK with conserved sequences may throw more light on the taxonomy and plasticity of phenotypes of the seagrasses.

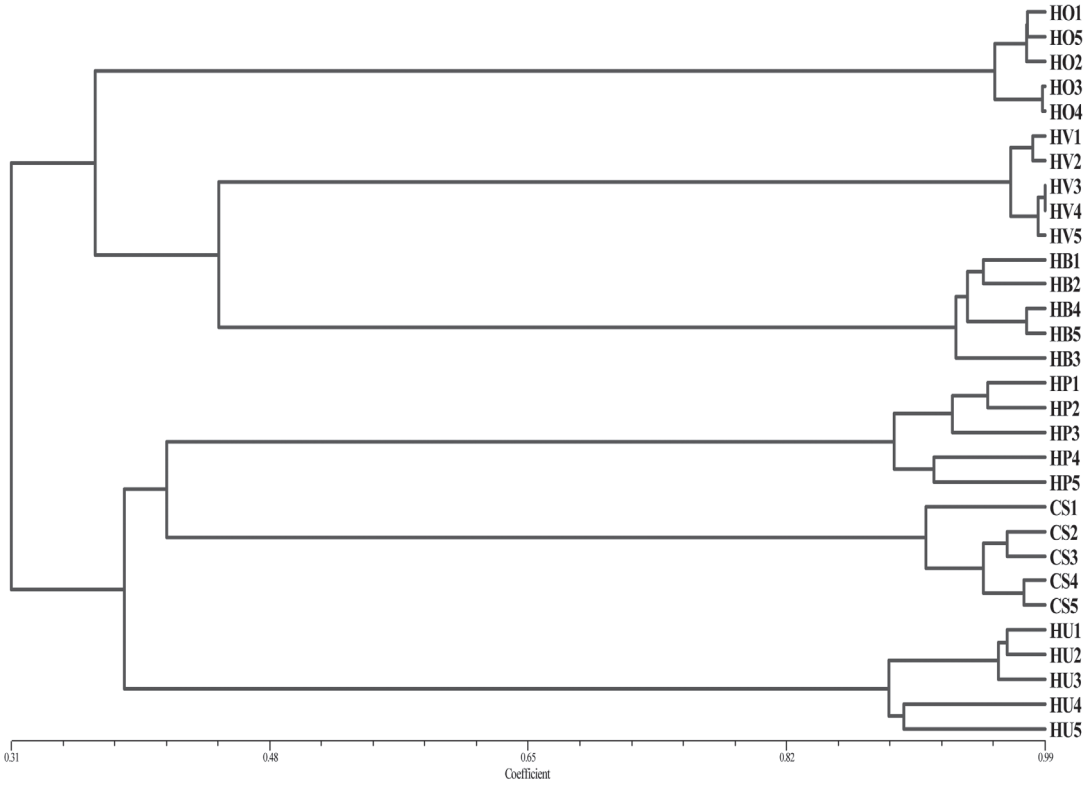

Fig. 6. Dendrogram showing the clustering pattern of 30 accessions of 6 species of seagrass as revealed from ISSR markers

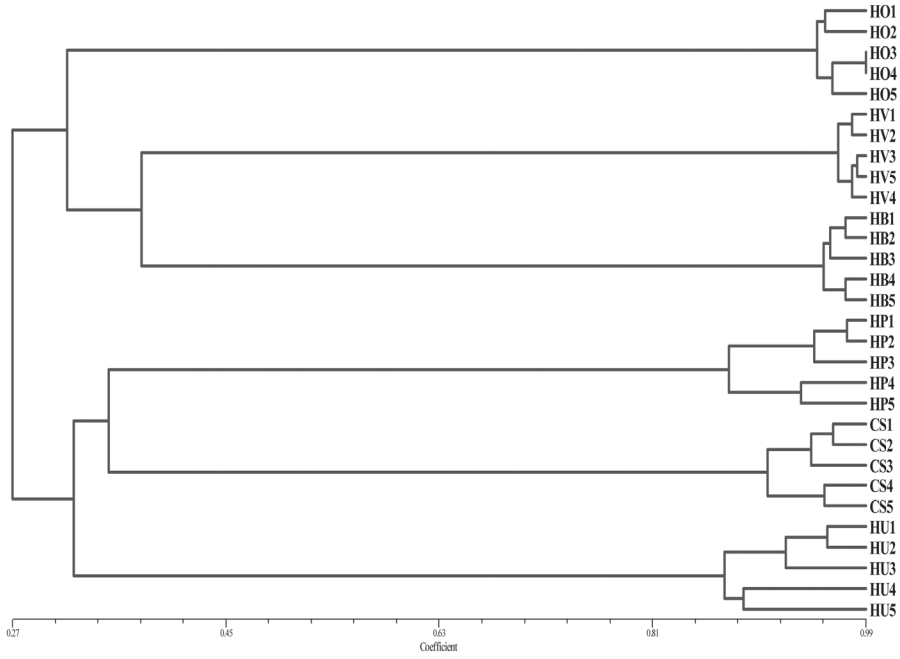

Fig. 7. Dendrogram showing the clustering pattern of 30 accessions of 6 species of seagrass as revealed from combination of RAPD and ISSR markers

Acknowledgements

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Taxonomic enumeration of the tribe *Aristideae* (Aristidoideae: Poaceae) in India

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ABSTRACT

Based on literature survey, herbarium consultation and authors' fieldwork, an updated checklist of 17 species of the tribe *Aristideae* (Poaceae) under two genera reported to occur in India has been prepared and presented in this paper. General note on the tribe and key to genera and species have been provided along with correct botanical name, synonym (s), citation of nomenclatural type, chromosome number (wherever available) and distribution of each species in the enumeration. Two species namely, *Aristida redacta* and *Aristida stocksii* are endemic to India.

1. Introduction

The grasses (Poaceae) are undoubtedly the most important group of plants in view of their economic and ecological significance. They provide all the major cereal crops for human consumption and forage for wild and domestic herbivores. Till date, 12 subfamilies, 51 tribes, 80 sub-tribes, 771 genera and ca. 12074 species have been recognised in the family Poaceae (Soreng *et al*., 2014). Among them, members of the tribe *Aristideae* (subfamily: Aristidoideae) are wide spread in the tropical and subtropical regions of the world. In general, many species of the genera *Aristida* and *Stipagrostis* are adapted to arid and semi-arid environments, such as deserts and sand dunes (Danin, 1996) and are conspicuous components of some vegetation types. The subfamily Aristidoideae Caro (1982) contains only one tribe *Aristideae* C. E. Hubbard (1960) with three genera: *Aristida* with 290 species, *Stipagrostis* with 50 species and *Sartidia* with 5 species and they are distributed throughout the world (Balkwill *et al*., 2011; Bourreil, 1967; Cerros *et al*., 2011; Clayton & Renvoize, 1986; Ghasemkhani *et al*., 2008; De Winter, 1965; Watson & Dallwitz, 1992). Members of *Aristideae* are characterised by their paniculate inflorescence, spikelets with bisexual florets, 2 glumes, female-fertile floret one, which consist of a single cylindrical, elongated floret per spikelet; and ligule with a dense fringe of hairs. The lemma is with three-awns (the awns separate from each other), palea short, less than half the length of lemma, involutes or with overlapping margins and often have a sharp-pointed callus and the floret disarticulating above long glume, lodicules present or rarely absent, stamens 1 to 3, ovary glabrous, styles 2, free, close, stigmas 2, caryopsis with the hilum short or long linear, endosperm hard (Cerros *et al*., 2011).

The first comprehensive account of the genus *Aristida* was provided by Hooker (1897) in Flora of British India, which enumerated 11 species. Subsequently, Bor (1960) reported the occurrence of 21 species from the Indian subcontinent. Karthikeyan *et al*. (1989) reported 10 species in Florae Indicae Enumeration and Moulik (1997) enumerated 11 species and 2 varieties of *Aristida* and 9

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species of *Stipagrostis* from India. An enumeration of 10 species of *Aristida* and 7 species of *Stipagrostis* have been provided below along with correct botanical name, synonym (s), citation of nomenclatural type, chromosome number (wherever available) and range of distribution of each species. Besides, the keys to genera and species have been provided for easy identification of taxa. Two species namely, *Aristida redacta* and *Aristida stocksii* are found to be endemic to India.

Key to genera of *Aristideae* C. E. Hubb.

1 Awns all or only the central awn plumose ..... *Stipagrostis*

1 Awns never plumose. ..... *Aristida*

Enumeration of taxa

ARISTIDA L. Gen. Pl. ed. 5, 35 (1754) *et* in Sp. Pl. ed. 1, 82 (1753)

[Type: *Aristida adscensionis* L. Gen. Pl. ed. 5: 1754; Sp. Pl. ed.1, 82 (1753). *Kieboul* Adans., Fam., Pl. 2: 31. 1763. *Chaetaria* P. Beauv., Ess. Agrostgr., 30. t. 8. f. 5, 6. 1812. *Curtopogon* P. Beauv., Ess. Agrostogr., t. 8. f. 7. 1812. *Arthratherum* P. Beauv., Ess. Agrostogr., t. -8. f. 8., *Streptachne* H.B.K., Nov. Gen. Et Sp. 1: 124. t. 40. 1816. *Trixostis* Rafin. in Ser. Bull. Bot. 1: 221. 1830. *Moulinsia* Rafin. in Bull. Bot. Geneva 1: 221. 1830. *Schistachne* Fig. et De Not. in Mem. Acad. Torin, ser. 2. 12: 252. 1852.

Cytology: chromosome base number, x=11 & 12, 2n= 22, 24, 36, 44, 48 & 66. 2, 4 & 6 ploid.

Distribution: Temperate and subtropical. Xerophytic.

Key to species of *Aristida* L.

1 No articulation between the lemma and the awns

2 Lateral awns much reduced and of a different texture from the third awn .....*A. redacta*

2 Lateral awns well-developed, if shorter than the central awn, of the same texture

3 Annual; awns unequal .....*A. cumingiana*

3 Annuals or perennials; awns equal

4 Panicles contracted

5 Spikelets more than 12 mm long; floret callus 1.5 mm long .....*A. setacea*

5 Spikelets up to 12 mm long; floret callus 0.5 mm long .....*A. adscensionis*

4 Panicles effuse; often as broad as long

6 Spikelets green or yellow at maturity; lower glume 12 mm long with an awn 2 mm long; upper glume 18 mm long, shortly awned; lemma 12 mm long with callus 2 mm long; panicle 20 cm long ..... *A. hystrix*

6 Spikelets purplish at maturity; lower glume 15 mm long; upper glume up to 20 mm long; inclusive of an awn 5 mm long; lemma up to 15 mm long with callus 1 mm long; panicle 30-40 cm long .....*A. cyanantha*

1 Articulation between the lemma and the awns

7 Articulation between the awns and the lemma situated at the top of the column, i.e. just below the spreading awns .....*A. mutabilis*

7 Articulation is between the tip of the lemma and the base of the column

8 Lower glume shorter than the upper; callus forked at the base ..... *A. hystricula*

8 Lower glume always longer than the upper; base of the callus pointed

9 Lower glume 25-28 mm long; the upper glume 6 mm long ..... *A. stocksii*

9 Lower glume 20 mm long; the upper glume 15-19 mm long ..... *A. funiculata*

Aristida adscensionis L. Sp. Pl. 82. 1753. *Aristida interrupta* Cavan., Ic. et Desr. Pl. 5: 45.t. 471.f.2.1799. *A. canariensis* Willd., Enum. Pl. Berol. 99. 1809. *A. divaricata* Jacq., Eclog. Gram. Rar. 7.t.6. 1813. *A. coarctata* H.B.K., Nov. Gen. et Sp. 1: 122. 1816. *A. fasciculata* Torr. in Ann. Lyc. Nat. Hist. 1: 154. 1824. *A. nigrescens* J.S. Presl ex C.B.Presl. Rel. Haenk. 1: 223. 1830. *A. curvata* Nees var. *abyssinica* A.Rich. Tent. Fl. Abyss. 2: 392. 1851. *A. maritima* Steud., Syn. Pl. Glum. 1: 137. 1854. *A. schaffneri* Fourn., Mex. Pl. 2: 78. 1886. *A. heymannii* Regel in Act. Hort. Petrop. 7: 649. 1881. *A. debilis* Mez in Fedde, Rep. Sp. Nov. 17: 151. 1921. *A. pumila* Dene in Ann. Sci. Nat. Ser. 2. 40: 85. 1835.

Distribution: INDIA (Jammu & Kashmir, Kerala, Maharashtra, Punjab, Rajasthan, Tamil Nadu and Uttar Pradesh), BURMA, PAKISTAN and SRI LANKA.

Aristida cumingiana Trin. & Rupr., Sp. Gram. Stipac. 141. 1892. *A. capillacea* Caran., Ic. et Descr. Pl. 5:43. 1799. *A. trichodes* (Nees) Walpers in Ann. Bot. Syst. 3: 753. 1853. *Chaetaria trichodes* Nees ex Lindl. in Hook., Kew J. Bot. 2: 101. 1850. *Aristida delicatula* Hochst. ex A.Rich., Tent. Fl. Abyss. 2: 393. 1851.

Distribution: INDIA (Madhya Pradesh, Bihar), CHINA, INDONESIA, LASOS, MYANMAR, NEPAL, NEW

GUINEA, PHILIPPINES, THAILAND, VIETNAM, AFRICA and NORTH AUSTRALIA.

Aristida cyanantha Steud., Syn. Pl. Glumac. 10 (2): 141. 1854; Boiss., Fl. Or. 5:492. 1884. Hoo.f. Fl. Brit. Ind. 7: 225. 1896; Bor. Grasses Burma Ceyl. Ind. Pak. 409. 1960.

Distribution: INDIA (Himalayas, Jammu Kashmir, Uttar Pradesh), AFGHANISTAN, NEPAL and PAKISTAN.

Aristida funiculata Trin. et Rupr., Sp. Gram. Stipac. 159. 1842. *A. stipacea* Ehrenb. et Hempr. ex Trin. et Rupr. Sp. Gram. Stip. 159. 1892. *A. macranthera* A.Rich., Tent. Fl. Abyss. 55. 2: 393. 1851. *A. kotschyi* Hochst. ex Steud., Syn. Pl. Glum. 1: 142. 1854; Hook.f. Fl. Brit. Ind. 7: 226. 1896; Blatter & McCann, Bombay Grasses, 214. 1935; Bor. Grasses Burma Ceyl. Ind. Pak. 410. 1960.

Distribution: INDIA (Punjab, Rajasthan and Tamil Nadu), ARABIA, NORTH AFRICA and PAKISTAN.

