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## Submergence and Salinity Tolerance in Sweet Potato (*Ipomoea batatas* L.): Progress and Prospects

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Sweet potato is grown in most of the states of India in a wide range of agro-ecological conditions. It is a high energy low input food crop. In addition to possessing a high starch content the recent varieties of orange and purple flesh sweet potato provide the cheapest source of antioxidants. Despite its wide adaptive nature, production of sweet potato is challenged by salinity and submergence stresses especially in coastal areas. To overcome these limitations, *in vitro* methodologies have been developed for rapid screening and large scale evaluation of sweet potato for salinity tolerance. Besides, paclobutrazol (PBZ) and CaCl<sub>2</sub> mediated submergence tolerance have also been studied. Outcome of such investigation towards stress tolerant sweet potato will help enhance productivity, promote food, nutrition security and economic sustainability especially in developing countries.

**Key words:** Salinity, stress tolerance, submergence, sweet potato

### Introduction

Sweet potato (*Ipomoea batatas* L.) is the world's fifth most important crop and is a major source of food and nutrition in developing countries (Food and Agriculture Organization, 2002). The advantages of sweet potato are well documented (Woolfe, 1993). Being extra-rich in food value this crop can play a major role in curbing nutritional deficiency. Sweet potato can produce more edible energy per hectare per day than wheat, rice or cassava and require less water and agrochemicals to grow within 90-120 days. It is a hexaploid ( $2n=6x=90$ ), cross pollinated crop which propagate vegetatively (Mukherjee *et al.*, 1998). This crop is gaining importance not only for high dry matter production per unit area per unit time but also a ready source of antioxidants. The  $\beta$ -carotene rich orange and anthocyanin rich purple flesh sweet potatoes are the cheapest source of antioxidants.  $\beta$ -carotene of orange flesh sweet potato (0.1 mg/1 g) is comparable to that of carrot and much higher than genetically transformed golden rice (1.6  $\mu$ g/1g). Anthocyanin content of sweet potato is recorded as 80-90 mg/100 g. To combat malnutrition, VITAA (Vitamin A for Africa) project coordinated by the International

Potato Center (CIP, Lima, Peru) has been launched and progressing well with orange flesh sweet potato. Vitamin A deficiency is one of the major cause of child blindness in India and about 40,000 children are affected every year. Being rich in  $\beta$ -carotene and anthocyanin orange and purple flesh sweet potato are most suited as a biofortified crop to combat malnutrition in many developing nations.

In India sweet potato is grown in an area of 0.14 m ha producing 1.17 million tones of tubers with a productivity of 8.3 t/ha. It grows in most of the states starting from the Himalayan foot hills to the southern plains of Peninsula. Though the crop can grow in wide agro-ecological conditions, the yield is affected severely due to abiotic stresses. Drought, salinity and water logging or submergence are the major abiotic constraints in successful agriculture. Sweet potato is relatively resistant to moisture stress because of its deep-root system but it is highly sensitive to salinity and waterlogging. In recent years such abiotic stresses are becoming predominant as a consequence of global warming.

Salinity is one of the major factors reducing plant growth and productivity worldwide and affects about 7%

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of the world's total land area (Flower *et al.*, 1997). The percent of cultivated land affected by salinity is even greater. 23% of the cultivated land is saline and 20% of the irrigated land suffer from secondary salinization (El-Hendawy *et al.*, 2005). Furthermore, there is also a dangerous trend of a 10% increase per year in the saline area throughout the world (Ponnamieruma, 1984). Salinity effects are more conspicuous in arid and semi-arid areas. In those areas, plant growth is severely affected by salinity through water deficit, salt-specific damages (Munns and Termaat, 1986) or oxidative stress (Hernandez *et al.*, 1995). It is claimed that abiotic stress is the primary cause of crop loss, reducing average yields of most major crops by more than 50% (Wang *et al.*, 2003). This proportion will rise with increasing irregularity of climate and frequency of extreme climate events.

In India 23.8 million hectares of land is affected by salinity. The total waterlogged area is reported to be 11.6 million ha (ICAR, 2006) and is expected to increase steadily due to global warming. Agricultural productivity on such vast wet land is affected due to submergence and salinity stresses. Such abiotic stresses are important growth-limiting factor for most non-halophytic plants (Villa-Castorena *et al.*, 2003) and sweet potato is not an exception. However, diversity in species, varieties and practices has permitted agriculture to withstand moderate change in climate over the past 10,000 years. One of the most effective ways to overcome abiotic stress problems is the biological approach to identify and grow stress tolerant plants. Extensive research have been carried out on salinity tolerance of cereals (Nabors and Dykes, 1985) and leguminous crops (Gosal and Bajaj, 1984). Research has significantly advanced in developing hardier crop varieties through conventional breeding methods, and molecular biology tools are accelerating the process. Rice researchers have succeeded in identifying a rice gene (*sub1A*) that enable plants to survive submergence for up to two weeks, while most rice survives underwater for only three days (Russell, 2007). However, substantial effort in developing salinity and submergence tolerance in sweet potato is lacking. Work in this line mostly reported the development of salt tolerant tissues and salinity induced changes in isozyme, organic and inorganic constituents (Chang *et al.*, 1984; Nyman *et al.*, 1989). In Taiwan, Lin *et al.*, (2006; 2008) reported submergence tolerance on sweet potato when pretreated with paclobutrazol (PBZ) and  $\text{CaCl}_2$ . However, in India work

on salinity tolerance of orange and purple flesh sweet potato is limited to the work carried out at the Central Tuber Crops Research Institute (Mukherjee, 2001 and 2002a; Mukherjee, 2005; Mukherjee *et al.*, 2007).

Salinity stress results in accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  ions which are detrimental to plants. Flooding stress results in reduction of oxygen at root zone that inhibit aerobic processes, damages roots causing rapid wilting and death of plants. Understanding plant responses to the external environment is of great importance and also a fundamental part for making the crops stress tolerant. To isolate stress tolerant lines, the knowledge on influence of salinity and submergence stresses and progress made in stress tolerance are prerequisite to augment genetic improvement programme in sweet potato.

This paper attempts to review the work carried out on antioxidative system, screening and evaluation for submergence and salinity tolerance in sweet potato. The review also projects future strategies towards development of stress tolerant lines.

### Submergence tolerance

Plants are severely affected by flooding, because under such submergence stress the production and the quenching of the reactive oxygen species (ROS) in plants can not be kept in a balanced state (Crawford and Brandle, 1996). Oxygen limitation and scavenging of reactive oxygen species (ROS) is the primary stress to plants growing under saline and submerged soils. Such environmental stress can throw the production out of balance (Gratao *et al.*, 2005).

In plant cells, stress-induced reactions are associated with free radicals being produced from reduction of molecular oxygen. These toxic radicals can be removed both enzymatically and chemically to protect plant cells against oxygen toxicity and counter the hazardous effects of ROS under stress (Gratao *et al.*, 2005). ROS scavenging is an important step in developing tolerance against waterlogged stress (Noctor & Foyer, 1998). Some antioxidative enzymes are increased to impart submergence tolerance, many of such enzymes have been identified and characterized. The amount of free radicals and the level of antioxidative enzymes in the cells share a direct proportionality (Pinhero *et al.*, 1997; Rout and Shaw, 2001). The antioxidative defense system in plants is composed of antioxidative enzymes



vit. ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) and metabolites such as ascorbic acid (ASA), reduced glutathione (GSH), oxidized glutathione (GSSG) and vitamin E (Sairam *et al.*, 2002; Gratao *et al.*, 2005; Lin *et al.*, 2008). In sweet potato, development of submergence tolerance have been found to be attributed by increased activity of SOD and CAT enzymes in leaf (Hwang *et al.*, 1999; Lin *et al.*, 2006; Lin *et al.*, 2008). Therefore, use of some oxidative enzymes or oxidants have been found to be useful in screening for flooding / submergence tolerant plants (Lin *et al.*, 2004). Thus, to impart flooding tolerance in sweet potato, experiments were carried out in Taiwan to boost up antioxidant system with paclobutrazol (PBZ) and  $\text{CaCl}_2$  which are discussed below.

#### Effect of paclobutrazol (PBZ)

Paclobutrazol (PBZ) is a member of the triazole family. Triazoles have both fungitoxic and plant growth regulatory effects. They can protect plants against various stresses, including drought, low and high temperatures, UV-B radiation, air pollutants, and fungal pathogens. Therefore, the triazoles have been characterized as plant multi-protectants (Kraus and Fletcher, 1994; Pinhero and Fletcher, 1994; Lin *et al.*, 2006). Paclobutrazol protects plants by boosting antioxidant defense systems. Paclobutrazol mediated changes in antioxidant enzymes and metabolites have already been studied in other crops (Lurie *et al.*, 1994; Paliyath and Fletcher, 1995). PBZ mediated stress tolerance might be attributed to the enhancement of antioxidative system. Such pretreated plants reported to have higher free-radical scavenging system to confer crop protection against oxidative stress (Kopyra and Gwozdz, 2003).

Changes of antioxidants and antioxidative enzymes in the flooding stressed sweet potato leaf, as affected by paclobutrazol treatment 24 h prior to stress imposition was studied by Lin *et al.* (2006). Under flooding stress conditions, the level of 'Taoyuan 2' sweet potato variety's antioxidative system found to have been influenced by PBZ treatment. Pre-treating with PBZ would have increased the levels of various components of antioxidative systems after exposure to different durations of flooding and drainage, thus inducing flooding tolerance (Lin *et al.*, 2006).

Enhancement on SOD, CAT and APX activity, and total glutathione, oxidized ASA and total ASA content

was reported when added with PBZ prior to flooding stress. Hence, PBZ could protect the sweet potato from submergence stress through its effect on the plant's oxygen detoxifying system. Lin *et al.*, (2006) suggested that pre application of 0.5 mg/plant of PBZ 24 h prior to flooding could mitigate flooding stress in sweet potato. Their findings encourage sweet potato farming in frequently flooded areas.

#### Effect of $\text{CaCl}_2$

Calcium is known to protect the integrity of cell membranes, reduce membrane permeability and prevent ion leakage caused by environmental stress (Lin *et al.*, 2008).  $\text{Ca}^{2+}$  emerges as a key secondary messenger and signal transducer of various environmental stress stimuli. The stimulating effect of  $\text{Ca}^{2+}$  depend on the particular environmental stress, the rate of stress development, previous exposure to stress conditions and the tissue type (Bin, 1995). Moreover, the effects of  $\text{Ca}^{2+}$  on elongation, senescence process and photosynthetic activity are dependent on its cytosolic concentration, which is governed by the activity of channels in the plasma membrane (Knight, 2000). It plays a crucial role in various adaptive cellular responses, as well as in regulating diverse physiological processes including those affected by abscisic acid (Gong *et al.*, 1998). Calcium has been reported to confer protection to plants experiencing stress by reducing the oxidative damage through the elevation of antioxidant contents or oxidative enzyme activities (Nayyar & Kaushal, 2002; Lin *et al.*, 2008).

The observation on enhanced antioxidative system with pretreatment of  $\text{CaCl}_2$  under stress found to have conformity when compared with PBZ treated plants. Pretreatment of sweet potato plants with  $\text{CaCl}_2$  could enhance tolerance to flooding stress by enhancing reduced glutathione (GSH). In an experiment in Taiwan (Lin *et al.*, 2008) three sweet potato varieties Taoyuan 2, Simon 1 and Sushu 18 were pretreated with four levels of  $\text{CaCl}_2$  (0, 60, 120 and 180 kg/ha) at weekly intervals for 50 days from planting before being subjected to flooding stressed conditions. The ascorbate peroxidase, superoxide dismutase, glutathione reductase, reduced ascorbate, total ascorbate, reduced glutathione and malondialdehyde contents of the three sweet potato varieties found to increase significantly under flooding stress owing to pretreatment with 60 and 120 kg/ha of  $\text{CaCl}_2$  concomitant with an enhanced flooding tolerance in all the three tested varieties. Thus, the elevated level



of activity of the antioxidative system in sweet potato leaves might have been induced by  $\text{CaCl}_2$  (Lin *et al.*, 2008).

### Salinity tolerance

Work on salinity tolerance in sweet potato at national and international level have been reported which also include some exotic collection introduced from International Potato Center, CIP, Lima Peru (Ekanayake and Dodds 1993; Ahmed *et al.*, 1999). *In vitro* method resulted in development of NaCl resistant cells tolerant to 1% NaCl for three passages (Salgado *et al.*, 1985).