Aristida hystricula Edgew. J. Proc. Linn. Soc. Bot. 6, 208. 1862; Bor. Grasses Burma, Ceyl. Ind. Pak. 410. 1960.

Distribution: INDIA (Punjab) and PAKISTAN.

Aristida hystrix L.f., Suppl. Pl. 113. 1781. *Aristida rigida* Roth, Nov. Pl. Sp. 42. 1821 non Cavan 1799. *Chaetaria hystrix* (Linn.f.) P.Beauv., Ess. Agrost. 30. 1812.

Distribution: INDIA (Madhya Pradesh, Tamil Nadu and Kerala) SRI LANKA.

Aristida mutabilis Trin. & Rupr., Sp. Gram. Stipac. 150. 1842. *Aristida longeradiata* Steud., Syn. Pl. Glum. 1: 140. 1854. *Aristida articulata* Edgew. J. Linn. Soc. Bot. 6: 209. 1862; Hook. f. Fl. Brit. Ind. 7: 226. 1896; Blatter & McCann, Bombay Grasses 212. 1935; Bor. Grasses Burma, Ceyl. Ind. Pak. 411. 1960.

Distribution: INDIA (Punjab, Rajasthan & Tamil Nadu), ARABIA, TROPICAL AFRICA, and PAKISTAN.

Aristida redacta Stapf in Bull. Misc. Inform. Kew 85. 1892. *Stipa aristoides* Stapf ex Lisboa in J. Bomb. Nat. Hist. Soc. 7: 358. 1892.

Distribution: INDIA (Karnataka, Madhya Pradesh, Maharashtra, Rajasthan, Tamil Nadu & West Bengal), Endemic.

Aristida setacea Retz., Observ. Bot. (Retzius) 4: 22. 1786. *Chaetaria setacea* (Retz.) P.Beauv., Ess. Agrost. 30: 158. 1812. *Aristida quinquesets* Steud., Syn. Pl. Glum. 1: 420. 1855.

Distribution: INDIA (Andhra Pradesh, Bihar, Gujarat, Himachal Pradesh, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Odisha, Rajasthan, Tamil Nadu, Uttar Pradesh & West Bengal), MYANMAR, SRIL LANKA, AFRICA, MADAGASCAR, ASIA-TROPICAL, BURMA, MALESIA and INDO-CHINA.

Aristida stocksii (Hook.f.) Domin in Biblioth. Bot. Heft 85: 338.1915. *Aristida funiculata* var *stockii* Hook.f., Fl. Brit. Ind. 7: 227. 1897.

Distribution: INDIA (Andhra Pradesh, Karnataka & Maharashtra), Endemic (Anil Kumar *et al*., 2016).

STIPAGROSTIS Nees. in Linn 7: 290. 1832.

[Type: *Stipagrostis capensis* Nees in Linnaea 7 (3): 291. 1832. *Stipagrostis* (Nees) Benth. & Hook.f. Gen. Plan. 3 (2): 1141. 1883. *Stipagrostis* (Nees) Trin. & Rupr. Sp. Gram. Stipac. 163. 1842. *Stipagrostis* (Nees) Henrad, Meded. Rijks-Herb. 38: 34, 45-46. 1929.]

Cytology: Chromosome base number, x=11. 2n= 22 and 44. 2 and 4 ploid.

Distribution: INDIA, AFRICA and SOUTHWEST ASIA.

Key to species of *Stipagrostis*

1 Central awn feathery throughout or at its upper part; lateral awns glabrous

2 Lowest leaf-sheaths pilose ..... *S. plumosa*

2 Lowest leaf-sheaths not pilose

3 Central awn plumose throughout

4 Glumes sub-equal, column of lemma about 8 mm long ..... *S. uniplumis*

4 Glumes unequal, column of lemma about 15 mm long ..... *S. paradisca*

3 Central awn plumose in upper half, naked below

5 Leaf-blades filiform ..... *S. obtusa*

5 Leaf-blades lanceolate ..... *S. ciliata*

1 All the awns including lateral plumose

6 Lower leaf sheaths pilose; central awn much longer the laterals ..... *S. griffithii*

6 Lower leaf-sheaths glabrous; central awn as long as the laterals ..... *S. pungens*

Stipagrostis ciliata (Desf.) De Winter in Kirkia 3: 133. 1963. *Aristida ciliata* Desf. In Schrad. Neues J. Hot. 3: 255. 1809. *Aristida schimperi* Hochst. ex Steud. Ex Steud., Syn. Pl. Glum. 1: 143. 1854.

Distribution: INDIA (Punjab), AFGHANISTAN, ARABIA, EGYPT, ETHIOPIA, NORTH AFRICA, PAKISTAN and SUDAN.

Stipagrostis griffithii (Henrard.) De Winter in Kirkia 3: 134. 1963. *Aristida griffithii* Henrd. in Meded. Rijks Herb. Leiden 54: 213. 1926.

Distribution: INDIA (Punjab), ASIA- TEMPERATE, AFGHANISTAN and WESTERN ASIA.

Stipagrostis obtusa (Delile) Nees in Linnaea 7: 293. 1832. *Aristida obtusa* Delile, Fl. Egypt. 175. t. 13. f. 2. 1792.

Distribution: INDIA (Jammu & Kashmir), AFRICA, ARABIA, EGYPT and PAKISTAN

Stipagrostis paradisea (Edgew.) De Winter in Kirkia 3: 135. 1963. *Aristida paradisea* Edgew. J. Asiat. Soc. Beng. 16: 1219. 1848.

Distribution: INDIA (N.W. Himalayas, Jammu & Kashmir), ARABIA, BALUCHISTAN, PAKISTAN and SOMALIA.

Stipagrostis plumosa Munro ex T.A nderson in J. Linn. Soc. Bot. 5 (Suppl.1): 40. 1860. *Aristida plumosa* Linn. Sp. Pl. ed. 2: 1666. 1763.

Distribution: INDIA (Punjab), AFGHANISTAN and PAKISTAN.

Stipagrostis punges (Desf.) De Winter in Kirkia 3: 135. 1963. *Aristida punges* Desf., Fl. Atlant. 1: 109. t. 35. 1798.

Distribution: INDIA (Rajasthan), AFGHANISTAN, AFRICA and EGYPT.

Stipagrostis uniplumis (Litcht.) De Winter in Kirkia 3: 136. 1963. *Stipagrostis pogonopila* (Jaub. & Spach) de Winter in Kirkia 3: 135. 1963. *Arthratherum pogonoptilum* Jaub. et Spach, Illus. Pl. Orient. 4: 56. 1851. *Aristida pogonopila* (Jaub. et Spach) Schweinfurth et Ascherson, Beitr. Fl. Aethiop. 306. 1867.

Distribution: INDIA (Punjab) and PAKISTAN

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Mathematical modelling and simulation of a rice straw-based biogas plant

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ABSTRACT

India's failure to curb the current practice of burning millions of tons of rice crop residue each year is criticised worldwide as it is associated with environmental pollution and human health hazards. The aim of this work is to use a mathematical model to determine the biogas potential of rice straw when fed into a full-scale biogas plant. The Anaerobic Digestion Model No. 1 (ADM1) was utilized to explore the ideal operating conditions to run a rice straw-based plant for 60 days. The model predictions suggested feeding the industrial plant with rice straw co-digested with manure. Stable methane production was projected when 5 and 25% of the feed was rice straw and the rest was manure. An average methane content of 865 m^3 and 1100 m^3 was predicted by the model when 20m^3 of manure was fed with 1m^3 and 5m^3 of rice straw respectively. This mathematical model could be a useful, efficient and cost-effective tool for predicting energy generation from different organic waste materials.

1. Introduction

Every year nearly 90-140 million tons of rice crop residues are being burnt in India (Bisen, 2017). The burning of paddy stubbles and other residues in certain parts of India has been responsible for deterioration in air quality and is a serious national concern (Singh *et al*., 2017). Despite its adverse consequences, unfortunately till date, this is the most common practice followed to dispose the enormous volume of rice crop wastes. As an alternative to such unsustainable and hazardous practices, many researchers strongly advocate generating biogas, a carbon neutral 'clean' renewable source of energy and treat the carbon-rich rice waste as a resource instead (Rahman*et al*., 2017; Mussoline, 2013; Teghammar *et al*., 2012; Naresh, 2013). Biogas production from carbohydrate-rich substrates like that of rice residues offers an added advantage due to their high C/N ration resulting in higher methane production (Kataki *et al*., 2017; Daiem *et al*., 2018).

When operating biogas plants, it is extremely important to understand the process dynamics and deriving the ideal design and operational parameters applicable to the specific substrate fed. Mathematical models have proven to be useful tools to address this issue and are reliable for designing a biogas plant and the processes involved, for determining the optimal operating conditions and also enabling the users to effectively control the anaerobic digestion processes for a longer durations (Donoso*et al*., 2011).

1.1. Anaerobic Digestion Model No. 1

The Anaerobic Digestion Model No. 1 (ADM1) was developed by the International Water Association's (IWA) Task Group and is revered as the most advanced and reliable model for simulating biogas systems (Batstone *et al.*, 2002). This widely applicable model includes the major processes involved in the bioconversion of complex organic materials into CH_4, CO_2 and inert by-products along with various metabolic intermediates (Biernacki *et al*., 2013). Numerous steps describing the biochemical as well as the physio-chemical processes are considered by the model. The ADM1 model considers the several processes occurring simultaneously and incorporates 31 processes, 19 of which

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are algebraic differential equations and 2 remain for the physio-chemical reactions (Batstone *et al*., 2002).

Organic substrates are characterized mathematically to degrade further to fractions of carbohydrate (XCh), proteins (XPr), lipids (XLi) and inerts (XI). The model's strength is also enhanced due to its mass conservation approach where the input is converted to solid, liquid and gaseous forms as output. The four stages of anaerobic digestion *i.e.* hydrolysis, acidogenesis, acetogenesis and methanogenesis are well interpreted considering the microbial activities in the model with the help of seven different biomass fractions and their decay (Biernacki, 2014). The strength of the model also remains in its applicability to different types of complex substrates under different conditions and for designing and operating biogas reactors of any size.

2. Materials and methods

2.1. Designing the system in the model

A lactate-included ADM1xp model designed specifically for carbohydrate-rich reactors by Satpathy *et al*. (2016a) was utilized in this study. A real-scale biogas reactor previously designed by Biernacki*et al*. (2013) using SIMBA was considered for this study (Fig.1). The industrial biogas plant had two parallel fermenters each with a capacity of 3500 m^3 and has a provision to feed the reactor hourly. SIMBA simulation software enables an appropriate representation of the real biogas plant and the hygienization, mixing tank, gas storage etc. was aptly presented in the model. This virtual design was used in an attempt to determine the biogas potential when rice straw co-digested with cattle manure is fed into industrial reactors in real-life scenarios. Simulations were performed using SIMBA 6 based on MATLAB R2013a (The MathWorks, 2011) for 60 days to study the performance of the biogas plant over a longer duration run.

2.2. Model's input parameters

The animal manure considered in the model as inoculum was derived from the experimental data from Biernacki *et al*. (2013). Experimental results from Daiem *et al*. (2018) was utilized for characterizing rice straw as feed. The characteristics of rice straw and manure are outlined in Table 1 and these data were put into the convertor block of the model. This step of incorporating the characterized substrate into the convertor block is highly significant in order to sensitize the mathematical model to the specific substrate. When the substrates considered during simulations are well characterized, the model's prediction capabilities also get strengthened.

The manure block in the model was additionally provided with the acid contents from experimental data derived from Satpathy *et al*. (2016a) and were kept to be 0.5, 0.9, 0.64, 0.69 kg COD/m^3 for acetic acid, propionic acid, lactic acid and butyric acid respectively. After incorporating these data, several trial and error tests were performed to determine the ideal proportion of mixing the rice straw with animal manure of this composition. After the model indicated the manure volume to remain at least 20 m^3 when simulating the reactor, failing which the reactor showed instability, different volumes of rice straw were tested to determine the ideal combination ratio.

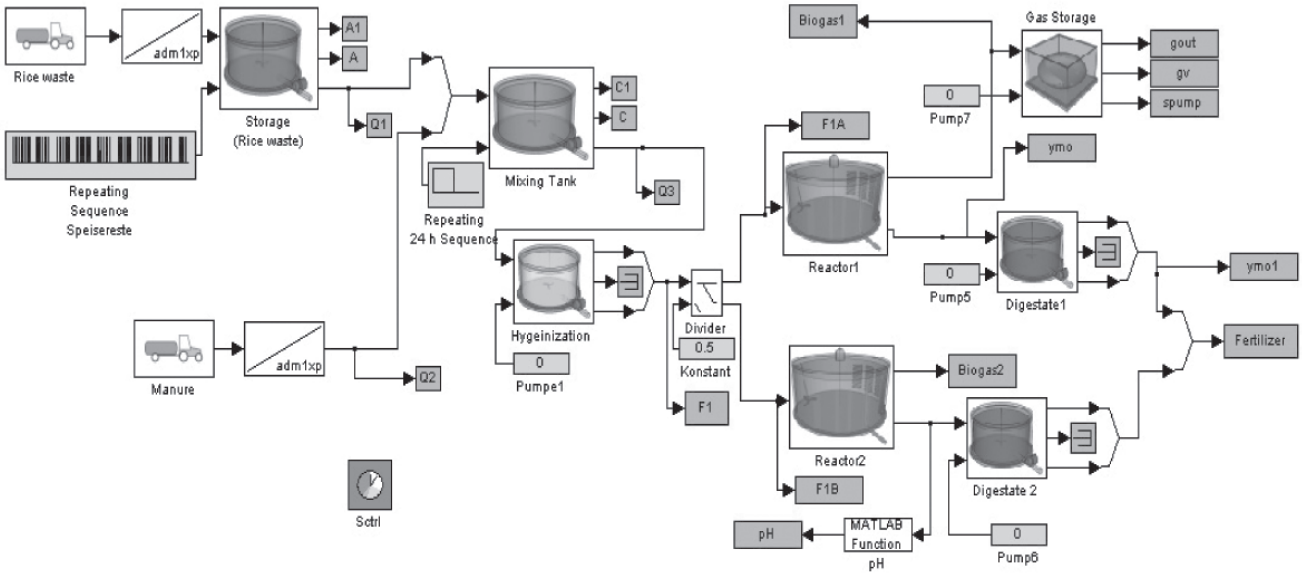

Fig 1. Model description of a full-scale industrial biogas plant prepared using SIMBA 6 simulation software derived from Satpathy *et al*. (2016b).

Table 1

Characterization of the rice straw and animal manure considered in this study (from experimental data of Daiem *et al*. (2018) and Biernacki *et al*. (2013) respectively).

Parameter (%)	Rice straw	Manure
Dry Matter (DM)	93.63	9
Organic Dry Matter (ODM)	69.38	80
Nitrogen (N)	0.52	3.5
Ammonium Nitrogen (NH_4)	0.01	2.2
Raw Protein	0.04	0.74
Raw Lipid	0.085	0.17
Raw Fibre	0.6	1.15
Inert fraction	0.3	0.509

3. Results and discussion

The ADM1 model demonstrated rice crop wastes to be a favourable substrate for biogas generation. A stable performance was predicted when the reactors were fed with 5 & 25% rice straw per day mixed with animal manure. Manures are significant since these provide effective buffering capacity and a healthy consortium of micro-organisms to generate biogas. The total CH_4 production daily on an average was determined to be 865m^3 and 1100 m^3 respectively when 1m^3 and 5m^3 of rice straw was mixed with 20m^3 of manure. Approximately, 50-51% of the biogas was predicted to be CH_4 with generation of 0 Hydrogen and the pH was maintained at 7. This in fact, is ideal for such carbohydrate-loaded biogas systems where maintaining stability is a challenge due to the increasingly available organic acids like acetic, propionic, butyric acid, valeric acid etc. resulting in further decrease in pH (Thamsiriroj *et al*., 2012; Satpathy, 2016).

When the digester was fed with 1m^3/day of rice straw (Fig. 2), the model displayed an immediate decrease in the amount of biogas which further revealed to have balanced with time. This could be due to the readily available intermediate organic compounds from the already degraded and hydrolysed rice straw and manure. This condition favoured the methanogens that ultimately resulted in a high methane formation at the initial stage. However, once the intermediate metabolic products were converted to biogas, degradation of a complex substrate like rice straw was slow as it is rich in lignin, cellulose and hemi-cellulose (Rahman *et al*., 2017). This leads to reduced availability of intermediate acids for different groups of bacteria and methanogens involved in the process of biogas formation. This is reflected in the decreased biogas production during the simulation trials. It is interesting to note that with time, the biogas production eventually starts improving. Such behaviour can be attributed to increased availability of the organic acids as the hydrolytic, acidogenic. acetogenic and methanogenic bacteria thrive and function efficiently while the conditions inside the digester become favourable with time (Luostarinen *et al*., 2011).

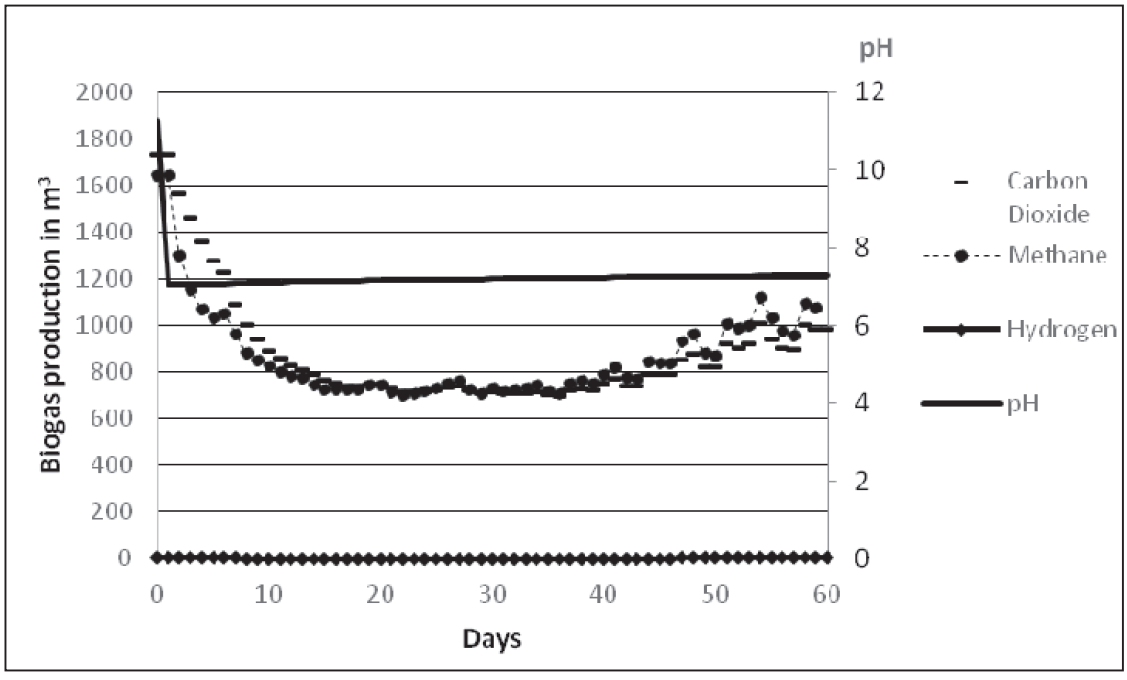

Fig. 2. Biogas formation predicted by the ADM1 model when 1m^3 of rice straw was fed with 20m^3 of animal manure.

With a rice straw feed of 5m^3/ day, the CH_4 production showed similar trend (Fig. 3). One remarkable difference was that despite the decrease in biogas production, the recovery period was relatively faster compared to the system fed with 1m^3/day. This is thought to be due to the increased C-content with the increased loads because of which the availability of the intermediate products for the methanogens and bacteria improved. The model's ability to predict the dynamic biological behaviour and simulate the processes reflecting the different stages and the several physio-chemical processes involved during formation of biogas production is noteworthy.

Digester instabilities are a common problem and there are many instances where initially the biogas reactors show extraordinary performance but with time there is an abrupt breakdown. The cause of such irregularities especially in carbohydrate-rich digesters can mostly be due to accumulation of organic acids which eventually lead to sudden drop in pH, thus disturbing the whole system (Seadi *et al*., 2014). During our simulation trials with an increased proportion of rice straw and comparatively less manure, we also observed such pH fluctuations and reactor failures. On the contrary, when simulated with a heavy manure load, the model clearly demonstrated steady operation over a longer duration. Thus, it is recommended to co-digest the carbohydrate-rich crop residue with higher volumes of animal manures to attain favourable conditions for successful biogas production.

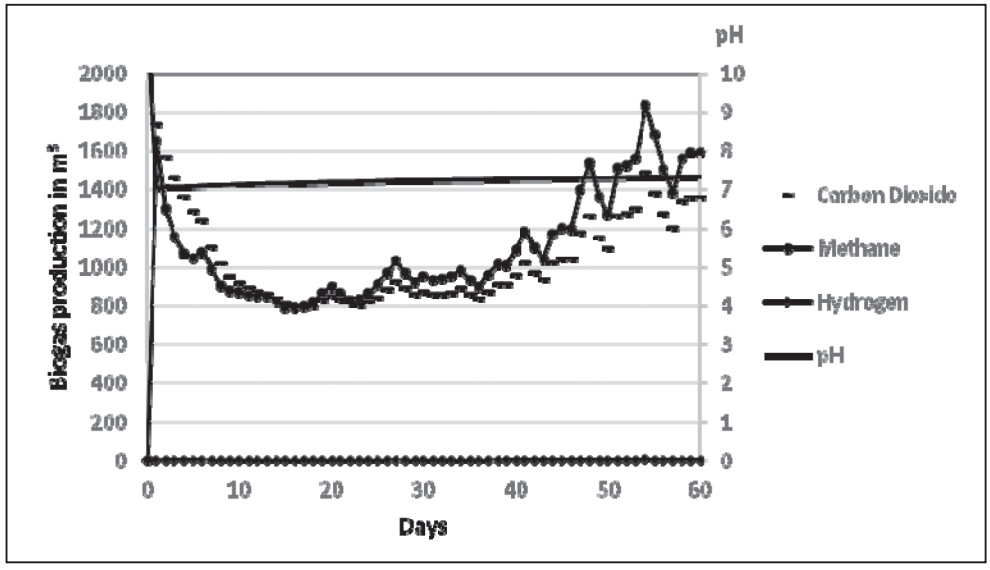

Fig. 3. Biogas formation predicted by the ADM1 model when 5m^3 of rice straw was fed with 20m^3 of animal manure.

4. Conclusion

Rice crop residues, being abundantly available and not finding applications either as a burning fuel or fodder due to its high silica content, offer additional scope for biogas generation (Rahman *et al*., 2017). The ADM1 model provided substantial insight into the biogas potential of rice straw to generate methane that can be utilized directly as a cooking gas or converted to electricity. The digestate attained from the output channel of such plants can also be effectively utilized as organic fertilizers. Thus, as an alternative to burning the millions of tons of the crop residues, generating the 'clean' carbon neutral biogas with minimal waste generation from such agricultural waste is strongly recommended. This would not only check the soil nutrient loss, environmental pollution and human health hazards but also help the farmers to generate additional revenues from the organic fertilizers.

The advantage of utilizing the ADM1 model is that it was instrumental in determining the ideal set of operational parameters specific to the substrate for maximum methane generation. The simulations also helped in understanding the possible inhibitions that might occur when operating with such complex substrates, thus pointing at taking necessary precautions beforehand. Biogas reactors have frequent encounters with reactor failures and sudden breakdowns. Such mathematical models could be effective in determining the ideal operating conditions and foresee the physio-chemical dynamics and performance of any type of biogas digester over a longer span of time *i.e*. even for several years. ADM1 model is thus recommended for designing and determining the ideal operating conditions for generating energy from different organic wastes that are otherwise discarded. Such models can be beneficial in determining the biogas potential on a virtual set-up without

performing elaborate experimental work, thus saving both time and money.

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Sustainable development and biodiversity conservation in North-East India: A review

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ABSTRACT

The present paper reviews the developmental activities of North-Eastern region of India and analyses the threats being experienced on biodiversity loss and ecosystem degradation. The north eastern states are rich in biological diversity and associated traditional knowledge and almost every part of the life and livelihood of local people is intricately connected with natural environment. Most of their livelihood earnings come from natural areas. The developmental pressure has resulted in denudation and conversion of large forest areas thus affecting the survival of flora and fauna. Several species in the region are endemic and critically endangered. We suggest sustainable measures to ensure that development is achieved with an inbuilt mechanism of biodiversity conservation with community participation. The region has potential of becoming knowledge partner with the development authorities through the existing good academic and research institutions. These institutions are capable of analysing and solving various environmental and biodiversity related issues and by providing a proper institutional framework by integrating the universities and research institutions of the region in to a research consortium, the primary goal of undertaking research on ecological problems and biodiversity conservation can be achieved. The development and institution interface as a long term strategy is needed to ensure implementation of biodiversity conservation programmes.

1. Introduction

North-Eastern Region of India comprises of Arunachal Pradesh, Assam, Tripura, Meghalaya, Manipur, Mizoram, Nagaland and Sikkim, covering an area of 262,179 sq kilometres with a population of 45 million as per 2011 census. The region is very rich in natural resources and is well recognised for its diversity of people, plants and animal life. These assets with socially, culturally complex and diversified region in the country, are now acknowledged as having the greatest potential for growth and development of the Region. The North-Eastern Region can be physiographically divided into the eastern Himalayas, the North-Eastern hills, and the Brahmaputra and Barak valley plains. At the confluence of the Indo-Malayan and Palearctic biogeographical realms, the region contains unique habitats characterized by diverse flora and fauna with a high level of endemism. The region is also home to more than 200 out of 450 of India's tribes with the culture and customs intricately linked to biodiversity conservation (World Bank, 2007).

1.1. Overview of the North-Eastern Region

Arunachal Pradesh is the largest state (area-wise) in the North-Eastern Region with rich alpine geographical diversity and a wide variety of wild life- flora and fauna. About 35% of the population of Arunachal Pradesh depends on agriculture and 17% of total cultivated area is under irrigation. Deposits of dolomite ore, limestone, graphite, quartzite, kyanite, mica iron-ore, copper ore have been reported (Behera, 2004). The state can be divided into five river valleys: the Kameng, the Subansiri, the Siang, the Lohit and the Tirap. The Arunachal Pradesh can be divided into three cultural groups on the basis of their socio-politico-religious affinities.

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Assam is known for its vast rolling plains, rarest flora and fauna, lofty green hills, mighty waterways and a land of fairs and festivals. Broadly, the inhabitants of Assam can be divided into three categories, namely the tribal population, the non-tribal population and the scheduled castes. About 63% of the state's population is engaged in agriculture and allied activities. About one sixth of the world's entire tea production comes from the state of Assam and the tea gardens contribute over half of the country's tea production. The state has extensive deposits of oil, coal, limestone, refractory clay, dolomite and natural gas. The oil reserves were discovered in the 19th century and Digboi became Asia's first oil refinery (Sadangi, 2008). Manipur is the land of rich valleys surrounded by beautiful hills and lakes. The picturesque valley of Manipur spreading over an area of 22,327 km. is an isolated hill state. The natural vegetation occupies an area of nearly 67% of the total state geographical area (FSI, 2017). Its economy is primarily agriculture, fisheries, cottage and forestry driven (Gonmei, 2013). Manipur has the highest number of handicrafts units as well as the highest number of craft persons comprising skilled and semi-skilled artisans in the entire North-Eastern region. Handloom is the largest cottage industry in Manipur and the state ranks among the top five in terms of the number of looms in the country (IBEF, 2018).

Meghalaya has historic, geographic and strategic significance for India. It is bound on north and east by the state of Assam, and on south and west by Bangladesh. The three physical divisions in the state are Garo (Western), Khasi (Central) and Jaintia (Eastern) hill divisions. The forest cover in the state is 76.45% of the state's geographical area (FSI, 2017). Meghalaya is basically an agricultural state with about 80 % of its total population being dependent on agriculture for livelihood. The state has a vast potential for developing horticulture due to agro-climatic variations, which offer much scope for the cultivation of temperate, sub-tropical and tropical fruits and vegetables. The mineral deposits in the state include limestone, coal, and granite among others.

The literacy rate in Mizoram is the second highest in the country. Agriculture is the mainstay for about 50-60% of the population of the state. Maize, paddy, pulses, oilseeds, millets are the crops grown in the state (Government of Mizoram, 2013). In Nagaland, almost all areas are in hilly region which is a continuation of the Burma Arc being joined with the Sub-Himalayan ranges in the north and stretching into the hills of Manipur. Unlike other NE states, the absence of Lakes and waterfalls in Nagaland can be conspicuous with some areas where water accumulates during rainy season and dries up in lean season. Life in Nagaland abounds with festivals throughout the year as all the tribes have their own festivals, which they greatly cherish. Most of these festivals revolve around agriculture, which is still the mainstay of the Naga society. Over 85% population of Nagaland is directly dependent on agriculture. Naga inhabits the wild, wide-open pastoral countryside.