In India, information on salinity tolerance is virtually confined to the work carried out at the Regional Centre of Central Tuber Crops Research Institute, Bhubaneswar (Mukherjee, 1999a, b, 2001, 2002, 2005; Mukherjee *et al.*, 2006, 2007; Dasgupta *et al.*, 2008.)

*In vitro* multiplication studies have resulted in enhanced rate of propagation of sweet potato with supplementation of NaCl in culture medium (Mukherjee, 1999a, b, 2001, 2002). Though rate of propagation in all possible routes of *in vitro* propagation was higher in NaCl supplemented medium, level of NaCl tolerance was quite high in case of embryogenic regeneration pathway. Despite the variation in degree of tolerance, all the tested

genotypes responded to somatic embryogenesis even with 10 g/l NaCl supplementation. Regenerated plants recorded a high rate (90-100%) of survival in the field. The somatic embryos produced with NaCl supplementation were quite hardy and could also tolerate low temperature (8 °C) stress during storage even without the protective covering by expensive encapsulating chemicals such as sodium alginate (Mukherjee and Debata, 2002).

The hydroponic cultures and NaCl mediated *in vitro* regeneration protocols developed in sweet potato and other tuber crops (Mukherjee *et al.*, 1994; Mukherjee *et al.*, 1998; Mukherjee, 1999a and b; Mukherjee, 2001; Mukherjee, 2002; Dasgupta *et al.*, 2002; Mukherjee *et al.*, 2006) have facilitated faster screening of large collection for stress tolerance. Isoenzymes and RAPD marker studies carried out in sweet potato (Mukherjee, 2002; Mukherjee *et al.*, 2003) have hastened their characterization.

The varied growth response, morpho-physio-biochemical changes were studied under *in vitro* NaCl mediated stress and under *in situ* saline conditions (Mukherjee *et al.*, 2007).

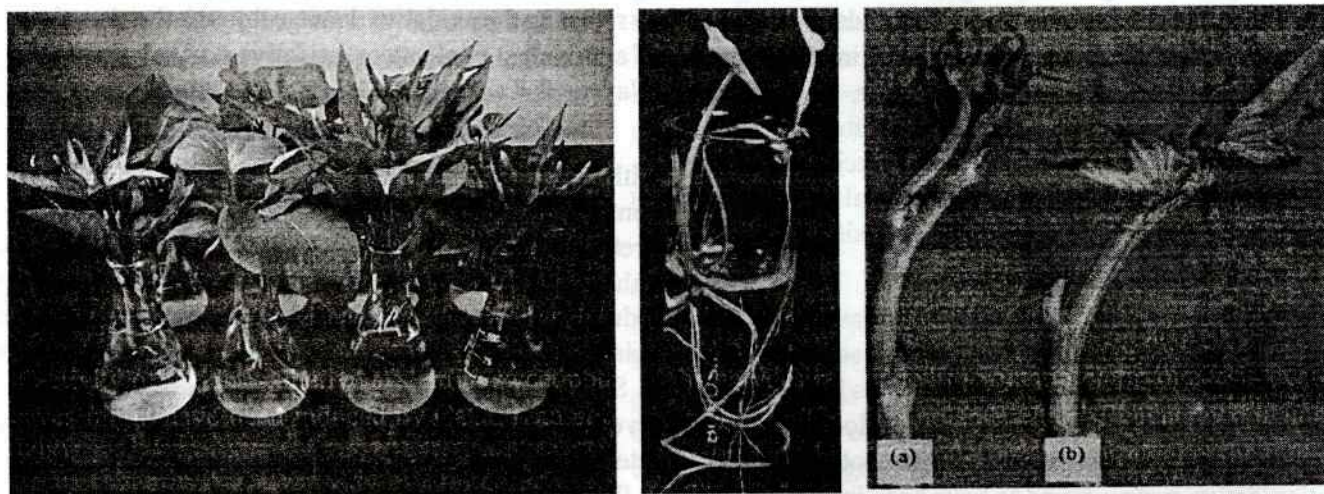


Fig. 1. Screening genetic resources of sweet potato for salinity tolerance.

- A. Screening in hydroponics.
- B. Sweet potato accessions grown in hydroponics culture with NaCl.
- C. Necrosis under salt stress in susceptible (a) and tolerant (b) accessions.



### Effect of NaCl on *in vitro* shoot development & rooting

Roots are among the first organs affected by salt stress and are most sensitive. A well marked difference was noticed in rooting response in all tested genotypes (Fig. 1A & B). Delayed rooting response (4-6 days) was observed at higher doses of NaCl (24 dS/m) as compared to the control (2-4 days). Based on rooting response and minimum percentage of leaf necrosis (Fig. 1C) at higher dose of NaCl (24 dS/m), 15 genotypes were screened out of 47 and evaluated subsequently under *in vitro* and *in vivo* conditions.

A wide variation in the growth parameters studied were observed among the genotypes tested with respect to different levels of salinity (Mukherjee, 2005). The analysis of variance for growth parameters *in vitro* indicated that there were significant effects of NaCl treatment, genotypes and NaCl x genotypes interaction for all the studied parameters. The results revealed that the growth parameters decreased significantly with increase in salinity stress. Plantlet growth was reduced remarkably in all the genotypes under 0.5 and 1.0% NaCl stress, compared with their control. Particularly, survivability of the explants was reduced to a great extent under salinity. Of the 15 genotypes tested, nine could survive at 0.5% NaCl. However, at higher (1.0% NaCl) stress, growth was completely suppressed and only six genotypes viz., CIP 420027, ST 13, Gouri, ST 14, CIP 187017.1 and SB-198/115 could survive, while rest of the genotypes showed necrosis and died in between 2-3 weeks of inoculation. At relatively higher stress (1.0% NaCl), no shoots emerged even after 21 days of culture and the explants consequently necrosed in nine genotypes (Dasgupta *et al.*, 2008).

*In vitro* screening and induction of stress tolerance showed varied growth responses among tested genotypes when cultured on MS medium with growth regulators and NaCl. Under 1.0% NaCl, shoot length was decreased by 12.98% to 75.46% among the responded genotypes. The reduction in leaf number was reported at 0.5% NaCl and the same was pronounced at 1% NaCl stress over stress free control. The six genotypes, which produced shoots at higher salt stress could develop roots even when cultured at 1.0% NaCl mediated stress.

### Influence of NaCl on antioxidative enzymes

Antioxidative enzyme activities increase in diverse environmental stress situations (Mittler 2002), as a

response related to detoxification of reactive oxygen species (ROS). Among many enzymes regulating the intracellular level of  $H_2O_2$ , peroxidases and catalases are considered to be most important. Such studies have been carried out in sweet potato under NaCl stress.

Stress induced increase in CAT and GPX activities have been reported in sweet potato (Dasgupta *et al.*, 2008). Leaf SOD activity was greater in salt stressed plantlets than that in controls. CAT activity in leaves of control and salt stressed plants varied significantly. The increase in CAT activity under NaCl stress over control was reported in the range of 60-70% increase in tolerant genotypes than 30-40% increase in susceptible genotypes. A similar pattern was observed for GPX activity, which also increased significantly in all the tested genotypes under induced salt stress condition. At higher stress, tolerant genotypes exhibited 40-60% increase in GPX over control. Rout and Shaw (2001) suggested that CAT and GPX as the most important  $H_2O_2$  scavenging enzymes leading to salt tolerance in aquatic macrophytes. In sweet potato, it could be presumed that CAT is more important  $H_2O_2$  scavenging enzyme in leaves than GPX.

### *In situ* evaluation of *in vitro* screened sweet potato

*In situ* evaluation is the most practical approach to validate the results of *in vitro* screening. The *in vitro* selected fifteen sweet potato genotypes out of 47 were grown under *in situ* salt stress (6-8.0 dS/m) in farmers' field of coastal Kendrapara district of Orissa state in India. The trial showed varied yield and biochemical responses (Mukherjee *et al.*, 2006, 2007). The yield ranged from 5.2 t/ha to 14.99 t/ha (Table 1). The highest yield was recorded by CIPSWA-2 (14.99 t/ha) followed by Gouri (14.80 t/ha). The genotypes viz. ST-13, ST-14, 420027 & SB 198/115 gave reasonably higher yield under salt stress in the range of 6-8.0 dS/m. Salinity induced biochemical changes showed decrease in reducing sugar and total soluble sugar (Fig. 2 & 3) in the range of 7.31-28.38% and 9.91-31.10% respectively in the tested genotypes. The protein content (Fig. 4) was observed to be decreasing 9.54-46.49% due to salinity stress. Increasing level of proline accumulation (Fig. 5) was observed in all the tested genotypes under stress. However, extent of increase was more than 78% in six different genotypes viz. 420027, ST-14, ST-13, CIPSWA 2, Gouri and SB-198/115. Nitrate reductase activity under stress was also high. Isozyme and DNA polymorphisms were quite pronounced in these tolerant genotypes under



*in situ* salt stress (Mukherjee *et al.*, 2007). Of the above identified salt tolerant six genotypes, five are rich in carotene and ST-13 is rich in anthocyanin. Carotene (Table 2) and anthocyanin contents of the identified salt tolerant genotypes were reported to be as good as tubers grown under non-stressed condition (Mukherjee *et al.*, 2007).

Moreover, performance of these *in vitro* screened salt tolerant genotypes with reasonably good yield coupled with high  $\beta$ -carotene (5-14 mg/100 g) and anthocyanin (85-90 mg/100 g) contents even under *in situ* salt stress (6-8 dS/m) have greater impact towards sustainable agriculture for coastal food and nutrition security (Mukherjee *et al.*, 2008; Mukherjee *et al.*, 2009).

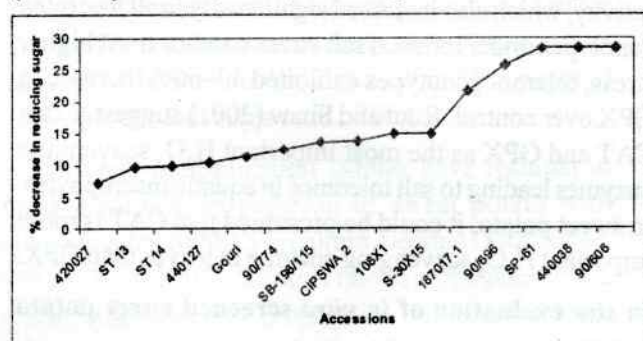


Fig. 2. Percentage reduction in reducing sugar content of leaves due to salinity stress.

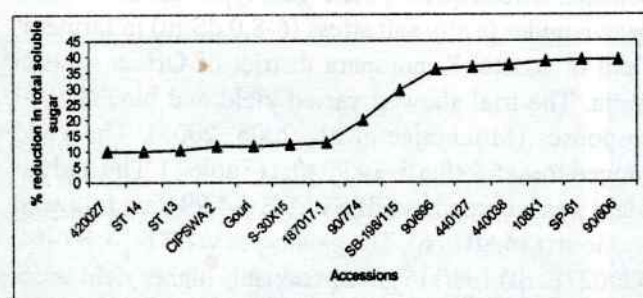


Fig. 3. Percentage reduction in total soluble sugar content of leaves due to salinity stress.

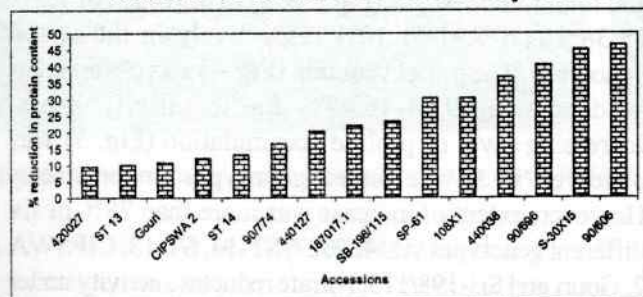


Fig. 4. Percentage reduction in protein content of leaves under *in vivo* salt stress.

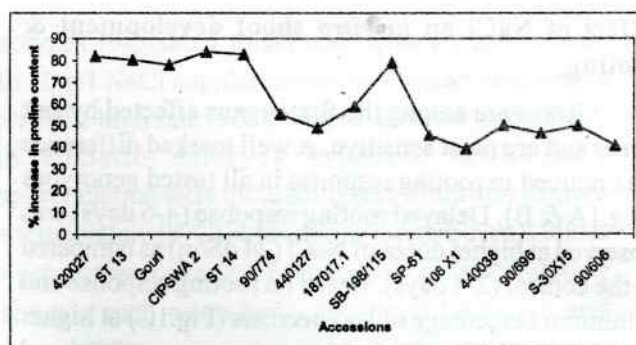


Fig. 5. Percentage increase in proline content of selected accessions of sweet potato under *in vivo* salt stress.