Tripura is situated between the river valleys of Myanmar and Bangladesh. Encircled almost on the three sides by Bangladesh, it is linked with Assam and Mizoram in the northeast. Tripura lacks an industrial base except for some cottage industries (handicrafts and handloom) and small scale manufacturing units (Govt. of Tripura, 2012). Sikkim is the second smallest state of India bordering Bhutan, Tibet and Nepal. This Himalayan state has a dramatic landscape that includes India's highest mountain, the Kangchenjunga (8,586 m). The Mt. Kangchendzonga, Rivers Teesta and Rangit are magnificent assets of Sikkim with their deep ravines and valleys. The economy of Sikkim is mainly based on agricultural and animal husbandry. Approximately, 11% of the total geographical area is under agriculture. Agriculture is of the mixed type and still at the subsistence level rather than commercial level. There is a vast potential for hydro-electric power generation for which many river valley projects around Teesta and Rangit rivers have been established (ENVIS Centre Sikkim, 2007).

1.2. Ecological Importance of the region

The land area in Arunachal Pradesh encompasses mountains with the Himalayan range along the northern borders criss-crossed with ranges running north-south. The state has more than 500 species of birds h of which several birds are highly endangered and endemic *e.g.* white winged duck, Sclater monal, Bengal florican, etc. The rich and varied wildlife available in the state are Elephants, Tigers, Leopards, Jungle Cats, White Gibbon, Red Pandas, and Musk. The "*Mithun*" (*Bos forntails*) is domesticated in large numbers as well as available in the wild.

In Assam, endangered species of wildlife found includes Golden Langur, Hoolock Gibbon, Pygmy Hog, Hispid Hare, White-Winged Woodduck, Tiger, Clouded Leopard, Swamp Deer, Gangetic Dolphins (Sadangi, 2008). During winter season, flocks of resident and migratory birds make Assam their natural habitat. Guwahati, on bank of the Brahmaputra, is the city which is regarded as the commercial capital of the North-East. Kaziranga, the world-renowned ecosystem in Golaghat and Nagaon district is the home of the great Indian one-horned Rhino. Manas National Park is the only Tiger Reserve of Assam and is also a UNESCO's world heritage site. The Hoolock Gibbon, the Slow Loris, the clouded Leopard, the spotted Linsang, Tragopan, and

four different types of Hornbill are part of the rich natural heritage of Manipur.

The available records on biodiversity of Meghalaya revealed the occurrence of about 3,128 species of flowering plants which contribute to about 18% of the total flora of the country, including 1,237 endemic species (Khan *et al.,* 1997). A wide variety of wild and cultivable plants, edible fruits, leafy vegetables and orchids are found in the forests of Meghalaya. Meghalaya is endowed with a rich orchid flora of nearly 352 species belonging to 98 genera representing 27.08% of the country's orchid flora. A botanical wonder, the pitcher plant (*Nepenthes khasiana*) - an insectivorous species, is found in Jaintia hills, West Khasi hills and South Garo hills of the state (http://megbiodiversity.nic.in/floral-biodiversity.html). The wild animals and birds of the state include elephants, tigers, bear, wild buffalo, Hoolock Gibbon etc. (Choudhury, 2003). In Mizoram, forest accounts for nearly 26.76 % of its geographical area (FSI, 2017). The tropical forest of Mizoram has rich variety of flora and fauna. The bamboo groves dominate the lower altitude and orchids of various hues, pinkish-white *Bauhinia*, sparkling Rhododendrons, yellow sunflowers and many other colourful wild flowers are found in the state. Nagaland abounds in serene natural beauty and panoramic views of the hills which in fact are the eastern offshoots of the mighty Himalayas (Sadangi, 2008)

According to the State of Forest Report (FSI, 2017), 59.96 percent of the total geographical area of the state of Tripura is forest, which can roughly be divided into four types, viz. Sal, Garjan, Bamboo and miscellaneous species. The State has rich natural resources. There are 90 mammal species in Tripura and in the aquatic ecosystem 47 species of fish have been found. Endangered species like spectacle monkey, or Chasma Bandar which the state boasts of, is found only in Sepahijala Wild life Sanctuary in Tripura (MoEF&CC, 2012).

The Himalayan state of Sikkim is the storehouse of natural beauty and unique ecosystems like glaciers, alpine meadows and thousands of varieties of wildflowers. Sikkim forest and wilderness areas are inhabited by the Snow-Leopard, Yaks, Bharal or Blue Sheep, Shapi and the endangered Red Panda. Sikkim is a multi-ethnic state. Broadly, the population can be divided into tribal and non-tribal groups. Lepchas, Bhutias, Sherpas are categorized as Scheduled Tribes. Sikkim is a part of hot spot zone, gifted with abundant natural resources and is enriched with about 4500 species of flowering plants, 363 species of ferns and its allies, 11 species of oaks, 28 species of bamboos, 550 species of orchids and 36 species of *Rhododendron*. The state is also rich in fauna with 144 species of mammals, 550 species of birds and over 600 species of butterflies.

2. Initiatives towards sustainable development

2.1. Research and Development Infrastructure

Host of reputed research and academic institutions are available in NE region. We have only highlighted a few major institutions involved in ecosystem and biodiversity research. The Institute of Biodiversity and Sustainability Development (IBSD), Imphal, Manipur was established in 2001 under the Department of Biotechnology, Ministry of Science and Technology, Government of India with the main mandate of research on conservation and sustainable utilization of bioresources for the socio-economic development of the region. Its main goal is to work for the scientific management of bioresources in the Indian region falling under Indo-Burma Biodiversity Hotspot, to study and document the unique biodiversity of bio-geographic junction of the Indian and oriental landmasses, to develop biotechnological interventions for sustainable development and utilization of bioresources and to undertake capacity building programmes (human resource development) in bioresources conservation and management. The institute has now established its research centres in the states of Sikkim, Mizoram and Meghalaya. The thrust areas of research of the institute includes study of medicinal plants and horticultural resources, *in vitro* conservation of selected plant resources for sustainable utilization, characterization of plant bioactive compounds and molecular characterization. Bio prospecting of microbial resources such as study of molecular microbial ecology of food and gut fermentation, microbial risk assessment and development of molecular diagnostic platforms, modulation of gut microbiota by fermented foods and building up of a microbial consortium for development of functional foods and bio-ingredients, wildlife conservation using biotechnological tools are other areas being pursued by the institute. IBSD has initiated Entrepreneurship Development Programme for unemployed youth by conducting training programme on sustainable utilization of microbial and botanical products for promoting organic farming, fish hatchery, seed production technology and hands-on training on rearing and post cocoon technology in composite sericulture (IBSD, 2016).

The North Eastern Region Community Resource Management Project (NERCORMP) is a rural development project for six districts of three states of North-East India viz., Assam (Karbi Anglong and North Cachar Hills), Manipur (Ukhrul and Senapati) and Meghalaya (West Garo Hills and West Khasi Hills) with the overall objective of improving the livelihood of vulnerable groups in a

sustainable manner through improved management of resources. It is a joint initiative of the North-Eastern Council, Ministry of DoNER, Govt. of India and International Fund for Agricultural Development (*IFAD*). The aims of NERCORMP include involving the communities more in decision making and planning, to make communities more responsible for the management of their development programmes in order to generate a greater sense of ownership of development interventions and to be more responsive to community's perspectives on needs and priorities. NERCORMP has been concentrating on building up community organisations at the grassroots level called Natural Resources Management Groups (NaRMGs) and Self Help Groups (SHGs). Some major activities include economic and livelihood activities such as promoting viable income generation activities for poor households through production of field crops, horticulture, forestry, livestock fisheries and non-farm activities using sustainable and environmentally friendly activities. It also undertakes programmes involving community based biodiversity conservation, natural resource management and communication activities where communities are assisted to conserve their unique and natural resources and biological diversity, strengthening indigenous institutions and institutionalising new conservation practices and strengthening the information sharing system and documentation of good practices (NERCOMP, 2017).

The CSIR- North East Institute of Science and Technology (NEIST), Jorhat was established in the year 1961 as one of the multidisciplinary laboratories of Council of Scientific & Industrial Research (CSIR) and has been engaged in multidisciplinary R&D work. The mandate of the Institute is to put to effective use the immense material resources of North Eastern region and to provide R&D inputs for developing the economy of the region with the goal to design, develop and deliver knowledge-bases and technologies for gainful application based on natural resources available in region and to improve the quality of life in rural areas. The institute has taken up projects such as bioprospecting, assessment of genetic diversity of medicinal, aromatic and economic plants of North-East India, science and technological interventions in combating malnutrition in women and children. The institute undertakes scientific research on raising of clonal microgarden and validation of microclones of 'Assam Teak' (*Phoebe goalparensis*) through multi-locational trials in North-East Region. The institute provides consultancy services to industries on Environmental Impact Assessment (EIA), biodiversity conservation plan for environmental protection etc. (NEIST, 2017).

The North Eastern Regional Institute of Science and Technology (NERIST) is a science and technology oriented higher education institute in Nirjuli, Itanagar, Arunachal Pradesh funded and controlled by the Ministry of Human Resource and Development, Government of India. The institute has been working for imparting quality education and training to cater to the needs of the region, developing entrepreneurship base in the region and strengthening research and development activities. The Forestry Department of the institute is equipped with infrastructure designed to produce professional personnel with management capacity and ability to take up the future challenges in the field of forestry. The Forestry courses with specializations in the field of forest ecology, wood science and technology, horticulture, taxonomy, ethnobotany, silviculture, biodiversity conservation, wildlife management, biotechnology, microbiology, forest genetics, forest economics and management, environmental restoration etc. have been framed to generate intellectual human resources with better understanding of the conservation aspects and sustainable management of the large forest cover in the North-Eastern states (NERIST, 2014).

The State Forest Research Institute (SFRI) of Arunachal Pradesh was established at Itanagar in 1993 with an aim to increase the understanding and information levels on biodiversity, its conservation and sustainable use recognizing the contributions of local and indigenous communities to the conservation and sustainable utilization of biological diversity through traditional knowledge, practices and innovations. Research and Development platform of the institute includes inventory and documentation of the biological diversity, genetic improvement using biotechnology tools, development of appropriate technological package ensuring bio-safety, silvicultural practices of important forest crops and development of appropriate cultivation and management package along with establishment of referral Herbarium and Museum. It's conservation and management activities involve *ex-situ* conservation and evaluation of germplasms of useful forestry species, establishment of germ-plasm banks, model nurseries, farms and silvicultural plots and *in situ* conservation through tissue culture and cryo-preservation techniques. It also works towards creation of State Biodiversity Information System, including Networking and Information Dissemination System, provides Consultancy Service to public functionaries and user agencies, technology transfer through training and extension services including eco-awareness education.

ICAR Research Complex for North Eastern Hill Region (ICAR RC NEH), Umiam, Meghalaya was established in

1975 by the Indian Council of Agricultural Research (ICAR). Its mandate includes the improvement and development of sustainable farming systems for different agro-climatic and socio- economic conditions of the region, to improve crops, livestock, fishery and to impart training for development of local competence for management of resources to enhance agricultural productivity, to maintain, analyse and project data base resources for perspective planning, to collaborate with the state departments of the region for testing and promotion of improved farming technologies. A web based software SFAR (Soil Fertility Assessment and Recommendation) for assessing the status of soil fertility (poor/medium/high) from the field test results of 12 soil parameters was developed. The institute has all the disciplines of agriculture, horticulture, animal sciences, agricultural engineering, agroforestry, fishery and social sciences to cater to the research needs of the tribal areas of NEH Region including Sikkim. It operates 15 Krishi Vigyan Kendras (KVKs) attached to different centres and HQ for providing on/off campus training to the practising farmers, school dropouts and farm women in the field of agriculture and allied sectors. Considering the entire NEH Region as one unit, the research centres have been so located as to represent the varying altitudes (60-1800 m above MSL) and agro-climates of the region. The research findings of the institute at different centres are being utilized for specific altitudinal range and agro-climatic conditions. It also acts as a repository of information on different farming systems of the region and also provides consultancy (ICAR-RC-NEH, 2016).

There are number of Central and state Universities in the North-East Region. North Eastern Hill University (NEHU), Shillong was set up with the objectives to disseminate and advance knowledge by providing instructional and research facilities in different branches of learning and to pay special attention to the improvement of the social and economic conditions and welfare of the people of the hill areas of the North-Eastern region, and, in particular, their intellectual, academic and cultural advancement. NEHU is a leading University of North East India which attracted students not only from North-East India but from all over the country, as well as scholars from foreign countries. We have now at least one central University in each state besides several state funded and private universities. Various departments and schools have been established in these Universities, which are generating intellectual human resources including research scholars involved in research in different aspects of social, economic, environmental, biological, physico-chemical research platforms pertaining to the North-East region. The department of Environment/ Forestry sciences in these institutions are capable of carrying out research on areas of natural resource management, environmental monitoring and remediation of degraded ecosystems, forest biodiversity assessment, monitoring and management, forests policies and laws, environmental impact assessment, watershed management, integrated pest management, land reclamation and rural development etc. They can also work on global positioning system studies and micro-seismicity of North-East Region. These institutions are also receiving funding from organisations such as MoEF & CC, DST, DBT, UGC and state governments. The School of Life Sciences, Biochemistry, Biotechnology & Bioinformatics, Botany and Zoology also continue to work on ecosystem-based research and Faculty members are in various Project Advisory Councils/ Task Forces of the funding agencies (NEHU, 2017). These institutions have to undertake studies on impact of development projects on structure and functioning of NE ecosystems.

2.2. Regulatory provisions and guideline for sustainable development

The sustainable development is an approach that aims to ensure that environmental, social and economic progress in the society is achieved without depletion of the natural resources. Therefore, the focus should be on ensuring a strong, healthy and just society. This means that the diverse needs of all people in existing and future communities, personal wellbeing, social cohesion and inclusion, and creating equal opportunity are the components of sustainable development as defined by the United Nations Conference on Environment and Development in Rio de Janeiro in 1992. The summit marked the first international attempt to draw up action plans and strategies for moving towards a more sustainable pattern of development. Despite the vast natural resources which could potentially make this region one of the wealthiest regions of India, there are several indications pointing towards natural resource degradation and low economic growth. There are several policy interventions and programmes for economic development of the NE region. Fig. 1 provides details of developmental projects which have been given environmental clearance under EIA Notification of 2006. The setting up of development projects such as Oil Refineries, cement plants, mining, Hydro Electric Power projects, construction of National Highways has been reported to cause ecological degradation and also displace large number of people which can ultimately bring about a drastic change in the demography of North East India. While according environmental clearance (EC), the authorities look at EIA report prepared by the project on likely impact to be created during project implementation in the area. Table 1 provides details of some safeguards stipulated in EC letters for ecosystem improvement and biodiversity conservation.

The Biological Diversity Act, 2002 was India's attempt to realise the objectives enshrined in the United Nations Convention on Biological Diversity (CBD), 1992 which recognises the sovereign rights of states to use their own biological resources. The act aims at the conservation of biological resources and associated knowledge as well as facilitating access to them in a sustainable manner and through a just process. All the NE states have enacted rules to impose restriction on activities related to access to biological resources. The State Biodiversity Boards (SBBs), if deems necessary and appropriate, can take steps to restrict or prohibit the access to biological resources for the following reasons:

(i) If it is a threatened species, or species that is likely to become threatened due to such access,

(ii) If it is an endemic species,

(iii) If the request for access is likely to result in adverse effect on the livelihoods, culture, or indigenous knowledge of the local people,

(iv) If the request for access is likely to result in adverse environmental impact which may be difficult to control and mitigate,

(v) If such access would cause genetic erosion or affect the ecosystem function,

(vi) If the purpose of resource use is contrary to national interest and other related international agreements entered into by the country

Table-2 provides a list of Endangered Species as compiled from National Biodiversity Authority (NBA) for some NE states of the country. The environmental impact assessment reports of some projects brought out significant information on some very important aspects of species and habitat status. On that basis, it was emphasized that Meghalaya has been recognized as a cradle for several endemic species and an important constituent of the biodiversity hotspots spread over North East India. Therefore, as a conservation measure for rich biodiversity of the region, the projects should be initiated on population inventory, propagation and reintroduction of threatened plants and establishment of conservation plots/ areas in respect of following endangered and endemic plants reported to have been occurring in the region:

1. *Pteracanthus griffithianus* (Acanthaceae)
2. *Nepenthes khasiana* (Nepenthaceae)
3. *Argostemma khasianum* (Rubiaceae)
4. *Fimbristylis nigrobrunnea* (Cyperaceae)
5. *Trivalvaria kanjilali* (Annonaceae)
6. *Begonia rubravenia* (Begoniaceae)
7. *Ceologyne ovalis* (Orchidaceae)

Table 1

Important safeguards stipulated under Environmental Clearance for conservation of biodiversity

Sectors	Stipulations for biodiversity conservation
River Valley projects	Biodiversity Management plan, Compensatory Afforestation programme, Identification of species endemic to the project region (*e.g.* Orchids in Sikkim) and their conservation, Construction of fish hatchery, Documentation and Identification of local aquatic fauna, Biodiversity and Habitat Conservation; Central level Multidisciplinary committee to monitor implementation of environmental safeguards including Biodiversity conservation during project execution.
Mining projects	Precautionary measures to be taken during mining operation for conservation and protection of endangered flora as well as fauna. Action plan for conservation of flora and fauna to be prepared and implemented in consultation with the State Forest and Wildlife Department. Necessary allocation of funds for implementation of the conservation plan to be made and the funds so allocated should be included in the project cost.
Industry projects	Green belt development consisting of plantation with native species in project site and surrounding villages in consultation with local DFO
Refinery projects	Preparation of contingency plan to mitigate adverse impact of increased human activities on wildlife habitats around project area,
Thermal projects	Afforestation and Green belt development consisting of plantation with native species in project site and surrounding villages in consultation with local DFO

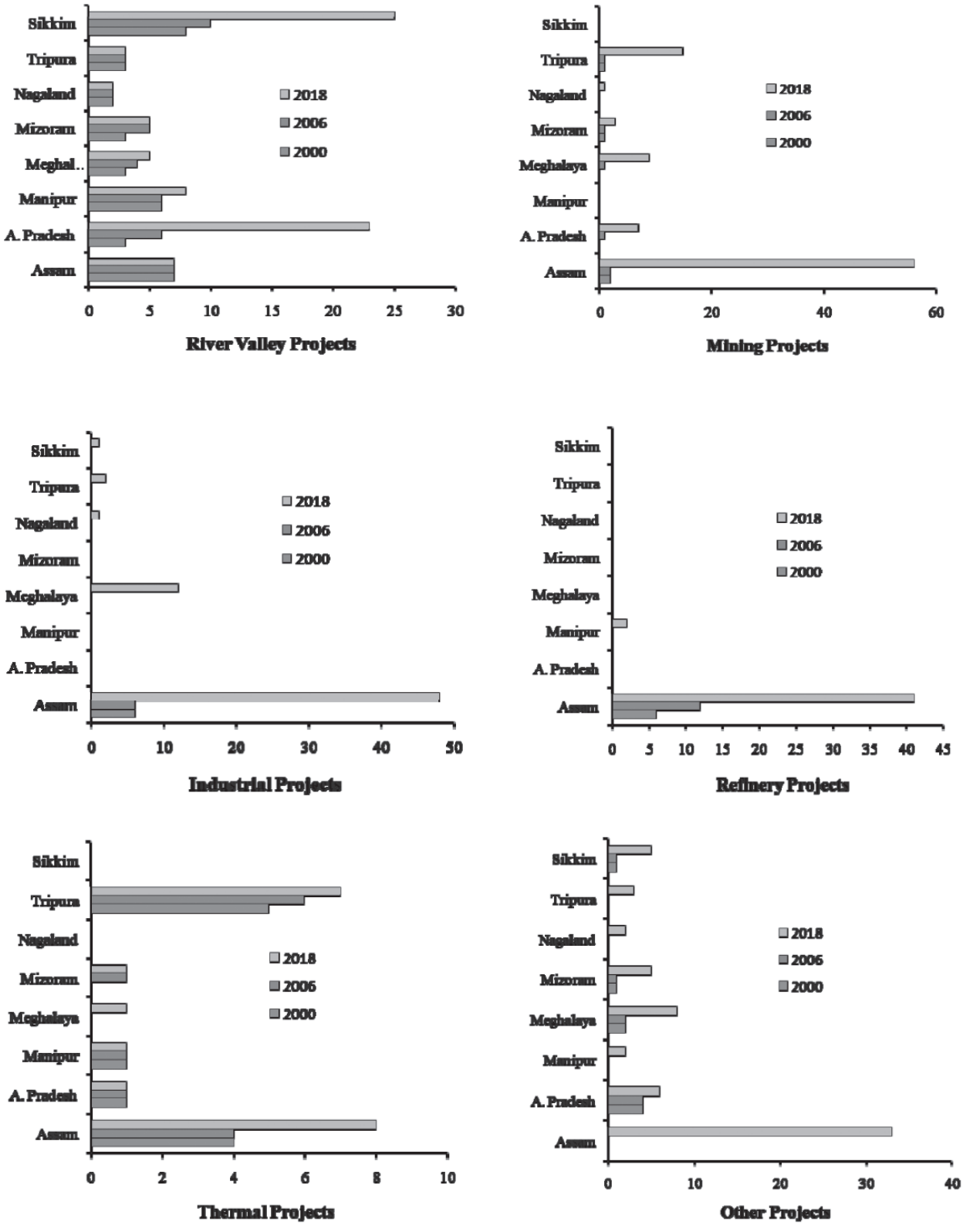

Fig 1. Number of environmental clearances given for construction of developmental projects in states of NE region.

Table 2
List of Endangered Species as compiled from National Biodiversity Authority

Sl. No.	States	Plants	Animals
1.	Meghalaya	*Aquilaria khasiana*	*Rhinoceros sondaicus*
		Gymnocladus assamicus	*Gyps bengalensis*
		Ilex khasiana	*Gyps tenuirostris*
		Jasminum adenophyllum	*Sarcogyps calvus*
		Nepenthes khasiana	*Philautus shillongensis*
		Nymphaea tetragona	
2.	Mizoram	*Jasminum wengeri*	*Rhinoceros sondaicus*
		Mantisia wengeri	*Dicerorhinus sumatrensis*
		Paphiopedilum spicerianum	*Felis marmorata*
		Paphiopedilum villosum	*Felis timmincki*
		Aquilaria malaccensis	*Arctictis binturong*
		Hydnocarpus kurzii	*Prionodon pardicolor*
		Dalbergia pinnata var. acaciaefolia	*Mustela kathiah*
		Cinnamomum cassia	*Helarctos malayanus*
3.	Manipur	*Lilium davidii*	*Rhinoceros sondaicus*
		Kalanchoe roseus	*Dicerorhinus sumatrensis*
		Rhododendron macabeanum	*Cervus eldi eldi*
		Malus baccata	*Ardea insignus Hume*
		Rhododendrn wattii	*Gyps bengalensis*
			Sarcogyps calvus
4.	Tripura	*Stichoneuron membranaceum*	*Gyps bengalensis*
			Sarcogyps calvus

The project proponent should sponsor research and development for conservation of threatened species occurring locally such *Hedychium dekianum* (Zingiberaceae), *Cymbidium eburneum* (Orchidaceae), *Dendrobium devonianum* (Orchidaceae) which, would be carried out by a research or academic institution located in Meghalaya. Further, Conservation Action Plan for preservation of wild fauna shall be formulated and implemented.

3. Challenges for sustainable development in North-East India

From the above discussion, it is clear that development challenges in NE region are quite complex compared to other regions of the country. The biodiversity of plants and animals needs conservation interventions, ethno-diversity needs to be protection and development should be sustainable. Tables 3 and 4 provide lists of research projects funded by MoEF, Government of India under two schemes in NE region. While projects in Table 3 relate to development impact to some extent, there are not many projects in the list addressing the current issues of environmental degradation. We have highlighted a few thrust areas on the basis of our monitoring feedback from the states.

There is strong need to initiate programmes for training and research on environment and development sectors and provide exposure to students and research scholars with latest information in their respective fields of activities and bring them closer to development perspective as far as scientific advancements are concerned. The NE institutions should also involve themselves in consultancy for EIA and biodiversity conservation and monitoring studies for development projects in the region. Although, these institutions continue to produce intellects in academia for the North-East Region and have been directly and indirectly shaping the academic, social and cultural features of the region, the field application and problem solving strategies with a clear vision of sustainable growth and development for the North East region is the need of the hour.

Table 3

List of research projects funded by MoEF&CC under Environmental Research Programme (ERP) in North-East Region (2006-2010).

Sl. No.	Title of the Project	Name of the Institute
1	Phytoremediation of hydrocarbon contaminated soil of Assam.	Dr. S. Deka, Resource Management & Environment Division, Institute of Advanced Study in Science & Technology, Guwahati, Assam.
2	Impact of Natural gas flare on growth and yield development in Rice (*Oryza sativa* L.).	Dr. K.K. Baruah, Department of Environmental Science, Tezpur University, Assam.
3	Impact of coal mining on water quality and microbial communities in Jaintia Hills District, Meghalaya.	Dr. H. Kayang Department of Botany, North Eastern Hill University, Shillong, Meghalaya.
4	Trace element analysis of Loktak lake and rivers draining into it and its impact on health.	Prof. N Rajmuhon Singh Dep. Chemistry, Manipur University, Canchipur, Imphal Manipur.
5	Studies on pollution of pond/fishery water of Guwahati with respect to fish health: Remediation by Zeolitic Action.	Dr. Anup Kumar Talukdar Department of Chemistry, Gauhati University, Gauhati, Assam
6	Assessment of toxic elements in water of semi-urban areas of Assam and investigation of the disease related contaminants.	Prof. A.K. Mishra, Department of Chemistry, Gauhati University, Guwahati, Assam
7	Development of environmentally benign process technology for extraction of natural dye of N.E. Region.	Dr. P. G. Rao, North East Institute of Science & Technology, Jorhat, Assam
8	Assessment of anthropogenic activities on soil/water and certain medicinal plants species in and around Bharalu river in Guwahati city.	Dr. Jibon Kotoky, Institute of Advanced Study in Science & Technology, Guwahati, Assam
9	Impact of stone mining on water quality of Tlawng river in Aizawl, Mizoram: Strategy for management of river through eco-restoration of abandoned mine areas.	Dr. B.P. Mishra, Department of Forest Ecology, Biodiversity & Environmental Sciences, Mizoram University, Aizwal, Mizoram.
19	Development of eco-friendly strategies for removal of phenols and other organic compounds form water using highly ordered porous carbon made from tree leaves.	Dr. Prodeep Phukan, Guwahati University, Guwahati, Assam.
11	Selective recovery of ethanol from ethanol-water mixture having the composition of biomass fermentation broth by pervaporation: the efficacy of hydrophobic polyurethane-zeolite mixed matrix membranes.	Dr. Dilip Kumar Kakati, Guwahati University, Assam.
12	Development of a low cost process for fluoride removal from fluoride contaminated water specific to NE Region for public use.	Dr. (Mrs.) Aradhana Goswami, North East Institute of Science & Technology, Jorhat, Assam.
13	Atmospheric deposition of nitrogen and productivity of winter cereal crops in North East India.	Dr. Bhagawan Bharali, Department of Crop Physiology, Assam Agricultural University, Jorhat, Assam.

Table 4

List of projects funded by MoEF&CC under Eco-system Research Scheme (ERS) in North-East Region (2008-2010).

Sl. No.	Title of the Project	Name and address of the Institute
1	Floristic studies on Macrophytic diversity of Nameri National Park (Assam) and Pakke Tiger Reserve (Arunachal Pradesh.	Dr. Nilakshee Devi, Lecturer Deptt. of Botany Guwahati University Guwahati, Assam
2	Studies on the diversity and distribution of soil microarthropod fauna of grassland and adjoining cultivated fields in subtropical ecosystems of Cachar, Assam.	Dr. D.C. Ray, Dept. of Ecology and Environmental Sciences, Assam University, Silchar, Assam.
3	Icthyofaunal diversity and studies on the biology of certain indigenous ornamental fishers of Meghalaya.	Sh. S.N. Ramanujam, Deptt. of Zoology, School of Life Sciences North Eastern Hill University, Shillong, Meghalaya.
4	Algal flora from different habitats of central Assam and conservation of collected strains.	Dr. Farishta Yasmin, Senior Lecturer in Botany, Deptt. of Botany, Nowgong College, P.O. Nagaon, Assam.
5	Distribution and Conservation Strategy of an endangered ornamental fish species *Chaca chaca* (Ham.- Buch) in Arunachal Pradesh.	Dr. Keshav Kumar Jha, Head, Deptt. of Zoology, Jawahar Lal Nehru College, Pasighat, Arunachal Pradesh.
6	All India Coordinated Research Project on reproductive biology of four rare endangered and threatened (RET) tree species namely; *Michelia punduana* Hook. f & Thoms.; *Rhododendron macabeanm* Watt. f. ex Balf; *Rhododendron wattii* Cowan and *Acer oblongum* Wall ex. DC. var. *microcarpum* Hiern of Nagaland & Manipur (NE).	Dr. S.K. Chaturvedi, Head, Department of Botany, Nagaland University, Hqs. Lumami, PO Mokokchung, Nagaland.
7	All India Coordinated Research Project on reproductive biology of four rare, endangered and threatened (RET) tree species namely: *Aquilaria malaccensis* Lamk. (Syn = *Aquillaria agallocha*) (Thymeleaceae), *Gymnocladus assamicus* Kanj. ex P.C. Kanjilal (Caesalpinaceae), *Ilex khasiana* (Aquifoliaceae) and *Illicium griffithii* Hook. f & Thoms. (Illiciaceae) of North East India particularly in Arunachal Pradesh and Meghalaya.	Prof. N. Venugopal, Professor, Centre for Advanced Studies, NEHU, Shillong, Meghalaya.