Table -1

Evaluation for salinity tolerance in sweet potato under *in situ* salt stress (EC: 6-8 dS/m) at coastal Kendrapara of Orissa state, India.

| Genotypes  | Yield (t/ha) |
|------------|--------------|
| SB 198/115 | 11.21        |
| S30X15     | 6.38         |
| 90/606     | 5.2          |
| 440127     | 10.25        |
| ST-14      | 12.25        |
| 440038     | 9.93         |
| 108X1      | 8.61         |
| GOURI      | 14.80        |
| 187017.1   | 9.11         |
| SP 61      | 5.95         |
| ST-13      | 12.49        |
| 90/774     | 6.94         |
| 420027     | 11.85        |
| 90/696     | 6.11         |
| CIPSWA-2   | 14.99        |
| CD at 5%   | 2.96         |

Table -2

Carotene content of identified salt tolerant genotypes grown under *in situ* salt stress (EC:6-8 dS/m).

| Genotypes  | Carotenoid (mg/100g tuber) |
|------------|----------------------------|
| ST-14      | 13.2                       |
| CIP-SWA 2  | 6.5                        |
| 420027     | 5.9                        |
| GOURI      | 5.2                        |
| SB 198/115 | 4.8                        |



### Present and future strategies towards development of tolerant lines

The tuberous crop sweet potato has an immense potential to meet the challenges of today's food demand. Diversity within its genotypes, wide distribution and potential to adapt in harsh environmental condition advocates for its further exploitation to develop genetically improved lines. Genetic improvement through conventional breeding alone is difficult in sweet potato due to hexaploidy coupled with self incompatibility. Intensification of breeding through integration of conventional and non conventional methods is thus essential to develop stress tolerant plants at a faster pace.

With the realization of potential usefulness of development of stress tolerant sweet potato, research programme was taken up and carried out at Regional Centre of Central Tuber Crops Research Institute, Bhubaneswar under AP Cess fund scheme of Indian Council of Agricultural Research (ICAR). The programme was aimed to screen and evaluate the genetic resources of sweet potato for salt stress tolerance integrating both conventional and non conventional methods. Such studies resulted in the identification of orange and purple flesh sweet potato with salinity tolerance through large scale screening and evaluation of sweet potato genotypes. Moreover, the increasing demand for food with increasing population growth demands climate resilient crops which could grow in vast wetland facing frequent waterlogging or submergence stresses. In this context, the results on PBZ and calcium chloride mediated flooding tolerance have greater implication to enhance productivity even on waterlogged conditions.

*In vitro* screening using stress chemicals would provide the efficient route to screen for stress tolerance. A detailed stress induced changes in morpho-physio-biochemical parameters of tolerant and susceptible lines would help characterize stress tolerant genes, which are prerequisite for development of desirable transgenics. Research over the past two decades has provided a better understanding of the molecular biology of stress responses in plants (Wang *et al.*, 2003). Many genes and gene products have been identified which get induced upon exposure of plants to various abiotic stresses like salinity, submergence, drought, low and high temperature exposure etc. Studies on molecular basis of stress

tolerance are being continued in many agri-horticultural crops. Unfortunately, such molecular studies in sweet potato are confined to determining its phylogenetic relationship (Huang *et al.*, 2000; Hwang *et al.*, 2002).

Future studies on molecular biology of stress tolerant and sensitive lines would help understand molecular mechanisms of stress tolerance with a view to identifying stress tolerant genes. Such knowledge would considerably facilitate gene pyramiding for metabolic engineering of high energy, nutrient rich sweet potato bestowed with multiple resistance against major abiotic stress factors.

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## Copper Toxicity Alters the Activities of Antioxidative Enzymes and Increases Lipid Peroxidation in Germinating Seeds

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The seeds of mungbean (*Vigna radiata* (L.) Wilczek) and black gram (*Vigna mungo* (L.) Hepper) were subjected to  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (1-100  $\mu\text{M}$ ) for 48 h and germination percentage of seeds, soluble protein content along with antioxidative enzyme activities and lipid peroxidation level of embryonic tissues were measured. The germination percentage was found to inhibited by higher concentrations of Cu (50  $\mu\text{M}$  onwards) and soluble protein content was decreased after initial increase at 1  $\mu\text{M}$  concentration. There was continuous increase in superoxide dismutase (SOD) activity with increase in Cu concentrations up to 50  $\mu\text{M}$ . Catalase (CAT) activity increased at 1  $\mu\text{M}$  and peroxidase (POX) activity was high up to 10  $\mu\text{M}$  and with subsequent increase in Cu concentrations, the activities of these enzymes declined. The measurement of higher level of lipid peroxidation indicated the imposition of oxidative stress in tissues due to higher concentrations of Cu. This was further substantiated by the neutralization of Cu-induced toxicity by antioxidant (ascorbate). The lower Cu concentration, (i.e., 1  $\mu\text{M}$ ) has been found to beneficial in both the seeds. The seeds could tolerate the concentrations up to 10  $\mu\text{M}$  and the concentrations at 50 and 100  $\mu\text{M}$ , toxic effects were more pronounced. The results suggest that higher concentrations of Cu impose oxidative stress in the tissues which might be one of the mechanisms behind Cu induced toxicity in germinating seeds.

**Key words:** Ascorbate, black gram, catalase, mungbean, peroxidase, soluble protein, superoxide dismutase

### Introduction

Environmental pollution due to heavy metals has got wide attention now-a-days. The different anthropogenic activities including mining, industrialization and urbanization have resulted in the environmental pollution due to different heavy metals. Copper is a group 1B metal used in industries like organic chemicals, fertilizers, iron and steel works, electrical works, pulp and paper industries, antifouling paints, pesticides, automobile industries etc. The metal is discharged into the environment especially to the fresh water environment in high concentrations through industrial effluents that severely affects the flora and fauna (Venkataramana and Radhakrishnaiah, 2001; Yruela, 2005; Lodhi *et al.*, 2006). As a trace element, Cu is essential for the physiological and biochemical functions of photosynthetic organisms (Baron *et al.*, 1995). But in high concentrations, Cu becomes toxic for phytoplanktonic communities. This metal can affect the electron transport system through photosystem II and modify or inhibit

enzymatic activities (Pinto *et al.*, 2003). According to Sunda and Guillard (1976), copper toxicity is related to cupric ion activity rather than total copper concentration. The Cu bioavailability is regulated by several factors including Cu speciation, which is linked to various parameters like pH, redox potential, alkalinity, organic and inorganic ligand concentration. (Van Hullebusch *et al.*, 2002).

Copper smelting gives rise to high localized concentrations of Cu near the smelting plants. For the last 100 years, copper sulphate (as Bordeaux mixture) has been used as a fungicide in agriculture (Thakur, 2006). More recently,  $\text{CuSO}_4$  has also been used in animal feed to increase the rate of weight gain by the piglets. Along with the industrial wastes, the use of this metal as pesticides also contributes significantly towards soil pollution. At concentrations above those required for optimal growth of plants, Cu has been reported to inhibit growth and interfere with important physiological processes like photosynthesis and respiration (Marschner,

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1995; Prasad and Strzalka, 1999). Presence of high levels of Cu normally shows reduced biomass and chlorotic symptoms in plants. Lower content of chlorophyll and alterations of chloroplast ultrastructure along with thylakoid membrane composition are generally found in leaves under high levels of Cu (Yruea, 2005).

Copper is known to catalyze the formation of hydroxyl radicals ( $\cdot\text{OH}$ ) from the non-enzymatic chemical reaction between superoxide radical ( $\text{O}_2^{\cdot-}$ ) and  $\text{H}_2\text{O}_2$ , via Haber-Weiss reaction (Halliwell and Gutteridge, 2007). Therefore, the excess of Cu can cause oxidative stress in the aerobic organisms that can weaken the natural antioxidative defense system. Germination is an important phase of growth and development in plant life. Even though the effect of toxic concentrations of Cu on different systems under different developmental phases have been reported largely, the studies on the effect of this metal during germination phase are rare. Therefore, in this study the effect of Cu on the activities of antioxidative enzymes like superoxide dismutase, catalase and peroxidase along with its effect on the level of lipid peroxidation was assessed in the germinating seeds of two locally available pulses: mungbean (*Vigna radiate* (L.) Wilczek) and black gram (*Vigna mungo* (L.) Hepper). The objective was principally to find the possible mechanism behind Cu toxicity which might help in formulating strategy for reduction of Cu toxicity in plants growing in polluted sites.

## Materials and Methods

### *Plant materials, germination conditions and imposition of Cu stress*

Mungbean (*Vigna radiate* (L.) Wilczek) and black gram (*Vigna mungo* (L.) Hepper) seeds were collected from local market and were selected for uniformity of size. Seeds were then surface sterilized with freshly prepared and filtered 3% solution of commercial bleaching powder (Calcium oxychloride,  $\text{CaOCl}_2$ ) for 30 min followed by thorough washings with distilled water for several times. The seeds were then spreaded in Petri dishes over filter paper moistened with 5.0 ml of different concentrations (1, 10, 50 and 100  $\mu\text{M}$ ) of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  solution separately. Distilled water was taken in another Petri dish as control. 25 nos. of surface sterilized seeds were spreaded in each Petri dish to maintain uniformity in stress imposition. The seeds were allowed to germinate in dark at  $30 \pm 2^\circ\text{C}$  and after 48 h of germination; the

seeds were collected separately for various analytical studies.

### *Germination study*

After 48 h of exposure to Cu, the no. of seeds germinated in each Petri dish was counted and percentage of seed germination was computed. This was compared with the percentage of seed germination in control Petri dish.

### *Biochemical analyses*

For biochemical analyses, the embryos from germinated seeds were excised carefully after thorough washing (only radicle and plumule portions were taken, discarding cotyledons). The extraction and estimation procedures were followed separately for different parameters.

For soluble protein extraction, the tissues were homogenized with sodium phosphate buffer of 50 mM, pH 7.5 under ice-cold conditions. The homogenates were centrifuged at 17,000 g for 10 min at  $-4^\circ\text{C}$  and to the supernatant, equal volume of 20% (w/v) trichloroacetic acid (TCA) was added. The samples were kept over night in refrigerator and washed subsequently with 10% TCA, absolute alcohol, alcohol and chloroform (in a proportion of 3:1), alcohol and ether (in a proportion of 3:1) and finally with ether. After washing, the pellets were air dried and re-suspended with 0.3 N NaOH solutions for 16 h at  $37^\circ\text{C}$ . Then the samples were centrifuged and supernatants collected separately for soluble protein estimation, after suitable dilutions. The protein content of the extract was estimated following the method of Lowry *et al.*, (1951), and calculated taking bovine albumin as standard.

For extraction of enzymes, the tissues were homogenized separately with extraction buffers under ice-cold conditions. The buffers used were sodium phosphate buffer, 50 mM, pH 7.4 for superoxide dismutase (SOD); and sodium phosphate buffer, 50 mM, pH 7.5 for catalase (CAT) and peroxidase (POX). The homogenates were centrifuged at 17,000 g for 10 min at  $-4^\circ\text{C}$  and the supernatants were collected separately and used for enzyme assays, after suitable dilutions.

Superoxide dismutase (EC 1.15.1.1) was assayed following the method of Das *et al.*, (2000) where inhibition of superoxide driven nitrite formation from hydroxylamine hydrochloride by SOD was measured under laboratory conditions. Activity of SOD was



calculated using the formula  $V_0/V$ , where  $V_0$  is the absorbance at 543 nm of the control (without enzyme) and  $V$  is the absorbance of sample (with enzyme) at the same wavelength. Catalase (EC 1.11.1.6) activity was assayed following the method of Aebi (1983) where the rate of decreasing concentration of  $H_2O_2$  at 240 nm due to CAT was measured and the activity was calculated using the extinction coefficient of  $40.0 \text{ mM}^{-1} \text{ cm}^{-1}$  for  $H_2O_2$  at 240 nm. To assay POX (EC 1.11.1.7) activity, guaiacol and  $H_2O_2$  were taken as substrates. The tetraguaiacol formation in the assay mixture increased the colour intensity which was recorded at 470 nm, as described by Kar and Feierabend (1984). The activity of POX was calculated using the extinction coefficient of  $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$  for tetraguaiacol.

Malondialdehyde (MDA), a decomposition product of peroxidized polyunsaturated fatty acid components of membrane lipid, was estimated in this study as thiobarbituric-acid-reactive material from tissue extracts. The extraction was done in 5% trichloroacetic acid and MDA was estimated as described by Heath and Packer (1968). Along with the tissues exposed to Cu, estimation of MDA was also done in the tissues exposed to  $\text{CuSO}_4 \cdot 5H_2O$  with added antioxidant (ascorbate). The unspecific turbidity in the reaction mixtures was corrected by subtracting the absorbance at 600 nm, and for absorbance at 532 nm originating from extract after incubation without thiobarbituric acid.

The SOD activity was expressed in unit, defined as the amount that inhibits the superoxide driven nitrobluetetrazolium reduction by 50% under the assay conditions. Activities of CAT and POX were expressed as katal, i.e., mole of substrate used up or product formed per second due to enzyme activities. All the experiments were performed at least for three times, with three replicates in each time. The mean values are presented with standard deviations.

## Results and Discussion

Copper is an essential micronutrient for plants and is known to be associated with many enzymes in redox reactions since this metal exists in multiple oxidation states *in vivo*. Under physiological conditions, Cu exists as  $\text{Cu}^{2+}$  and  $\text{Cu}^+$  and acts as a structural element in regulatory proteins. Among the different physiological roles the participation of Cu in photosynthetic electron transport,

mitochondrial respiration, oxidative stress responses, hormone signaling and cell wall metabolism are important (Marschner, 1995; Raven *et al.*, 1999). The concentration of Cu in the cell is usually maintained at low level since this element is extremely toxic in view of its high redox properties. In plant tissues, the average content of Cu is  $10 \mu\text{g.g}^{-1}$  dry weight (Baker and Senef, 1995) and the critical free Cu concentration in nutrient media (below which Cu deficiency occurs) ranges from  $10^{-14}$  to  $10^{-16} \text{ M}$ . The concentrations of Cu in soil solution range from  $10^{-6}$  to  $10^{-9} \text{ M}$  and hence plants find a variable supply of this metal in soil. Under its deficiency, different physiological anomalies in plants are found that are observed in the form of deficiency symptoms. It has been found that the redox cycling between  $\text{Cu}^{2+}$  and  $\text{Cu}^+$  can catalyze the production of highly toxic hydroxyl radicals ( $\cdot\text{OH}$ ). The hydroxyl radicals are known to be potentially toxic reactive oxygen species that can cause damage to proteins, lipids, DNA and other biomolecules (Halliwell and Gutteridge, 1984). Thus, at high concentrations, Cu can become toxic to plant growth and development (van Assche and Clijsters, 1990; Marschner, 1995).

In this study, the concentrations of  $\text{CuSO}_4$  to which the seeds were exposed during germination represented a wide range, i.e., from low to high ( $1\text{--}100 \mu\text{M}$ ). It was found that there was no significant inhibition in seed germination both in *Vigna radiata* and *Vigna mungo* up to  $10 \mu\text{M}$   $\text{CuSO}_4$  (Fig. 1A). But from  $50 \mu\text{M}$  onwards, there was inhibition in the germination rate which was more significant in case of *Vigna radiata* and at  $100 \mu\text{M}$ , there was complete inhibition of the germination in it. Thus, as per the findings of this report, Cu, like other heavy metals including Hg, Cd and Pb which are known to inhibit seed germination and early stages of seedling growth and development (Al-Yemeni, 2001), is a germination inhibitor at higher concentrations. The soluble protein content of the embryonic tissues of both the pulses were also found to altered due to Cu toxicity (Fig. 1B). There was slight increase in soluble protein content both in mungbean and black gram embryos at  $1 \mu\text{M}$   $\text{CuSO}_4$  and at  $10 \mu\text{M}$  the content again declined to almost control level. But at higher concentrations ( $50 \mu\text{M}$  onwards), the soluble protein contents declined significantly. This was a direct indication of the metabolic perturbation due to higher concentrations of Cu in germinating tissues.

Oxidative metabolism in aerobic cells involves the steady state formation of reactive oxygen species (ROS)



and their subsequent decomposition by endogenous antioxidative defense system. The fate of the organisms depends on the efficient scavenging of the toxic ROS. When their concentrations build up beyond the capacity of endogenous antioxidative system to scavenge them off, the system is exposed to a situation called oxidative stress (Halliwell and Gutteridge, 2007). The antioxidative defense system includes the enzymes like SOD, CAT, POX, glutathione reductase etc. along with several low molecular antioxidants like ascorbate, carotenoids, flavonoids, reduced glutathione,  $\alpha$ -tocopherol etc. Alterations in antioxidative system have been reported in several systems under different developmental conditions and also under exposure to biotic and abiotic stresses (Elstner *et al.*, 1988; Cakmak *et al.*, 1993; Verma and Dubey, 2003; Dey *et al.*, 2007; Dey *et al.*, 2009). Superoxide dismutase is an important antioxidative enzyme that dismutates superoxide radicals to  $O_2$  and  $H_2O_2$ . By reducing the superoxide radical concentration it also decreases the risk of hydroxyl radical generation via metal catalyzed Haber-Weiss reaction (Halliwell and Gutteridge, 2007). In this study, with increase in Cu concentration increased SOD activity has been observed in embryonic tissues of both the pulses (Fig. 2A). In *Vigna radiata* the increase was very sharp in comparison to that in *Vigna mungo*. Increase in SOD activity has also been reported in senescing mungbean cotyledons (Dey, 2008), in wheat seedlings exposed to Pb stress (Dey *et al.*, 2007) and also under Pb stress in rice plants (Verma and Dubey, 2003). The enhanced SOD activity, as reported herein, reduces the chances of accumulation of superoxide radicals. But at the same time it increases the accumulation of  $H_2O_2$  as a dismutation product. Thus, the increased SOD activity under Cu stress would increase the chances of imposition of oxidative stress in the embryonic tissues of both the pulses.

Catalase is the principal enzyme in the aerobic cells that decomposes  $H_2O_2$  to  $O_2$  and  $H_2O$  and thereby prevents its build up. The higher concentration of  $H_2O_2$  favours the catalatic mode of CAT action and the lower concentration of  $H_2O_2$  favours the peroxidatic mode of action. Enhanced CAT activity was found in this study only at  $1.0 \mu M$   $CuSO_4$  in both the cases and there after there was decrease in the activity (Fig. 2B) which indicated that the protection against  $H_2O_2$  has become weaker from  $10 \mu M$  onwards. Peroxidase is another antioxidative enzyme that also decomposes  $H_2O_2$  with co-oxidation of reduced co-substrates like phenols,

quinines, ascorbate, short chain alcohol etc. (Elstner, 1982). For assaying POX activity, guaiacol was taken in this study as reduced co-substrate and the results are presented in Fig. 2C. It was found that the increase of Cu concentrations up to  $10 \mu M$ , there was very sharp increase in POX activity in case of mungbean and from  $50 \mu M$  onwards, there was very sharp decline. But in case of black gram, enhanced POX activity was maintained up to  $10 \mu M$  and then there was decline in the activity. This indicated that up to the concentrations of  $10 \mu M$ , the concentration of  $H_2O_2$  was maintained below the injurious level due to higher activities of both CAT and POX. Probably because of this, there was no inhibition in germination in the seeds up to  $10 \mu M$   $CuSO_4$ . Towards higher concentrations, since there was decrease in the activities of both CAT and POX, the accumulation of  $H_2O_2$  in the tissues increased which favoured the chances of imposition of oxidative stress.

Among the different reactive oxygen species, hydroxyl radicals are the most potentially toxic to living systems. This free radical is known to generate from  $H_2O_2$  in presence of transition metal ions (Halliwell and Gutteridge, 2007). Due to decreased CAT and POX activities at higher concentrations of Cu in this study, the accumulation of  $H_2O_2$  in the tissues was favoured which increased the chances of hydroxyl radical formation. The unsaturated fatty acid components of membrane lipids are highly susceptible to hydroxyl radical attack and get peroxidized causing the loss of membrane integrity and cellular architecture. Consequently lipid peroxides and hydro peroxides are formed. Since lipid peroxidation is the consequence of free radical mediated reactions in aerobic cells, it is usually considered as an indicator of the prevalence of oxidative stress situation (Kappus, 1985). In this study, the level of lipid peroxidation was measured by estimating malondialdehyde (MDA), a decomposition product of peroxidized polyunsaturated fatty acid components of membrane lipid taking thiobarbituric acid as the reactive material. Only at  $1.0 \mu M$   $CuSO_4$ , there was decrease in lipid peroxidation in comparison to the control (Fig. 3) and towards higher concentrations, the lipid peroxidation level increased in both the cases. The increased lipid peroxidation in the embryonic tissues with increased Cu suggests the probability of imposition of oxidative stress in the tissues which might be the one of the mechanisms behind Cu induced toxicity in germinating seeds.



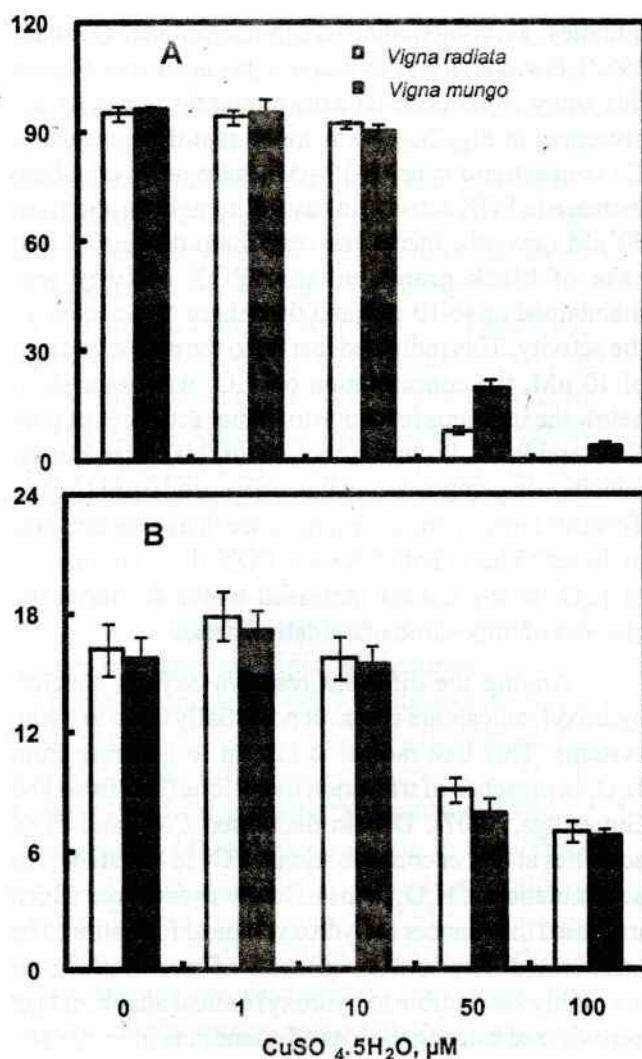


Fig. 1. Changes in germination percentage of the seeds (A) and soluble protein content of embryos (B) of *Vigna radiata* and *Vigna mungo* seeds germinated in presence of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  for 48 h.