As per the Strategy Report, 2007 (World Bank, 2007) by the Sustainable Development Department of the World Bank, the process of development and growth in North-Eastern Region of India is being met by many challenges. It reports that for a region to benefit from its natural resource wealth, certain institutional elements must be present which include clear institutional arrangements, participatory decision making by the different levels of stakeholders (ranging from state governments to water users and forest-dependent producers), clear and transparent rules and regulations and equitable enforcement. In addition, an integrated management system is required with regard to river basins and natural resources. However, these institutional arrangements do not fully exist in the North-East today. Certain developmental issues in all sectors has been taken up by the central government but due to weak state capacity, complex and incomplete institutional arrangements, incomplete knowledge base for the natural resource management and non-involvement of local stakeholders, the current approaches have not yielded the needed benefits to local populations. Therefore, there is a need for an integrated approach that enhances cooperation at and between regional and local levels for developing a shared vision of costs and benefits through strategic planning

and infrastructure interventions that create a sustainable ecosystem to improve the lives and livelihoods of communities and citizens. There is a strong need for better planning for the biodiversity, forestry and water-related sectors. Better management and development could generate the greatest impact by providing communities with assurance that investments in industries and infrastructure are sustainable. Hydropower sector also emerges as one of the best opportunities for development in the region as revenue from hydropower projects could potentially double the region's net state domestic product (NSDP).

The region's wealth of biodiversity has been well highlighted but there are still significant gaps in existing knowledge. The developmental pressure (Fig.1) for economic growth and biotic interferences have resulted in deforestation, mining and quarrying, jhumming, charcoal-making, construction of reservoirs and dams, overharvesting of medicinal plants, drying up of wetlands, overfishing and pollution of water bodies. This has impacted the ecosystems and their components like flora and fauna in the North-Eastern Region. In addition, conflicts between development and conservation are likely to have negative impacts on species conservation. No doubt, the biological resources can be used sustainably and beneficially only at both regional and local levels, by fully involving local communities in the region and make efforts to catalogue and document existing plants and animals and their potential uses. Proper institutional development is necessary by integrating the institutions to a research consortium consisting of the region's universities, to have long term ecological initiatives on biodiversity-related work, for which a considerable part of this can be achieved through implementation of biodiversity-oriented projects in existing universities and institutions of the region. The development and institution interface as highlighted above will go a long way to ensure implementation of biodiversity conservation in NE Region of India.

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Evaluation of cytotoxic potential of methanolic extract of Guduchi [*Tinosporia cordifolia* (Willd.) Hook. f. & Thoms.] on root meristematic tissues of *Allium cepa* L.

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ABSTRACT

Tinospora cordifolia (Menispermaceae), commonly known as 'Guduchi', is a robust climber with greenish yellow flowers and having a number of bioactive compounds such as berberine, palmatine, magnoflorine, syringin, furanolactone, jatrorrhizine with high medicinal activity. Extracts of *T. cordifolia* reported to have anti-oxidant, anti-tumour and anti-inflammatory activities. Present study deals with an investigation on isolation of secondary metabolites from bark in methanolic extracts and evaluation of its cytotoxic potential on *Allium cepa* root meristems. Three concentrations (20µg, 40µg and 100µg) of crude extracts of *T. cordifolia* were studied under 6h and 24h of treatment on root meristematic tissues. The cytotoxic compounds present in the bark extracts brought about significant reduction of mitotic index in 24h of treatment at 20 µg ml^{-1}, 40µg ml^{-1} and 100 µg ml^{-1} concentrations as compared to control. Different cytological abnormalities like clumping of chromosomes, DNA fragmentation, spindle arrest with scattered chromosomes, chromatin condensation, diplochromatin chromosome erosion, denucleation and chromosome break were observed. The preliminary investigation showed that this plant-derived bioactive compounds can destroy the cells at micromolar concentration and hence may be a potential drug for treatment of cancer.

1. Introduction

Since the beginning of human civilisation, plants played an important role in the protection of human and animal health against chronic degenerative diseases. Hence an impressive number of phytochemicals (bioactive compounds) have been isolated from nature. As per an estimate of WHO, about 80% of total inhabitants on earth rely on traditional medicines for their primary health care needs (Anonymous, 1948). About 8,000 species of medicinal plants belonging to 450 genera are reported to occur wild in India, which is about 50% of the known flowering plants of the country (Owolabi *et al.,* 2007). *Tinospora cordifolia,* commonly known as 'Guduchi' is a large, glabrous, deciduous climber belonging to the family Menispermaceae. It is distributed throughout the tropical parts of Indian subcontinent including Pakistan, Sri Lanka, Bangladesh and China, ascending to an altitude of 300 m (Garg *et al.,* 2007). In Hindi, the plant is commonly known as 'Giloi', which is a Hindu mythological term, refers to the heavenly elixir that has saved celestial beings from old age and kept them eternally young (Bhandari, 2006). In India, the species is distributed from Kumaon to Assam in north extending through West Bengal, Odisha, Bihar, Deccan, Konkan, Karnataka and Kerala (Wani *et al.,* 2011). A large number of compounds have been isolated from the aerial parts and roots of *T. cordifolia* which includes berberin, tinosporacide, tinosporin, tinocordifolioside, cordifolioside A, cordifolioside B, isocolumbin, magnoflorine (Mehra *et al.,* 2016). The presence of several terpenoids, alkaloids, lignin, carbohydrates, bitters, steroids and glycosides has also been reported in the species. One of the most important constituents present in the stems of *T. cordifolia* is berberine, an isoqunoline alkaloid, having various pharmacological

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actions which enhance the therapeutic values of the plant (Andola, 2000). In modern medicine, it is used for the treatment of general weakness, fever, dyspepsia, dysentery, gonorrhoea, urinary diseases, viral hepatitis and anaemia. Recently, it is also being used as an immunomodulatory, antioxidant, anti-neoplastic, anti-stress, anti-hyperglycemia, anti-diabetic agent (Joshi and Joshi, 2013). There are few reports on the cytotoxic properties of different extracts of *T. cordifolia* (Uddin *et al.,* 2011). The bark of *T. cordifolia* is used as an astringent and for treatment of fever and tumours, while the leaves are used to treat ulcers, sore throat, inflammatory conditions in traditional medicine (Adhvaryu *et al.,* 2008). Although chromosome number of *T. cordifolia* has been determined as 2n =22, no detailed study on genetic variability in terms of ploidy level and phytochemical constituents has been done till date (Rana *et al.,* 2012). In consideration of the facts that very scanty information on the cytotoxic and anti-inflammatory properties of the tinosporin derivatives are available in different parts, we report here the cytotoxic activity of bark extracts of *T. cordifolia* on *Allium cepa* root meristems.

2. Materials and methods

2.1. Plant materials

The plant material consists of dried powdered bark of *Tinospora cordifolia*, collected from Dhansole village of Baripada block, Mayurbhanj district, Odisha and identified with the help of Flora of Orissa (Saxena and Brahmam, 1995). The herbaria specimens and dried bark samples were deposited in the herbarium of the Department of Botany, Utkal University, Bhubaneswar, Odisha.

The test plant was onion *i.e*. *Allium cepa* L. (Liliaceae), which has 16 long chromosomes. This species is an excellent plant material to be used as a biomarker for environmental monitoring with many advantages, such as low cost, profuse rooting from the bulb, ease of storage and handling, short duration to conduct a test, large cells with easily visible long chromosome and the ease at which the abnormalities in chromosomes during mitosis can be observed (Banerjee and Giri, 2014).

2.2. Extraction of crude bark extract

The fresh bark of *T. cordifolia* was collected and dried under room temperature and powdered using a grinder. The powdered bark sample was kept in air tight containers until the time of use. The powdered bark (50 g) was exhaustively extracted with 200 ml 99.8% methanol (Merck) using Soxhlet apparatus at 40^{o}C. The methanolic extract was filtered and the filtrate was condensed under reduced pressure and concentrated to dryness under controlled temperature (40^{o}C) with the help of IKA RV10 Rotary Evaporator (Germany) fitted with IKA HB10 digital temperature controller, vacuum pump and water chiller (Cole-Parmer).

2.3. Cytotoxic study

Allium cepa var. Deshi was grown in sand in the green house of Botany Department, Utkal University, Vani Vihar, Bhubaneswar, Odisha and was used as experimental material for cytotoxicity study. After 5-6 days, bulbs with 3-4 cm long roots were washed in running tap water and subjected to treatment of 0µg ml^{-1} (Control), 20µg ml^{-1}, 40 µg ml^{-1} and 100µg ml^{-1} concentration of bark extract dissolved in dimethyl sulfoxide (DMSO) followed by double distilled water and kept for 6 h and 24 h at room temperature. A control experiment was conducted without any bark extract.

2.4. Mitotic index and chromosome study

Root tips from each treatment were collected and fixed in 1:3:: acetic acid: ethanol overnight at room temperature. Fixed root tips were treated with 45% glacial acetic acid for 15 min and were stained in 2% aceto-orecin: 1N HCl (9:1) for 4-5 h. Stained root tips were squashed in 45% acetic acid on a clean glass slide. For each treatment ~100 cells from root tips were scored at random from each slide and the data were pooled for each treatment. The mean data were taken from each treatment and each experiment was replicated thrice. Cells from each root tip were scored at different stages of chromosome under Olympus BX56 microscope (Japan) attached with a digital camera. All the observations were recorded for abnormalities during the cell and chromosome division under different concentrations of crude bark extract.

2.5. Cell death measurement

The cytotoxicity levels were measured for both treated and control roots by staining them in 0.25% Evan's Blue (w/v) for 30 min (Baker and Mock, 1994). Stained root tips were transferred to 1 ml of N, N – Dimethylformamide for 1 h at 37^{o}C. The absorbance of the dissolved Evan's Blue solution was measured at 600 nm in a UV Visible Spectrophotometer and plotted in a graph and calculated statistically.

3. Results and discussion

3.1. Mitotic index and chromosomal anomalies

The treated roots became brown in colour and growth was restricted in 24 h of direct treatment in 100 µg ml^{-1} as compared to control or 6 h of direct treatment. The concentration of the bark extract and the time of exposure played an important role in the reduction of mitotic index