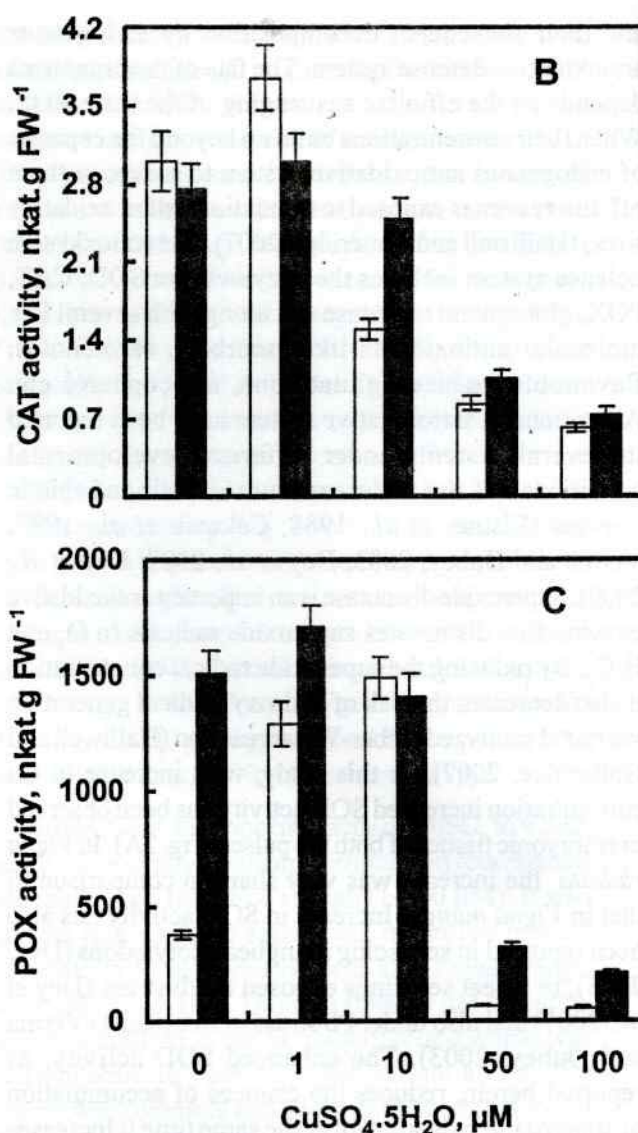
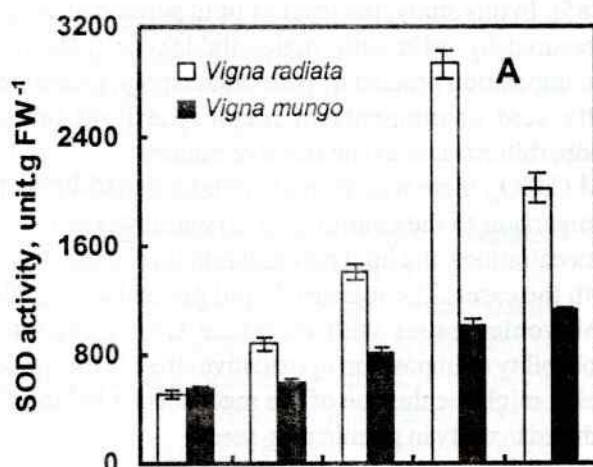


Fig. 2. Changes in the activities of superoxide dismutase (A), catalase (B) and peroxidase (C) of growing embryo of germinated seeds of *Vigna radiata* and *Vigna mungo* in the presence of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  for 48 h.

The inhibition of seed germination, alterations in the activities antioxidative enzymes and increase in lipid peroxidation suggest that the higher concentrations of Cu have imposed oxidative stress in the germinating seeds which might be one of the probable mechanisms behind Cu toxicity. In such a case, supplementation of antioxidant could neutralize the Cu-induced toxicity. To substantiate this presumption, antioxidant (ascorbate) was supplemented with toxic concentrations of Cu and lipid peroxidation level was measured in the embryonic



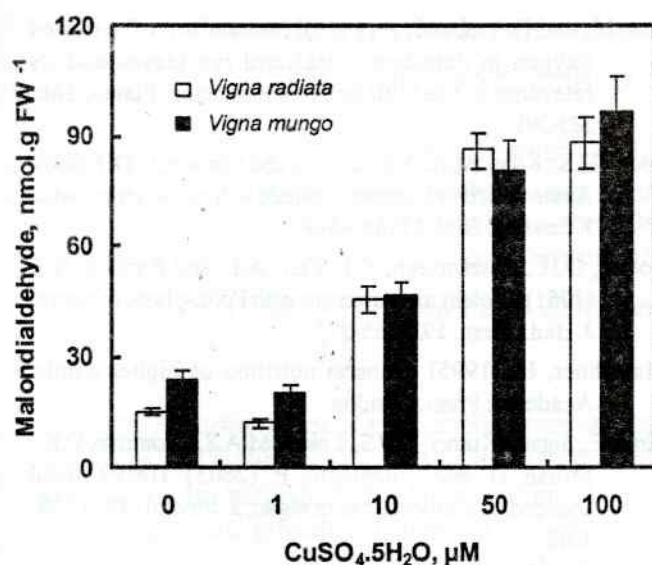


Fig. 3. Changes in the lipid peroxidation level in growing embryonic tissues of germinated seeds of *Vigna radiata* and *Vigna mungo* in presence of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  for 48 h.

by ascorbate, as reported herein, indicated the probability of involvement of these ROS in Cu toxicity in germinating seeds.

The results of this study suggest that  $1.0 \mu\text{M}$   $\text{CuSO}_4$  has beneficial effect in the germinating seeds, since there was increase in the soluble protein content in the embryonic tissues at this concentration, even though no enhancement in the germination percentage was noticed. Up to  $10 \mu\text{M}$ , significant inhibition in germination was not observed and probably this was because of the protection given by elevated CAT and POX activities. Thus, *Vigna radiata* and *Vigna mungo* seeds can tolerate Cu up to  $10 \mu\text{M}$  and among these two, *Vigna radiata* seems to have slight more resistance to Cu than *Vigna mungo*. The higher concentrations ( $50 \mu\text{M}$  onwards) had toxic effect which was probably due to imposition of oxidative stress in the tissues. This has been presumed on the basis of alterations in the activities of antioxidative enzymes, as found in this study.

Table-1

Effect of exogenous antioxidant (ascorbate) on the Cu induced lipid peroxidation in germinating embryonic tissues of *Vigna radiata* and *Vigna mungo*.

| Treatments                                                        | Malondialdehyde, nmol.g FW <sup>-1</sup> |                    |
|-------------------------------------------------------------------|------------------------------------------|--------------------|
|                                                                   | <i>Vigna radiata</i>                     | <i>Vigna mungo</i> |
| Control (Distilled Water)                                         | 15.2 ± 1.0                               | 24.2 ± 2.1         |
| 10 µM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$                   | 45.5 ± 3.7                               | 46.45 ± 3.5        |
| 10 µM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ + 1 mM Ascorbate  | 39.1 ± 2.8                               | 42.5 ± 3.2         |
| 10 µM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ + 10 mM Ascorbate | 35.4 ± 2.5                               | 36.2 ± 3.1         |
| 50 µM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$                   | 86.0 ± 5.0                               | 80.65 ± 8.1        |
| 50 µM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ + 1 mM Ascorbate  | 85.0 ± 6.1                               | 71.5 ± 7.9         |
| 50 µM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ + 10 mM Ascorbate | 61.9 ± 5.2                               | 52.0 ± 5.1         |

tissues after 48 h of exposure. A reduced level of lipid peroxidation was found in the embryonic tissues of both *Vigna radiata* and *Vigna mungo* exposed to 10 and  $50 \mu\text{M}$   $\text{CuSO}_4$  separately along with ascorbate at 10 mM level (Table 1). Of the two concentrations tested, 10 mM ascorbate was found to reduce the Cu-induced lipid peroxidation significantly. Thus, ascorbate has reduced the Cu-induced toxicity in germinating seeds. The supplementation of ascorbate had also increased the germination percentage (results not shown). Ascorbate is known to scavenge superoxide radical, hydroxyl radical and singlet oxygen (Bodannes and Chan, 1979) and reduction in the Cu-induced lipid peroxidation

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## Evolutionary Relationships in *Fusarium* species Based on Internal Transcribed Spacer (ITS) Sequences

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The genus *Fusarium* is one of most diverse group among fungi. They exist in varied environments as saprophytes, pathogens and symbionts. Most species are ubiquitous in soil and plant debris as saprophytes. But many species are also pathogenic to plants causing various diseases. Moreover, they are increasingly associated with opportunistic infections in humans and other animals, causing systemic infections with a high mortality rate. Species of *Fusarium* also exist as endophytes in many plant species, producing important bioactive metabolites. The existence of *Fusarium* in different forms is often confusing and methods are needed to distinguish such closely related species. Molecular techniques have been widely used to identify *Fusarium* species and for assessing genetic variation within collections and populations. In the present investigation evolutionary relationships of different *Fusarium* species have been assessed by considering their ITS sequences. The generated tree showed five clades establishing evolutionary relations among saprophytic, pathogenic and endophytic *Fusarium* species. However, consideration of other genetic loci of *Fusarium* may provide more information on evolutionary relationship of *Fusarium* species that exists in different forms.

**Key words:** Evolutionary relationship, *Fusarium*, ITS sequences

### Introduction

Among fungi, the genus *Fusarium* is extremely diverse and survives in all inhospitable conditions. They exist in varied environments as saprophytes, pathogens and endophytes. However, they are commonly known as pathogens causing diseases in both plants and animals. They are widespread in causing diseases in plants such as vascular wilts, root, stalk and cob rots, collar rot of seedlings and rots of tubers, bulbs and corms. Some of the pathogenic species also produce mycotoxins that contaminate grain. Moreover, they are increasingly associated with opportunistic infections in humans and other animals, causing systemic infections with a high mortality rate (Krcmery *et al.*, 1997) as well as localized infections in the skin and other body parts (Gupta *et al.*, 2000).

Most species of *Fusarium* exist as saprophytes and can be commonly isolated from soil and plant debris (Klaasen *et al.*, 1991). These saprophytic species grow quickly on isolation media and can be readily isolated

from diseased root and stem material, making it difficult to isolate the pathogen or pathogens that caused the disease. Therefore, it is important to test *Fusarium* isolates from diseased roots for pathogenicity to the plant. This is an important part of the diagnostic process, and is one of the reasons why the diagnosis of a root disease can be difficult. For example, *Fusarium* genera include many important pathogens that cause vascular wilt diseases and some root rots. *F. oxysporum* also includes many saprophytic strains which colonize diseased roots after the pathogen has killed root tissues. Some of these saprophytic species may also colonize the outer cells of the roots and plant tissues and exists as endophytes, causing no damage. At present many endophytic *Fusarium* species are found to harbor wide variety of plant species. Some of this endophyte has produced important bioactive metabolites for therapeutic applications (Deng *et al.*, 2009).

The existence of *Fusarium* in different forms (i.e. pathogenic, saprophytic and endophytic) is often

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confusing and many pathologists consider it to be a pathogen because of its virulence nature causing diseases mostly to plants and animals. Moreover species identification of *Fusarium* by morphological traits is problematic because mycelial pigmentation, shape and size of conidia are unstable and highly dependent on composition of media and environmental conditions. Further, entirely diverse groups as well as closely related species are very difficult to identify and distinguish. In such cases molecular techniques have become reliable and are highly suitable tools for identifying *Fusarium* species and for assessing genetic variation within collections and populations (Burgess *et al.*, 1996). Of the available molecular techniques, analysis of the Internal Transcribed Spacer (ITS) region has been especially useful for phylogenetic study of closely related *Fusarium* species at intraspecies and population level. (Peterson, 1991). This technique can also be applied to assess the evolutionary relationships among *Fusarium* species based on their ITS sequences. However, there is meager information about phylogenetic relationships of *Fusarium* species that exist in different forms.

Therefore the objective of this study is to investigate the phylogenetic affiliations of *Fusarium* species that exists in different forms in the environment and to find their evolutionary relationships at intraspecific level.

## Materials and Methods

### Sequence Retrieval

ITS sequences of *Fusarium* species that exists in different environment were retrieved from Genbank of NCBI database. During the retrieval process data containing the source, habitat and location of each *Fusarium* species were considered.

### Phylogenetic Investigation

Phylogenetic investigation of different *Fusarium* species was carried out using software MEGA4 (Tamura *et al.*, 2007). The sequences were aligned using clustalw and the evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973).

## Results and Discussion

In the present investigation 30 taxa of *Fusarium* species from various environment and locations that exist in different forms were considered for deriving phylogenetic affiliations (Table 1). In this study only ITS

sequences were considered because of its conservative nature and evolutionary significance (Duggal *et al.*, 1997). The generated tree showed four clades (Fig. 1) which has evolved from an endophyte with a pathogenic ancestry. The evolutionary relationships of different *Fusarium* species within each clade have been described.

### Clade-I:

In this clade there are seven *Fusarium* species consisting of two endophytes, three pathogens and two saprophytic isolates which radiated into two subclades indicating the possible evolution of saprophyte and pathogen from an endophyte and emergence of pathogenic form from saprotrophs. Many workers have suggested the relationship of endophyte with their respective host range from mutualistic to latent and virulent pathogens (Schulz *et al.*, 1998). Thus endophytes might become pathogens under certain circumstances. The transformation of endophytes to pathogens may be well supported from that fact that some dark-septate fungal endophytes that colonize the roots of many herbaceous plants and trees are mutualistic, whereas others become pathogenic (Sieber, 2002). Further, there are reports that endophytes can become parasites under certain conditions and vice-versa (Schulz and Boyle, 2005). Evolution of saprotrophs from endophytes could be explained from the assumption that after senescence and unavailability of specific host followed by unfavorable host-symbiotic relationship, the endophytic species might have evolved to saprophytic habit. Similar suggestion was made and evaluated by Promputtha *et al.* (2006) where they hypothesized that endophytes become saprotrophs at host senescence.

### Clade-II:

It includes six different species of *Fusarium* consisting of two endophytes and four pathogenic strains. The endophytes form a co-evolved lineage which is well supported by its bootstrap value. This clade also indicates emergence of pathogenic forms from endophytes. It is believed that host-microbe interactions can range from mutualism through commensalism to parasitism in a continuous manner (Redman *et al.*, 2001). The switching over of life style from endophyte to parasite can also be attributed to mutational frequency which is very commonly observed in genus *Fusarium* under different cultural and environmental condition (Miller, 1945). Such result has been observed in *Colletotrichum magna*, a



Table-1

List of *Fusarium* species and Genbank accession numbers of ribosomal RNA gene sequences used in this study.

| Species                    | Source                      | Habitat     | Country      | Genbank Accession |
|----------------------------|-----------------------------|-------------|--------------|-------------------|
| <i>F. equiseti</i>         | Soil                        | Saprophytic | UK           | FJ439592.1        |
| <i>F. solani</i>           | Soil                        | Saprophytic | China        | AB470903          |
| <i>F. solani</i>           | Soil                        | Saprophytic | China        | AB470904          |
| <i>F. oxysporum</i>        | Soil                        | Saprophytic | China        | EU326194          |
| <i>F. solani</i>           | Compost                     | Saprophytic | Greece       | EF621488.1        |
| <i>F. solani</i>           | Soil                        | Saprophytic | South Africa | DQ094700          |
| <i>F. oxysporum</i>        | Soil                        | Saprophytic | China        | AY928418.1        |
| <i>F. oxysporum</i>        | Soil                        | Saprophytic | Viet Nam     | EU076969.1        |
| <i>F. solani</i>           | Soil                        | Saprophytic | Japan        | AB518683.1        |
| <i>F. solani</i>           | Soil                        | Saprophytic | UK           | FJ460588.1        |
| <i>F. arthrosporioides</i> | <i>Taxus</i> sp.            | Endophytic  | S.Korea      | AY575714.1        |
| <i>F. aquaeductuum</i>     | Mangrove                    | Endophytic  | China        | FJ624268.1        |
| <i>F. oxysporum</i>        | <i>Dioscorea</i>            | Endophytic  | China        | DQ459007          |
| <i>F. sporotrichioides</i> | <i>Centaurea</i>            | Endophytic  | —            | EF682110.1        |
| <i>F. poae</i>             | <i>Centaurea</i>            | Endophytic  | —            | EF682109          |
| <i>F. mangiferae</i>       | Thai Medicinal Plant        | Endophytic  | Thailand     | GQ924893.1        |
| <i>F. oxysporum</i>        | <i>Juniperus recurva</i>    | Endophytic  | India        | EF591767.1        |
| <i>F. solani</i>           | <i>N. nimmoniana</i>        | Endophytic  | India        | FJ158121.1        |
| <i>F. solani</i>           | <i>Amona Squamosa</i>       | Endophytic  | China        | EF488413          |
| <i>F. solani</i>           | <i>Campotheca acuminata</i> | Endophytic  | China        | EF062312.1        |
| <i>F. chlamydosporum</i>   | <i>Theobroma</i> sp.        | Pathogenic  | Ghana        | FJ545402.1        |
| <i>F. oxysporum</i>        | Cotton                      | Pathogenic  | China        | GQ365156.1        |
| <i>F. culmorum</i>         | Wheat                       | Pathogenic  | Japan        | AB272115          |
| <i>F. culmorum</i>         | <i>Hypothenemus</i>         | Pathogenic  | Spain        | DQ655727.1        |
| <i>F. oxysporum</i>        | <i>Theobroma</i> sp.        | Pathogenic  | Ghana        | FJ545403.1        |
| <i>F. asiaticum</i>        | Wheat                       | Pathogenic  | Japan        | AB289550.1        |
| <i>F. lateritium</i>       | Tick                        | Pathogenic  | USA          | FJ861389.1        |
| <i>F. cerealis</i>         | Wheat                       | Pathogenic  | USA          | AJ246148          |
| <i>F. coeruleum</i>        | Potato/Tomato               | Pathogenic  | USA          | DQ164858.1        |
| <i>F. nelsoni</i>          | Human                       | Pathogenic  | USA          | GQ505434.1        |

\* — No data available

fungus pathogen that causes anthracnose in cucurbit plants, exerts an endophytic lifestyle after mutation and can even exhibit mutualistic effects (Redman *et al.*, 1999). Further, colonization of different hosts can cause a fungus to adopt contrasting lifestyle (Freeman *et al.*, 2001).

### Clade III:

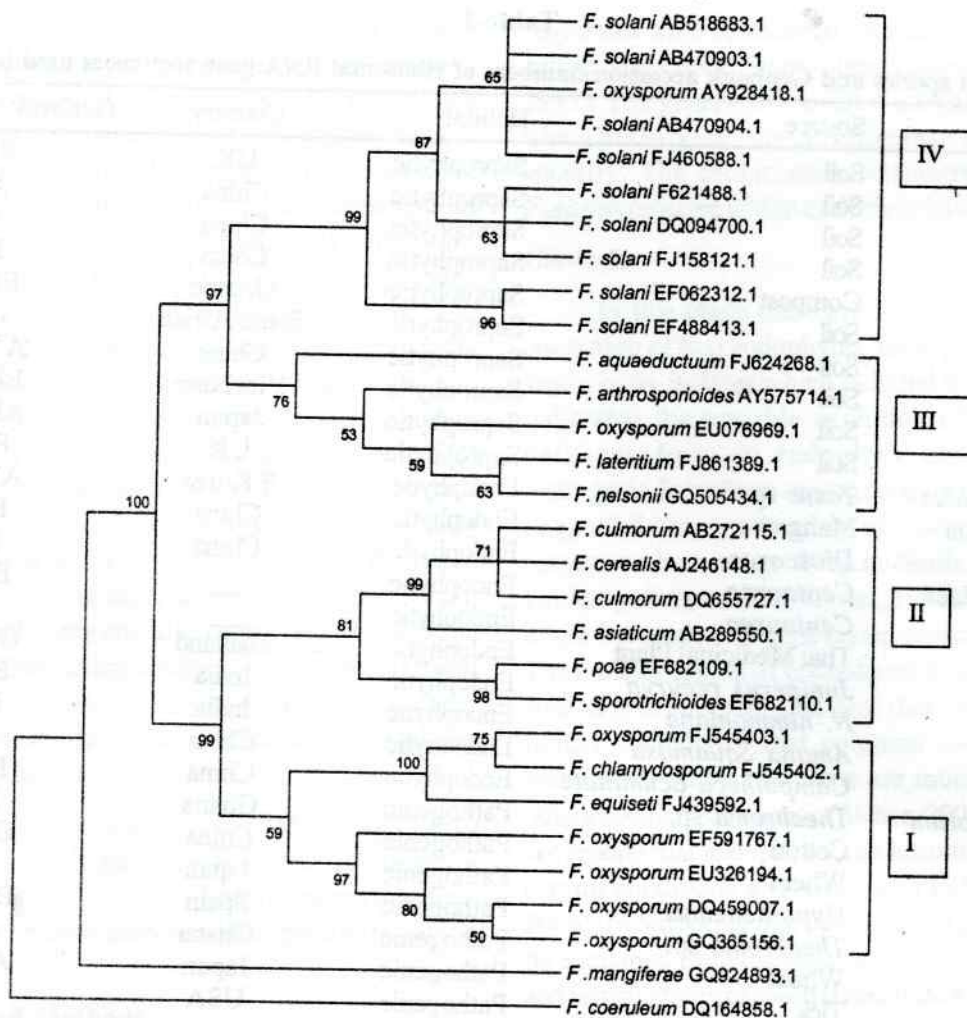
This clade includes five different *Fusarium* species constituting one saprophyte, two endophytes and two parallelly evolved pathogenic species. The gradual transformation of endophyte to pathogen via saprophyte is depicted in this clade. Therefore, it is believed that the endophyte might have transformed to saprotroph before it adopted pathogenic lifestyle. This could be supported

from the assumption that after senescence and unavailability of specific host followed by unfavourable host-symbiotic relationship, the endophytic species might have evolved to saprophytic habit. Such assumptions have been hypothesized by Promputtha *et al.* (2006) which have indicated that endophytes become saprotrophs at host senescence.

### Clade IV:

This clade comprises of three endophytes and seven saprophytes represented in three subclades, where one of the subclade is dominated only by saprotrophs. This clade also further validates the assumption that saprotrophs have evolved from endophytes. Many endophytic fungi colonize inner healthy tissues of plants





**Fig. 1.** Evolutionary relationships of 30 taxa of *Fusarium* species from different environment and locations. The tree was generated using the UPGMA method. The optimal tree with the sum of branch length = 2.48967713 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

without causing disease symptoms. In many instances these fungi produce secondary metabolites or hormone improving plant growth and health. Among several fungi reported as endophytes, species of *Fusarium* are commonly isolated as endophytes from several plants. The endophytic *Fusarium* that inhabit the upper tissue of plants might have gradually invade the below ground part and exist as saprophytes. because many non-pathogenic *Fusarium* species have been reported to colonize in the rhizosphere of several plants (Elias *et al.*, 1991). Moreover, such species have proved beneficial by solubilizing insoluble phosphorus supporting plant growth and health (Rajankar *et al.*, 2007) similar to the role played by endophytes.

The evolutionary relationships of *Fusarium* species existing in different forms and habitat still need to be elucidated at molecular level. The switching over of lifestyle of *Fusarium* species from endophytes to parasites or saprophytes have been attempted in our present investigation. The study reveals that both saprophytic and pathogenic *Fusarium* has evolved from endophytic species with a pathogenic ancestry. Many workers have described host-microbes interaction ranged from mutualism to parasitism and there are reports that endophytes can become parasites under certain conditions and vice versa which is seen in our rooted tree.



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## Biosystematic status of *Kyllinga* Rottb.

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Biosystematic study of three species of *Kyllinga* is carried out. Anatomy of stem and leaf blades corresponds to the radiate chlorenchyma in the family Cyperaceae. Peripheral vascular bundles are small in stem (culm). The median vascular bundle of the leaf is without direct abaxial strand. Palynologically, the group of species are having verrucate sculpture pattern, which is unusual to other genera of tribe Cypereae. The cytological study reveals high number and small sized chromosome.

**Key words:** Bulliform cells, Cyperaceae, radiate chlorenchyma, subepidermal strands, verrucate sculpture

### Introduction

The taxonomic position of *Kyllinga* Rottb. in the family Cyperaceae, appears to be very much controversial in view of its inclusion within the genus *Cyperus* Linn. (*Sensu lato*) by many authors. This genus *Kyllinga* Rottb. is included along with *Cyperus* Linn. in the tribe Eucypereae, on the basis of characters of rachilla of spikelet which is deciduous in the *Cyperus* and persistent in the Eucypereae. Though the researchers like Pax (1887), Clarke (1893, 1894), Cooke (1908), Segdwick (1918), Blatter & Mc Cann (1935) and Haines (1924) have considered it as a separate genus and it was later supported by Kukenthal (1936). The workers like Koyama (1961), Kern (1974), Saxena and Brahman (1996) also included this genus along with few other genera under *Cyperus* Linn.

However, during the present investigation three species viz. *K. brevifolia* Rottb., *K. monocephala* Rottb. and *K. triceps* Rottb. could be collected and biosystematic analyses were carried out from morphological, anatomical, palynological and cytological studies.

### Materials and Methods

Most of the members flourish quite well in the rainy seasons, some in winter and some in early summer. During present study 3 species of the genus have been collected from the state of Orissa. Field notes recording the gross morphological features and ecology were noted at the time of collection of plants. The specimens have been deposited in the departmental herbarium after

adopting the usual herbarium methods of pressing, drying, poisoning with mercuric chloride and mounting them on the herbarium sheets (42 cm x 28 cm). Field notes are finally transferred to herbarium sheets after the specimens are mounted.

The anatomical study deals with the transverse sections of different vegetative parts of sedge leaf and stem (culm) by the method devised by Sharma and Mehera (1969).

Inflorescence portion (spikelet) from which the pollen grains can be taken were collected from living plant, dehydrated in acetic alcohol (1:3) and after 24 h, preserved in 70% alcohol. The slides of the pollen grain were prepared by the acetolysis method by Erdtman (1952). Methods of pollen preparation and measurements were according to Erdtman (1969).