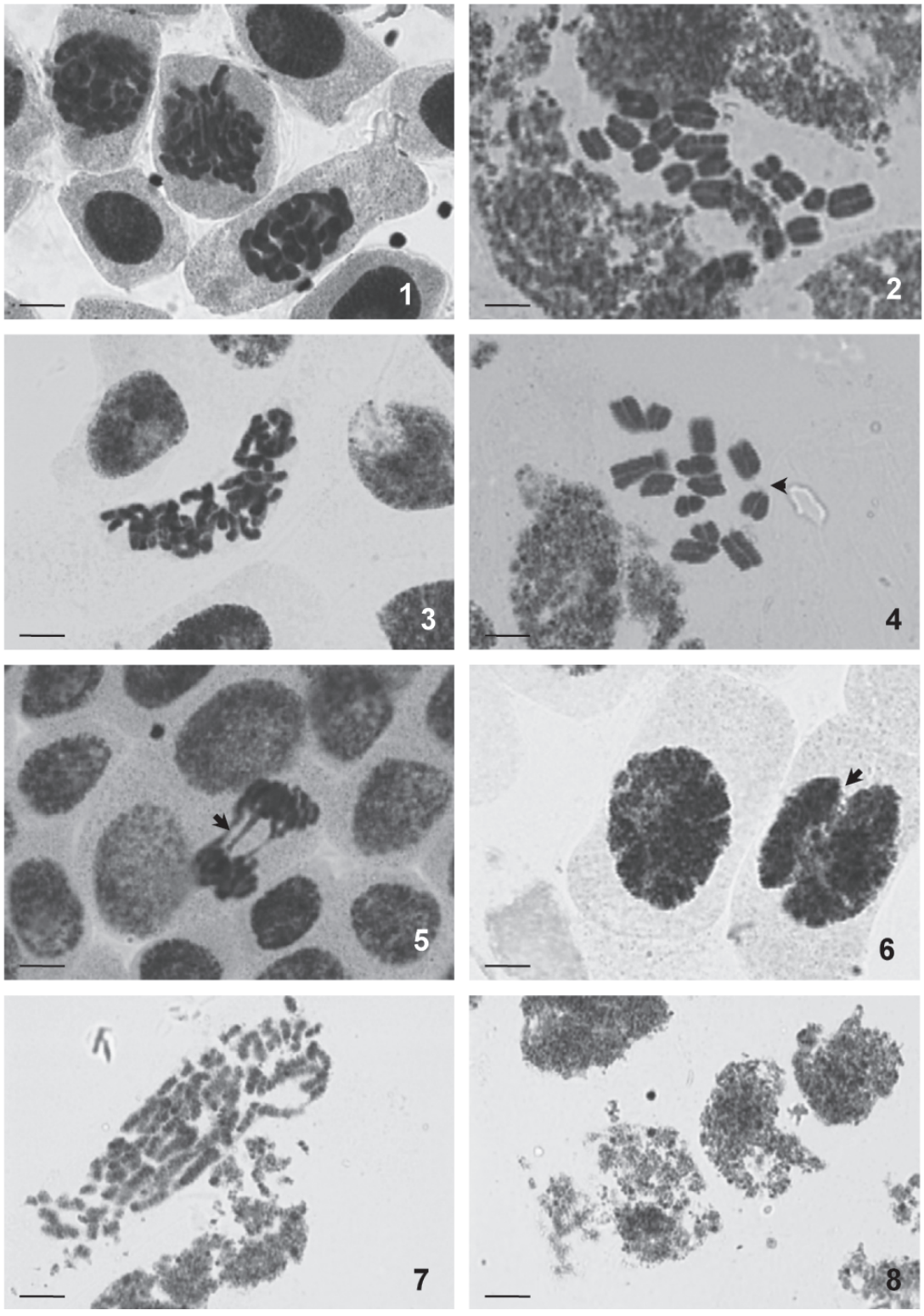
in a dose-dependent manner. Mitotic Index (MI) decreased progressively with increase in concentration as well as the duration of the treatment (Table 1). The MI was 41.20 in 6h and 29.48 in 24 h of treatment in 20 µg ml^{-1}, which was slightly higher than 40 and 100 µg ml^{-1} concentration. The mitotic index dropped significantly by about ~1.90 to ~2.78 fold in 24 h of treatment at 20 µg ml^{-1} and 100 µg ml^{-1} concentrations respectively as compared to respective control. Control root tip cells showed normal mitosis at all stages of divisions. However, C-mitosis, chromosome bridges, chromosome fragments, chromosomal clumping, chromosome break and chromosome erosions and spindle abnormalities with spread metaphase, denucleation and chromosome condensation were recorded (Figs. 1-8). Spindle fibre abnormality (SFA) included C-mitosis, chromosome stickiness, chromosomal clumping (Fig. 1) and metaphase chromosomal abnormality (Fig. 3) including chromosomal breaks (Fig. 4) and chromosomal bridges (Fig. 5). The frequency of chromosome break and chromosome erosions (Fig. 7) increased significantly with increasing concentration of bark extract and increase of treatment time. Very condensed chromosomes with mitotic effects in the cell as well as chromosome fragments were noticed in 100 µg ml^{-1} (Fig 2. 2 and 4). Sticky chromosome bridges were found in low doses *i.e*., 20 µg ml^{-1} of extract. Chromosomal stickiness as well as break and erosion (Figs. 4 and 7) are usually irreversible leading to denucleation of chromatin materials (Fig. 8) and could be due to the toxic effects of plant extracts leading to cell death. Moreover, high mitotic index with more percentage of cells in metaphase stage was noticed at lower concentrations compared to other concentrations. Likewise, the percentage of cells in anaphase stage also varied in different concentrations. The oxidative damage by bark extract might have induced various chromosomal abnormalities, which was found to be dose-dependent (Table 1). The highest percentage of abnormalities was noted with treatment of 100µg ml^{-1} of extract as compared to treatment with 20 µg ml^{-1}. A comparative analysis of all abnormalities with different hours of treatment showed that a comparatively high percentage of metaphase cells were damaged as compared to anaphase. Metaphase and anaphase displayed various types of abnormalities such as spindle fibre anomalies leading to pre-treatment effect, chromosome break with lagging chromosomes, chromosome erosion, chromosome clumping, sticky chromosomal bridge formation and C-mitosis formation. The maximum number of chromosome breaks and erosion was found with treatment of 100 µg ml^{-1} extract for 24 h. At 20 µg ml^{-1} treatment, significant percentage of abnormalities was also registered. The increase in percentage of chromosomal abnormality was dependent on dose- and time of treatment. The treated root tips showed abnormal spindle formation in low dose leading to metaphase arrest and separation due to direct bark extract treatment. Chromosomal break was observed in 40 µg ml^{-1} treatment, while 20 µg ml^{-1} treatments produce early separation of chromosomes in anaphase. Chromosomal damages were prominent resulting in erosion and intense break of chromosome at 100 µg ml^{-1} treatment. This clearly indicates that methanolic extracts of *Tinospora cordifolia* have potential carcinogenic chemicals, which can kill the cells. Hence, isolation and extraction of individual chemicals; separation and testing of their efficacy at cellular level are very important considerations for discovery of cytotoxic molecules. Since, this extract has serious impact on cell cycle, the extract in crude form may have potential anti-cancer activity.

Root is the most sensitive and accessible part of *Allium cepa*. The inhibition of root growth upon exposure to bark extract clearly showed cytotoxic effect of plant extract and consequent chromosomal aberrations similar to heavy metal stress (Zhang *et al.,* 2009). Chromosome stickiness is a lethal type of aberration besides chromosome fragments and bridges, which was also observed in the present study. Increased frequency of chromosome bridge and presence of more chromosome fragments in the cells might be due to chromosome replication and protein synthesis in roots induced under plant extract stress. The active molecules of bark extracts might be interfering with calmodolin, a calcium modulating protein, located in the mitotic spindle by influencing the uptake of Ca^{++} causing abnormal processes of chromosome movements leading to mitotic abnormalities (Liu *et al.* 1995).

There are not many reports on toxicity of plant extracts causing chromosomal abnormalities such as chromosome break, lagging, erosion and effect on cell division. Various types of abnormalities were noticed by treating the *Allium cepa* cells with methanolic bark extract of *Tinospora cordifolia* at the cell and tissue levels, affecting the elongation zones of root apex. The effect of low doses of methanolic bark extract was found to have significant effect on oxidative damage of chromosome structure. Chromosomal damage includes gross structural changes, which are initiated by chromosome breaks and erosion.

3.2. Cell death and cytotoxicity

Increased Evan's blue uptake of 10.25% was found with treatment of 20 µg ml^{-1} and 18.65% in 100 µg ml^{-1} at 24 h treatment as compared to control. However, in 6 h treatment with both the concentrations, no significant cell death was observed. Pronounced cytotoxic effect of bark extract on roots of *A. cepa* in both the concentrations was



Figs. 1 - 8 : Cytotoxic effects of crude extract of *T. cordifolia* on *Allium cepa* root tip cells; Fig. 1: Clumping of chromosomes at 100 µg ml^{-1} concentration at 6 h direct treatment; Fig. 2 & 3: Spindle abnormalities with metaphase chromosome effect and abnornal metaphse at 20µg ml^{-1} concentration at 24hr; Fig. 4 & 5: Condensation diplochromatin with chromosome break (arrow head) and sticky chromosome bridge at anaphase (arrow head) at 40µg ml^{-1} concentrationat 24 hr treatment; Fig. 6: Unequal binucleate nuclei without cell plate formation (arrow head); Fig. 7 & 8: Chromosome errosion and chromatin denucleation at 100µg ml^{-1} concentration at 24 hr treatment. Bar= 10µ.

Table 1

Effect of crude bark extract of *Tinospora cordifolia* on mitotic index of *Allium cepa* root apical meristems

	Concentrations					
	20µg ml^{-1}		40µg ml^{-1}		100µg ml^{-1}	
Treatment	Mitotic index (±S.D.)	Cell aberration percentage (±S.D.)	Mitotic index (±S.D.)	Cell aberration percentage (±S.D.)	Mitotic index (±S.D.)	Cell aberration percentage (±S.D.)
Control	49.16	1.23	47.26	1.03	46.24	1.01
	±1.25	±0.54	±0.96	±0.61	±0.94	±0.66
6h	41.20	55.26	37.46	62.45	35.38	67.36
	±2.15	±2.17	±2.44	±1.27	±2.56	±1.29
24h	29.48	69.23	24.31	74.13	22.30	88.73
	±1.53	±1.58	±2.10	±1.58	±2.08	±1.78

found to vary with duration of treatment. The uptake of Evan's blue stain by the samples with longer period of exposure was significantly more at higher concentrations in comparison to those exposed to low concentrations for short duration. The increase in Evan's blue colour uptake in the roots of *A. cepa* at different concentrations of bark extracts indicates its cytotoxicity effect even at micromolar concentrations, which may be due to mitotic arrest leading to cell death (Arya and Mokherjee, 2014). Our observation on DNA and chromosomal damage in *A. cepa* caused by the crude bark extract of *T. codiforlia* in the present investigation is comparable with the findings of Figueiro *et al.* (2016) using *Glandularia selloi* leaf extract. Root extract of *Coccinia grandis* also showed cytotoxic and pesticidal effect (Hasan and Sikdar, 2016). *Rhaphidophora korthalsii* - a root climber used in Chinese traditional medicine for cancer and skin diseases has been reported to have cytotoxic effect on NK cells against the NK sensitive target K562 cell line (Yeap *et al.,* 2013). The higher percentage of cell death might be due to higher lipid peroxidation activity that might be leading to membrane instability.

4. Conclusion

The findings of our study established the cytotoxic effect of the crude bark extract of *T. codifolia* at very low dose on root tip cells of onion (*Allium cepa*). It can be concluded that onion is sensitive to plant alkaloids similar to animal cells at very low concentrations and therefore, can be used as an indicator for cytotoxicity. The active principles of the methanol fraction of bark extract have high cytotoxic effect, which necessitates detailed study to elucidate the molecular mechanism of cell death.

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Effect of mercuric chloride on seed germination and seed respiration of *Vigna radiata* (L.) Wilczek and *Vigna mungo* (L.) Hepper

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ABSTRACT

Mercury is a typical toxic trace metal and its bioaccumulation in plants and subsequent entry into the food chain results in long-term health hazards in human beings. During the present investigation, the effect of mercuric chloride on seed germination, radicle length, hypocotyl length and seed respiration of two commonly consumed leguminous crops viz. *Vigna radiata* (mungbean) and *Vigna mungo* (blackgram) were studied. Considerable reduction in the percentage of seed germination, radicle length, hypocotyl length and respiratory O_2 consumption in germinating seeds was observed with the increase of concentration of mercuric chloride. The effect of mercury was found to be dependent on dose and duration of exposure besides genetic factors.

1. Introduction

In recent years, there has been an increasing ecological and global public health concern associated with environmental contamination by heavy metals. Human exposure has also risen dramatically as a result of an exponential increase of their use in several industrial, agricultural, domestic and technological applications. The multiple industrial, domestic, agricultural, medical and technological applications of heavy metals have led to their wide distribution in the environment; raising concerns over their potential effects on human health and the environment (Bradl, 2002).

Their toxicity depends on several factors including the dose, route of exposure, and chemical species, as well as the age, gender, genetics, and nutritional status of exposed individuals. Because of their high degree of toxicity, arsenic, cadmium, chromium, lead, and mercury rank among the priority metals that are of public health significance. These metallic elements are considered systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure (Chang *et al*., 1996). In biological systems, heavy metals have been reported to affect cellular organelles and components such as cell membrane, mitochondrial, lysosome, endoplasmic reticulum, nuclei, and some enzymes involved in metabolism, detoxification, and damage repair (Wang & Shi, 2001).

Mercury is a widespread environmental toxicant and pollutant which induces severe alterations in the body tissues and causes a wide range of adverse health effects (Sarkar, 2005). Both humans and animals are exposed to various chemical forms of mercury in the environment. Because mercury is ubiquitous in the environment, humans, plants and animals are all unable to avoid exposure to some form of mercury (Holmes *et al*., 2009). Bioaccumulation of mercury in plants and its entry into the food chain resulting in long term health hazard is of major concern.

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Generally, legumes are richer in proteins than cereal grains. Of these, green gram (*Vigna radiata*) and black gram (*Vigna mungo)* are two important tropical grain legumes, which provide humans with the significant amount of dietary proteins. In view of their wide-spread consumption as food items, the effect of the heavy metal *i. e*. mercury on seed germination, radicle length, hypocotyl length and seed respiration in *Vigna radiata and Vigna mungo* were studied.

2. Materials and methods

2.1. The test chemical

Mercuric chloride ($HgCl_2$) was taken as the test chemical to conduct the experiments to evaluate its toxicity on the plant species. The molecular weight of the chemical is 271 g/mole.

2.2. Preparation of the stock solution

271 mg of $HgCl_2$ was dissolved in 100ml sterilized distilled water to prepare 10mM stock solution. The stock solution was kept in a refrigerator for its subsequent use in the experiment. The required experimental concentration of the metal was achieved by diluting the stock solutions with distilled water. The concentrations of $HgCl_2$ for the experiment ranged from 0 – 5mM.

2.3. The test plants

Two leguminous species such as *Vigna mungo* (blackgram) and *Vigna radiata* (Green gram or Mung bean) were selected for the toxicity study. The mature seeds were collected from Centre for Pulses Research (OUAT), Bhubaneswar.

2.4. Experimental details

One hundred and fifty seeds of each of *V. radiata* and *V. mungo* were soaked in tap water overnight. Of the soaked seeds, 20 viable seeds of each species were selected and placed over pre-moistened cotton in two sets of petridishes. Three petridishes were taken as replicates of each test concentration and the control was grown in cotton soaked with tap water. Thirty milliliters of $HgCl_2$ solution of concentration range i. e. 100µM, 200µM, 500µM, 700µM and 1000µM were added to each petriplate and two sets were prepared. The petriplates were kept under continuous light intensity of 70µE/m^2S provided from cool white fluorescent tubes, relative humidity of 65% and constant day and night temperature of 29°±2°C for observation of rate of germination, rate of respiration and measurement of hypocotyl and radicle length.

The number of germinated seeds in each petridish was counted at the interval of 18h, 24h, 42h and 48h. After 48h, the hypocotyl and the radicle were separated in each seedling and their lengths were measured. The length of hypocotyl and radicle of all the seedlings in each petridish were measured and their average was determined.

Respiration of germinating seeds was measured after 48h using an Infra Red Gas Analyzer (IRGA) (PP Systems, UK). The measurement was done under ambient light, CO_2 and temperature. The measurement of respiration was done under the respiration measurement mode of the instrument using a soil respiration chamber (SRC). Prior to the measurement, the respiration was flushed with air for 15 seconds after which it was placed above the petriplate containing germinating seeds. The petriplates were kept on a rubber pad to ensure that the cuvette (SRC) is placed airtight on the petriplate. The measurement was recorded for 1 min and for each samples three readings were taken at an interval of 5 minutes between two consecutive readings.

3. Results

In the present study, mercuric chloride ($HgCl_2$) was selected as the contaminant and the phytotoxic effect was studied on seedlings of *Vigna radiata* and *Vigna mungo.*

3.1. Effect on seed germination

The rate of germination of seeds of *Vigna radiata* and *Vigna mungo* with treatment of different concentrations of $HgCl_2$ is presented in Table 1 and 2 respectively. Significant decrease in the rate of germination was observed with increasing concentration of $HgCl_2$. The percentage of seed germination increased constantly with prolongation of incubation period and decreased with increasing concentration of the applied $HgCl_2$. As compared to control, the rate of germination was invariably lower in all seeds treated with $HgCl_2$. However, at a concentration of 5000 µM, the rate of germination becomes nearly constant for *Vigna mungo* irrespective of the period of exposure. Similarly, for *Vigna radiata*, at 2000 µM concentration, the percentage of seed germination reached almost a constant value at 18, 244, 42 and 48 hrs of treatment. Thus from the result it could be derived that the seed germination process might have been influenced by the toxic effect of $HgCl_2$. The level of toxicity and time of exposure varied between *Vigna radiata* than *Vigna mungo* and the later was more tolerant to $HgCl_2$ toxicity.