Determination of chromosome number and study of other features of cytological interest were mostly done through meiotic and pollen mitotic analysis. Through out the year, all the three species were collected at different times according to their flowering time. For the purpose, very young spikelets often inside the sheath were collected and fixed in glacial acetic acid and ethyl alcohol (1:3) and were squashed in aceto-carmine (0.5%). For making the above squash preparation permanent both the slides and cover slips were separated in mixture of glacial acetic acid + xylol + ethyl Alcohol (1:1:1). The slides and coverslips were then passed through different gradations of ethyl alcohol and xylol mixtures and finally mounted them in canada balsam.

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## Results

The genus *Kyllinga* Rottb. is represented by 60 species throughout the world, of which 6 species occur in India. In the present investigation only 3 species could be collected out of 5 species occurring in Orissa.

## Anatomy

In *Kyllinga* the vascular bundles are superimposed in the mid rib of transsection of leaf. The peripheral vascular bundle of culm are much smaller than the remainder and are often circular than elliptical or oval in outline (Fig. 2.6).

## Leaf surface and transection of leaf blade

On observing the the species of the genus *Kyllinga* it is found that prickles are generally present at leaf margin but distribution is varying with species. Stomata are paracytic and these are mostly on abaxial surface but a few generally present near leaf margin on adaxial surface only. Subsidiary cells are triangular to dome-shaped. Papillae generally absent, silica-bodies are conical in shape with satellites on adaxial surface. In *K. brevifolia* number of silica bodies are 2-6 per cell on adaxial surface and up to 5 on abaxial surface. *K. monocephala* shows 1-6 silica bodies per cell on adaxial surface and up to 9 on abaxial surface arranged in one row. While *K. triceps* has 5 number of silica bodies per cell on both surfaces. Transverse veins are not distinct.

*K. brevifolia* Rottb. shows widely V shaped to almost flat outline with double adaxial rib in each half of lamina. Margin is sub-equal. Adaxial cells over sclerenchymatous strands are smaller than their neighbours, less pronounced adaxial ribs and lamina narrower (Fig. 2.1). Each half of lamina is provided with minute abaxial strands and 2 slightly crescentiform adaxial strands. Vascular bundles at leaf margins are provided with both pulviniform adaxial and abaxial strands. Chlorenchyma cells are slightly lobed and rounded to oval in outline. Air cavities are small and occasional. Bulliform cells in adaxial groove are slightly larger than other. Vascular bundles are 21-25 in each half of lamina, 3 of them are conspicuously larger than remainder. T.S. lamina of *K. monocephalous* Rottb. although similar to *K. brevifolia* in all respect, but it differs from the above having flanged V-shaped outline with double adaxial rib in each half of lamina (Fig. 2.2). Keel is prominent,

rounded, margins sub-equal and obtuse. Other differences include more number of vascular bundles (about 25-29) in each half of lamina. Similarly the outline of T.S of lamina of *K. triceps* Rottb. is V-shaped keeled (Fig. 2.3), slightly prominent margin abruptly tapered to rounded points. Adaxial hypodermis is scattered, mostly solitary with translucent cells. Sclerenchyma comprises 6-8 small pulviniform adaxial strands in each half of lamina and one adaxial strand is present near each leaf margin. Air-cavities are small. Bulliform cells are few in number. Vascular bundles are 9-18 in number half of lamina, two conspicuously larger than the remaining.

## Transection of culm

Epidermis is not papillose. Outline is variable. Central ground tissue is spongy and tending to break down. Air-cavities are peripheral, inconspicuous or absent. Assimilatory tissue is radiate chlorenchyma, present around the minor vascular bundles. Vascular bundles are embedded at inner boundary of chlorenchyma, relatively small and circular; Vascular bundles present in central translucent tissue are larger and more widely spaced, not penetrating towards culm centre. Sclerenchyma consists subepidermal numerous strands, varying in size and shape. Vascular bundles in translucent central tissue are usually with crescentiform sclerenchyma caps at xylem ends.

T.S of culm of *K. brevifolia* is triangular in outline. Central air cavities are large. Concave sides of culm is found apparently absent from angles (Fig. 2.4). Chlorenchyma is semi radiate around outer vascular bundles, cells rounded and slightly lobed. There are 50 minor vascular bundles and only a few cell beneath the epidermis 11-15 major vascular bundles in translucent ground tissue are present. Peripheral sclerenchymatous strands are angular, the largest is found in the angles of culm. Secretory cells are occasional. *K. monocephala* has subcircular to subelliptical in outline. Chlorenchyma is semi radiate around outer and assimilatory in function (Fig. 2.5). Cells are rounded and slightly lobed. Minor vascular bundles are 45 in number only a few cells beneath the epidermis and 10 major vascular bundles in translucent ground tissue. Peripheral strands are angular and sclerenchymatous in nature. Secretory cells are occasional. T.S of culm of *K. triceps* is triangular in outline (Fig. 2.6). Epidermis is single layered rectangular cells without presence of any prickles or hairs.



Table-1

Anatomical diagnostic characters of species *Kyllinga*

| Sl. No. | Species Examined      | Subsidiary cells | Dermal appendages        | Outline of blade | Adaxial Epidermis | Outline of stem | Aerating canals |
|---------|-----------------------|------------------|--------------------------|------------------|-------------------|-----------------|-----------------|
| 1       | <i>K. brevifolia</i>  | Triangular       | Prickles on leaf margins | Broadly V-shaped | Simple bulliform  | triangular      | In stem         |
| 2       | <i>K. monocephala</i> | Sub-triangular   | Prickles on leaf margins | Flanged V-shaped | Simple bulliform  | Sub circular    | -               |
| 3       | <i>K. triceps</i>     | Triangular       | Prickles on leaf margins | V-shaped keeled  | Simple bulliform  | triangular      | In blade        |

Assimilatory tissue comprises translucent cells, those are scattered in chlorenchyma situated at inner boundary of sclenchymatous strands. There are 35 minor vascular bundles in chlorenchyma and about 9 larger vascular bundles in peripheral part of central ground tissue, 3 much larger vascular bundles in translucent ground tissue. Central ground tissue is parenchymatous without intercellular space. Sclerenchyma composed of 25 subepidermal strands varying in size and shape, sometimes sub triangular or pulviniform. Secretory cells are present on the subepidermal region.

## Palynology

Morphologically *K. triceps* Rottb. is distinct as compared to two other species named *K. brevifolia* Rottb. and *K. monocephala* Rottb. Palynologically *K. brevifolia* Rottb. (Fig.3.1 & 3.2) is much distinct as compared to two other species. Morphological aspect of pollen like bilateral symmetry, heteropolar polarity are found in the former species against radiosymmetrical symmetry and isopolar polarity in the latter two species (Table-2). Similarly, both colpate and non colpate.

Table-2

Morphological aspects of pollen showing shape, symmetry, polarity, aperture and sculpture pattern in *Kyllinga*

| Sl. no. | Taxon                 | Shape               | Symmetry         | Polarity    | Aperture    | Aperatural Margin | Sculpture Pattern |
|---------|-----------------------|---------------------|------------------|-------------|-------------|-------------------|-------------------|
| 1.      | <i>K. brevifolia</i>  | Sub-spheroidal      | Bilateral        | Heteropolar | Non-colpate | Crassimarginate   | Psilate           |
|         |                       | Obovoidal           |                  |             | Colpate     |                   |                   |
|         |                       | Slightly triangular |                  |             | Colporate   |                   |                   |
|         |                       |                     |                  |             | Syncolpate  |                   |                   |
| 2.      | <i>K. monocephala</i> | Sub-spheroidal      | Radiosymmetrical | Isopolar    | Non-colpate |                   | Verrucate         |
|         |                       | Circular            |                  |             | Porate      |                   | Clyperate         |
|         |                       | Ellipsoidal         |                  |             | Annulate    |                   |                   |
|         |                       | Slightly triangular |                  |             |             |                   |                   |
|         |                       | Quadrangular        |                  |             |             |                   |                   |
| 3.      | <i>K. triceps</i>     | Spheroidal          | Radiosymmetrical | Isopolar    | Non-colpate |                   | Foveolate         |
|         |                       | Ellipsoidal         |                  |             | Annulate    |                   |                   |
|         |                       | Slightly triangular |                  |             |             |                   |                   |
|         |                       | Quadrangular        |                  |             |             |                   |                   |



Table-3

Details of pollen measurements of three species of *Kyllinga*

C=Colpus, P=Polar Axis, E= equatorial Axis, LC= length of colpus, BC= breadth of colpus, W= exine thickness

| Sl. No | Taxon                 | P/Range (Mean)         | E/Range (Mean)         | P/E   | LC/Range (Mean)       | BC/Range (Mean)     | W    |
|--------|-----------------------|------------------------|------------------------|-------|-----------------------|---------------------|------|
| 1      | <i>K. brevifolia</i>  | 16.16-21.21<br>(18.62) | 16.15-19.19<br>(17.87) | 1.041 | 15.56-18.84<br>(17.2) | 3.03-5.05<br>(4.22) | 0.92 |
| 2      | <i>K. monocephala</i> | 14.12-19.19<br>(16.11) | 12.16-17.22<br>(13.66) | 1.179 | -                     | -                   | 1.22 |
| 3      | <i>K. triceps</i>     | 14.14-20.20<br>(18.55) | 15.15-18.15<br>(16.22) | 1.143 | -                     | -                   | 1.01 |

All measurements in micron ( $\mu$ )

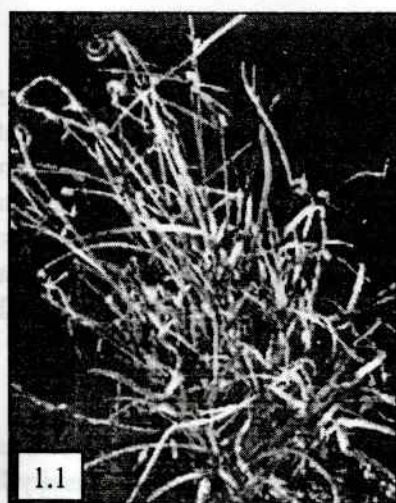
## Cytology

Three species of the genus collected during the survey have been worked out cytologically. The chromosome number of the members of this genus, when compared with other genera; it is found that, all the species are characterized by very high chromosome numbers with relatively small size. The aneuploidy is also evident in the genus. However, different workers report discordant chromosome numbers in three Indian species studied. This is presumably difficult in counting, that is experienced due to larger to smaller size through intermediate chromosome numbers i.e. heterogeneous size and large number of chromosomes in all the members of the genus studied so far. It is found that the range of chromosome number varies from  $n=51$  (Fig. 4.1) in *K. monocephala* to  $n=62$  (Fig. 4.2) in *K. triceps* and an intermediate number  $n=60$  in *K. brevifolia* (Fig. 4.3).

## Discussion

After Bentham and Hooker (1862-1883) two schools of thought have developed. Several other workers have made a lot of transfers, convergence and divergence of different generic and species status, considering the feeble demarcation line in between the two genera *Cyperus* and *Kyllinga*. Koyama (1961) amalgamating all the six genera i.e. *Pycneus*, *Juncellus*, *Eucyperus*, *Papyrus*, *Diclidium* and *Mariscus* described by Bentham and Hooker (1862-1883) under the tribe Cyperaceae, into one genus i.e. *Cyperus* (*Sensu lato*). During the present work attention has been given through different bio-systematic analysis to the genus *Kyllinga* Rottb. On morphological and embryological characters *Kyllinga* Rottb. is also very distinct from other genera of the tribe Cyperaceae. Anatomically, it has also been noted that chlorenchyma is predominately radiate and the

Plate-1

Fig.1.1. *Kyllinga brevifolia* Rottb.Fig.1.2. *Kyllinga monocephala* Rottb.Fig.1.3. *Kyllinga triceps* Rottb.