3.2. Effect on radicle and hypocotyl length

The lengths of hypocotyls were found to be almost double the length of radicles in both the species of *Vigna* as can be seen in Table 3. In untreated condition, the radicles and hypocotyls of germinating seeds of *Vigna radiata* were

Table 1

Rate of germination (%) of *Vigna radiata* seeds treated with different concentrations of Hg^{2+}

Concentration (μM)	Period of treatment			
	18 h	24 h	42 h	48 h
0	100 ± 2.42	100 ± 2.86	100 ± 3.18	100 ± 1.49
100	96 ± 3.59	98 ± 2.86	100 ± 2.95	100 ± 3.18
500	87 ± 2.62	88 ± 2.58	91 ± 2.68	93 ± 2.59
1000	76 ± 2.85	79 ± 2.18	79 ± 2.83	79 ± 2.63
2000	68 ± 1.63	68 ± 2.85	68 ± 1.93	68 ± 2.63
5000	52 ± 0.82	52 ± 0.86	53 ± 0.18	53 ± 0.95

Values are the means ±SD from 3 replicates

Table 2

Percentage of germination of seeds of *Vigna mungo* treated with different concentrations of Hg^{2+}

Concentration(μM)	Period of treatment			
	18 h	24 h	42 h	48 h
0	56 ± 1.83	66 ± 2.19	87 ± 2.86	97 ± 2.56
100	73 ± 1.86	75 ± 2.15	87 ± 2.86	98 ± 3.11
500	56 ± 0.63	57 ± 1.59	64 ± 2.04	72 ± 2.85
1000	32 ± 0.32	40 ± 1.28	48 ± 0.59	59 ± 1.38
2000	20 ± 0.25	29 ± 1.04	29 ± 0.85	32 ± 1.06
5000	20 ± 0.18	20 ± 1.01	20 ± 0.85	20 ± 0.63

Values are the means ±SD from 3 replicates

longer compared to that of *Vigna mungo*. The ratio of radicle length and hypocotyl length remained 1:2 approximately till the concentration reached 500 mM for *Vigna mungo*. Beyond this concentration, there was no noticeable growth of hypocotyls. In *Vigna radiata*, however, the length of hypocotyl and radicle became constant at 500 mM and at higher concentrations the growth of hypocotyls was negligible.

The results of the above experiment indicate that $HgCl_2$ has significant toxic effect on germination of seeds of *Vigna*

Table 3

Variation in length of hypocotyls and radicles of germinated *V. radiata* and *V. mungo* seeds treated with different concentrations of Hg^{2+}

Concentration(μM)	*Vigna radiata*		*Vigna mungo*	
	Root length (cm)	Shoot length (cm)	Root length (cm)	Shoot length (cm)
0	3.228 ± 0.013	6.442 ± 0.039	2.853 ± 0.018	5.289 ± 0.128
100	1.735 ± 0.032	3.685 ± 0.028	1.483 ± 0.063	3.049 ± 0.142
500	1.357 ± 0.042	1.328 ± 0.042	0.975 ± 0.042	1.216 ± 0.056
1000	0.813 ± 0.024	0	0.426 ± 0.017	0
2000	0.852 ± 0.026	0	0.421 ± 0.029	0
5000	0.438 ± 0.014	0	0.429 ± 0.013	0

Values are the means ±SD from 3 replicates

mungo and *Vigna radiata* and growth of hypocotyls was inhibited to considerable extent. The radicle length remained constant after 1000 mM concentration in *Vigna mungo,* while in *Vigna radiata* there was a gradual shortening of radicle length with increasing concentration of $HgCl_2$. This leads to the conclusion that the germination process was triggered before the accumulation of significant level of the toxic metal in the seeds leading to a higher rate of germination of seeds of both the test species. Subsequently, due to metal uptake and accumulation, the germinated seeds did not grow further and there was wilting and degeneration of the emerged radicles.

3.3. Effect on seed respiration

In each treatment, $HgCl_2$ caused significant decrease in the rate of respiratory O_2 consumption (Table 4) by the germinated seeds and emerged seedlings. The inhibitory effect increased with increasing concentrations of the metal solution for both the test species. At 5000 µM concentration, there was no measurable respiratory consumption of O_2. This indicates that the metal completely inhibited the activity of cells at 5000 µM concentration. The respiratory O_2 consumption at 1000 µM concentration of $HgCl_2$ was about 30% of that observed in control.

Table 4

Respiratory O_2 consumption of germinated seeds of *V. radiata* and *V. mungo* after 2 days of treatment with different concentrations of Hg^{2+}

Concentration (µM)	*Vigna radiata*	*Vigna mungo*
0	1.365 ± 0.005	1.025 ± 0.017
100	1.085 ± 0.038	0.956 ± 0.028
500	0.916 ± 0.049	0.427 ± 0.013
1000	0.439 ± 0.028	0.315 ± 0.015
2000	0.214 ± 0.017	0.104 ± 0.008
5000	0	0

Values are the means ±SD from 3 replicates

4. Discussion

It was observed that rate of seed germination in *V. radiata* and *Vigna mungo* decreased with the increase in concentrations of $HgCl_2$, which suggests that the seed germination process is influenced adversely due to toxic effect of mercury. Similar trend was also noticed in length of radicles and hypocotyls of germinating seeds. Further, it was observed that the respiration consumption at 0.1ml is about 30% compared to control. Possibly, with increase of concentration of $HgCl_2$ the amount of oxygen liberated increased and consumption also increased directly affecting the seed respiration and this has possibly resulted in reduction of the rate of seed germination and growth of radicles and hypocotyls. Mor *et al*. (2002) and few other researchers observed that the Hg contaminated soil retards the rate of seed germination and elongation of hypocotyls. The toxic effects of heavy metals such as lead, manganese and arsenic etc. have been reported by several workers (Chakrabarty *et al*., 1989; Joardar *et al*., 1988; Bandyopadhyaya *et al*., 1997).

Several studies have demonstrated that plant roots accumulate Hg when they are exposed to Hg-contaminated soils (Lenka *et al*., 1992) and plant roots accumulate more amount of Hg than shoots.

The result obtained during the present study revealed that heavy metal like mercury get accumulated in seeds and has profound toxic effects on seed germination and seedling establishment. The accumulation of the metal in seeds and other plant parts from soil and subsequent consumption by human beings is likely to have adverse effect on health.

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Macrolichen diversity of Mahendragiri Hills of Odisha state, India – II

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ABSTRACT

The present paper enumerates a total of 13 species of macrolichens belonging to nine genera and six families collected from Mahendragiri Hills of Odisha state. Of these, 11 species, namely *Coccocarpia erythrocardia*, *Heterodermia albicans*, *H. antillarum*, *H. formula*, *Hyperphyscia isidiata*, *Myelochroa aurulenta*, *Phaeophyscia hispidula, P. pyrrhophora, Phyllopsora manipurensis*, *Pyxine reticulata* and *Scytinium gelatinosum* are reported here as new distributional records for the state of Odisha.

1. Introduction

Mahendragiri Hills, located in Gajapati district of Odisha State in the Eastern Ghats, are a range of hills with highest peak of 1501 meter. This mountain range and adjoining areas are quite rich in terms of biological diversity, both plants and animals. In spite of fairly rich lichen diversity in Mahendragiri Hills, this taxonomic group has not received much attention from lichenologists. In recent years, Nayak *et al.* (2016) and Swarnalatha (2016 a&b, 2017) have contributed to the study of the lichens of Mahendragiri.

As a part of the research project on survey and documentation of lichens of Odisha, the author undertook three field tours to the Mahendragiri Hills between 2016 and 2018. Based on the collections, occurrence of 18 species of lichens have been reported in different publications (Swarnalatha, 2016 a&b, 2017). As an extension of the earlier work, some more taxa were identified subsequently and in the present paper, 13 more species of macrolichens under nine genera and six families are reported from the Mahendragiri Hills. Of these, 11 species such as *Coccocarpia erythrocardia*, *Heterodermia albicans*, *H. antillarum*, *H. formula*, *Hyperphyscia isidiata*, *Myelochroa aurulenta*, *Phaeophyscia hispidula, P. pyrrhophora, Phyllopsora manipurensis*, *Pyxine reticulata* and *Scytinium gelatinosum* are reported here as new distributional records for the state of Odisha. An enumeration of these 13 species is provided in this paper. In the enumeration, the species reported as new to the State of Odisha are marked with asterisks (*).

2. Materials and methods

The present work is based mainly on the collections made by the author from Mahendragiri Hills of Odisha during 2016-2018 which are deposited in the herbarium at Botanical Survey of India, Deccan Regional Centre, Hyderabad (BSID). Besides, a few specimens of lichens available in the herbarium of University of Lucknow (LWU) collected from Mahendragiri Hills by D. D. Awasthi and group during 1986 and currently housed in the Herbarium of National Botanical Research Institute, Lucknow (LWG) were also examined.

The morphological features were studied under stereo-zoom microscopes (Olympus SZ61) and anatomical characters were examined with compound microscope (Magnüs MLX-Tr). Colour spot tests were performed by using K, C and PD reagents. The lichen substances were

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identified by Thin Layer Chromatography (TLC) using solvent system A, following White & James (1985). All specimens were examined under UV light (365 nm).

3. Enumeration of Taxa

Coccocarpia erythrocardia (Müll. Arg.) Arv., Opera Botanica 67: 55. 1983. (COCCOCARPIACEAE)

Specimen examined: Odisha: Gajapati District, Paralakhemundi Forest Division, Mahendragiri Hills, N 18°582 22.23 , E 084°222 38.43 , alt. *c*. 1105m, 11 Sep. 2016, *G. Swarnalatha* 514D (BSID).

Coccocarpia pellita (Ach.) Müll.-Arg., Flora Regensburg 65: 320. 1882. (COCCOCARPIACEAE)

Specimen examined: Odisha: Gajapathi District, Parlakhemundi Forest Division, Mahendragiri Hills, N 18.97244°, E 084.36148°, alt. *c*. 1137m, 18 Aug. 2018, *G. Swarnalatha* 706 (BSID).

Heterodermia albicans (Pers.) Swinscow & Krog, Lichenologist 8(2): 113 .1976. (PHYSCIACEAE)

Specimen examined: Odisha: Gajapathi District, Parlakhemundi Forest Division, Mahendragiri Hills, N 18.96507°, E 084.36945°, alt. *c*. 1355m, 20 Aug. 2018, *G. Swarnalatha* 739A (BSID).

Heterodermia antillarum (Vain.) Swinscow & Krog, Lichenologist 8(2): 114. 1976. (PHYSCIACEAE)

Specimens examined: Odisha: Gajapathi District, Parlakhemundi Forest Division, Mahendragiri Hills, N 19°002 26.33 , E 084°212 58.23 , alt. *c*. 560m, 08 Sep. 2016, *G. Swarnalatha* 435C (BSID); Mahendragiri Hills, N 18.97287°, E 084.36082°, alt. *c*. 1105m, 18 Aug. 2018, *G. Swarnalatha* 702C, 703B (BSID).

Heterodermia firmula (Linds.) Trevis., Atti Soc. ital. Sci. nat. 11: 615. 1868. (PHYSCIACEAE)

Specimen examined: Odisha: Gajapathi District, Parlakhemundi Forest Division, Mahendragiri Hills, on fallen tree branch, 10 July 2016, *G. Swarnalatha* 399 (BSID).

Hyperphyscia isidiata Moberg, Nord. J. Bot. 7(6): 722. 1987. (PHYSCIACEAE)

Specimen examined: Odisha: Gajapathi District, Parlakhemundi Forest Division, Mahendragiri Hills, N 18.96753°, E 084.36868°, alt. *c*. 1392m, 21 Aug. 2018, *G. Swarnalatha* 753 (BSID).

Myelochroa aurulenta (Tuck.) Elix & Hale, Mycotaxon 29: 240. 1987. (PARMELIACEAE)

Specimen examined: Odisha: Gajapati District, foot hills of Mahendragiri, near Burukhat area, 04 Mar. 1986, *D.D. Awasthi, G. Awasthi, R. Mathur and P. Srivastava* 86.201A (LWU-LWG).

Parmotrema andinum (Müll. Arg.) Hale, Phytologia 28(4): 334. 1974. (PARMELIACEAE)

Specimen examined: Odisha: Gajapati District, foot hills of Mahendragiri, near Burukhat area, 04 Mar. 1986, *D.D. Awasthi, G. Awasthi, R. Mathur and P. Srivastava* 86.201B (LWU-LWG).

Phaeophyscia hispidula (Ach.) Essl., Mycotaxon 7(2): 305. 1978. (PHYSCIACEAE)

Specimen examined: Odisha: Gajapathi District, Parlakhemundi Forest Division, Mahendragiri Hills, N 18.95882°, E 084.37431°, alt. *c*. 908m, 19 Aug. 2018, *G. Swarnalatha* 724 (BSID).

Phaeophyscia pyrrhophora (Poelt) D. D. Awasthi & M. Joshi, Indian J. Mycol. Res. 16(2): 278. 1978. (PHYSCIACEAE)

Specimen examined: Odisha: Gajapathi District, Parlakhemundi Forest Division, Mahendragiri Hills, N 18.97253°, E 084.35698°, alt. *c*. 1035m, 21 Aug. 2018, *G. Swarnalatha* 767 (BSID).

Phyllopsora manipurensis (Müll.-Arg.) Gotth. Schneider, Bibl. Lichenol. 13: 177. 1979 (1980). (RAMALINACEAE)

Specimen examined: Odisha: Gajapathi District, Parlakhemundi Forest Division, Mahendragiri Hills, N 18.96553°, E 084.36848°, alt. *c*. 1378m, 20 Aug. 2018, *G. Swarnalatha* 743 (BSID).

Pyxine reticulata (Vain.) Vain. Suomal. Tiedeakat. Toim. (Ser. A) 6: 70. 1914. (CALICIACEAE)

Specimen examined: Odisha: Gajapathi District, Parlakhemundi Forest Division, Mahendragiri Hills, N 19°002 26.33 , E 084°212 58.23 , alt. *c*. 560m, 08 Sep. 2016, *G. Swarnalatha* 434 (BSID).

Scytinium gelatinosum (With.) Otálora, P.M. Jørg. & Wedin, Fungal Diversity 64: 290. 2014. (COLLEMATACEAE)

Specimen examined: Odisha: Gajapati District, foot hills of Mahendragiri, near Burukhat area, 04 Mar. 1986, *D. D. Awasthi, G. Awasthi, R. Mathur* and *P. Srivastava* 86.178 (LWU-LWG).

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