## Plate-2

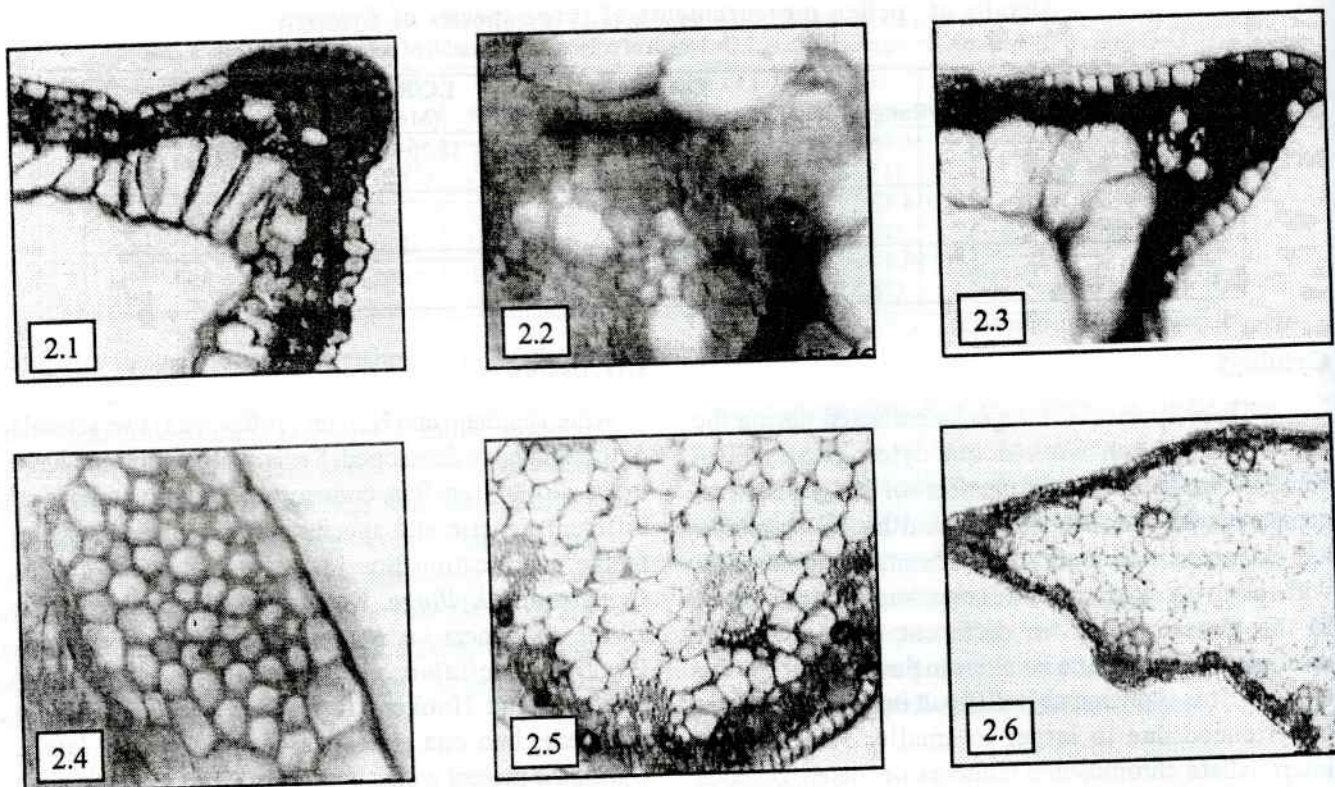


Fig. 2.1. *Kyllinga brevifolia*, T. S of lamina showing median vascular bundle with bulliform cells

Fig. 2.2. *Kyllinga monocephala*, T. S a portion of lamina showing median vascular bundle

Fig. 2.3. *Kyllinga triceps*, T. S of lamina showing median region

Fig. 2.4. *Kyllinga brevifolia*, T. S of stem showing pulviniform hypodermal strand

Fig. 2.5. *Kyllinga monocephala*, T. S of stem showing larger vascular bundles

Fig. 2.6. *Kyllinga triceps*, T. S a portion of stem with hypodermal strands

median vascular bundle of the leaf is without direct abaxial strands. The peripheral vascular bundle of the culm is smaller than the remainder. Padhey (1971) came to the conclusion that *Kyllinga* Rottb. differs markedly from *Cyperus* Linn. and its merger with *Cyperus* Linn. (*Sensu lato*) does not seem to be valid. Palynologically the group of species belonging to the genus *Kyllinga* Rottb. are having verrucate pattern of sculpture which is quite unusual in other genera of tribe Cyperaceae. The chromosomal pattern in *Kyllinga* Rottb. is also found to be different being characterized by high number and small sized chromosome. Considering all these factors the authors support the view of Rath (1980) to treat *Kyllinga* Rottb. as a separate and distinct genus. The

gradual specialisation of arrangement of floral structure from larger spikelets with many flowers to a capitate head is one of the most significant factors in this regard. Moreover the characters described under different disciplines like morphology, anatomy, palynology and cytology support strongly of the attainment of the genus *Kyllinga* Rottb., the most advanced condition within the cyperoid group of genera.

#### Acknowledgement

The authors are thankful to the Head of Post-Graduate Department of Botany, Utkal University for providing necessary laboratory facilities.



## Plate-3

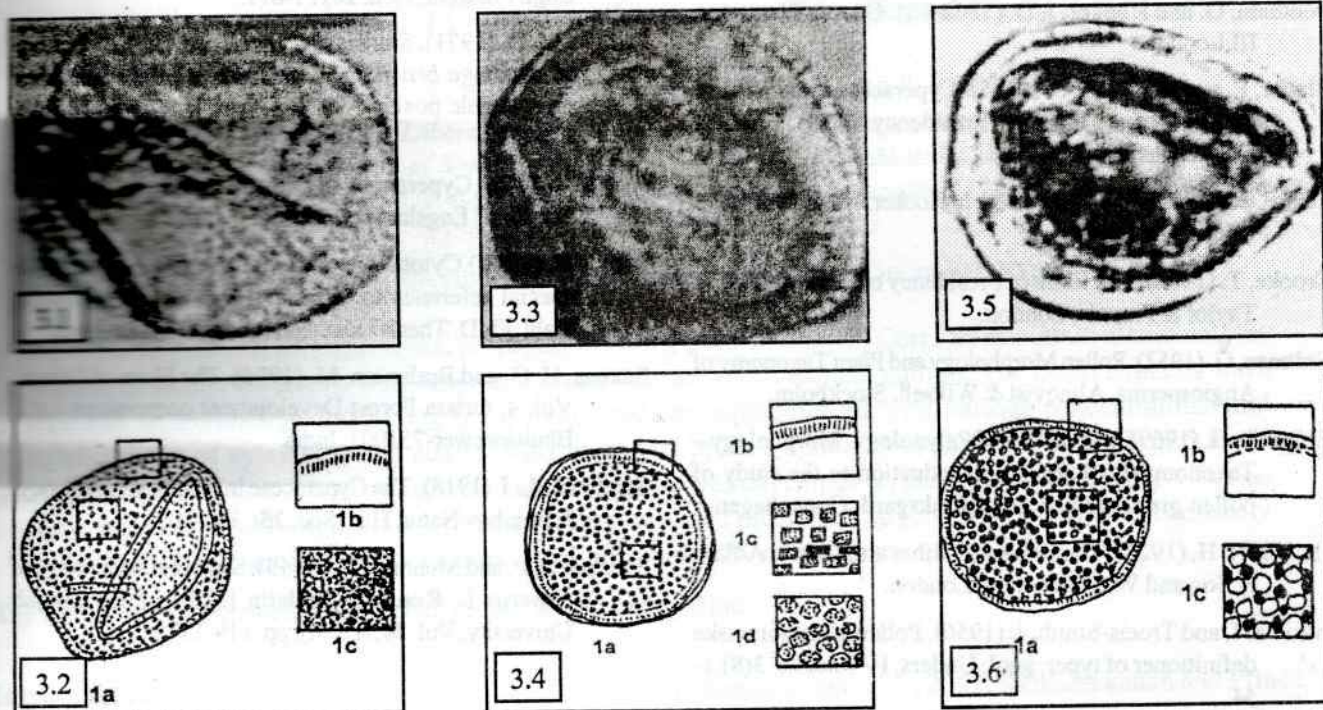


Fig. 3.1. Pollens of *Kyllinga brevifolia* showing syncolpate condition

Fig. 3.2. 1a. Slightly triangular pollens showing colpate and syncolpate condition in E. V

Fig. 3.2. 1b. Detail wall structure. 1c. Detailed sculpture pattern showing psilate condition

Fig. 3.3. Porate pollens of *Kyllinga monocephala*

Fig. 3.4. 1a. Sub-spheroidal pollens showing cleared three layer wall and non-colpate condition in E. V

Fig. 3.4. 1b. Detail wall structure. 1c. Detailed sculpture pattern showing verucate and clypeate condition

Fig. 3.5. Non-porate pollens of *Kyllinga triceps*

Fig. 3.6. 1a. Spheroidal pollens showing non-colpate condition in E. V and P. V respectively.

Fig. 3.6. 1b. Detail wall structure. 1c. Detailed sculpture pattern showing foveolate condition.

## Plate-4

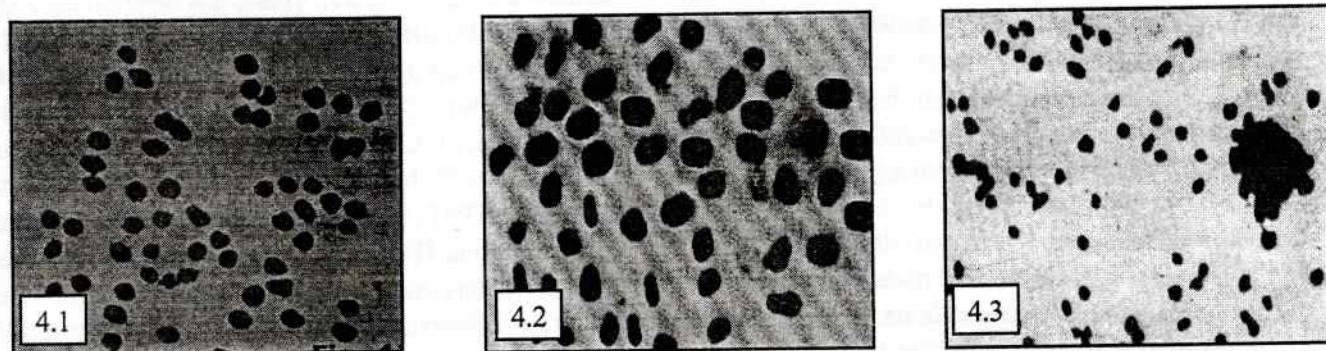


Fig. 4.1. Microphotograph showing chromosome numbers of *Kyllinga brevifolia* showing  $n=60$

Fig. 4.2. Microphotograph showing chromosome numbers of *Kyllinga monocephala* showing  $n=51$

Fig. 4.3. Microphotograph showing chromosome numbers of *Kyllinga triceps* showing  $n=62$



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## ***In vitro* Culture of Mature Nodal Explants for Micropropagation of *Aegle marmelos* (L.) Corr., an Important Medicinal Tree Species**

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An efficient protocol for rapid clonal propagation of *Aegle marmelos* (L.) Corr., an important medicinal tree species of family Rutaceae, was achieved through mature nodal explant culture. The nodal explants were cultured on Murashige and Skoog (MS) basal medium supplemented with various concentrations and combinations of cytokinins and auxins. Maximum bud break response was observed on BAP (0.5 mg/l) supplemented medium. An average of 9.0 shoots/explant was developed on the same medium after 4 weeks. *In vitro* shoots were rooted on IBA (3.0 mg/l) supplemented medium. The rooted shoots were successfully acclimatized and established under natural condition, with 90% survival rate.

**Key words:** *Aegle marmelos*, medicinal plants, micropropagation

### **Introduction:**

*Aegle marmelos* (L.) Corr. is a spiny medicinal tree belonging to the family Rutaceae. It is distributed throughout India in dry forests and is also cultivated (Warrier *et al.*, 1994). This plant is listed in earliest ayurvedic (an ancient Indian system of preventive health care) medicinal texts such as Charaka samhita (600 BC) and Sushruta samhita (500 BC) and has been widely used in Ayurveda for variety of ailments. All parts of the tree like roots, barks, leaves and fruits are highly medicinal (Raghu *et al.*, 2007). The ripe fruits are astringent, sweet, aromatic, cooling, febrifuge, laxative and tonic and are good for the heart and brain (Sivarajan and Balachandran, 1994; Warrier *et al.*, 1994). Antioxidant activity (Kamalakkanan and Prince, 2003 a), hepatoprotective effect of leaf (Singanan *et al.*, 2007), protective effect on pancreas (Kamalakkanan and Prince, 2005), antidiarrhoeal activity (Shoba and Thomas, 2001), antiulcer activity of seeds (Goel *et al.*, 1997), antifungal activity of leaves (Rana *et al.*, 1997) and antitumour and antimutagenic activity (Annapurani and Priya, 1999; Lampronti *et al.*, 2003) of *A. marmelos* plant are clinically evaluated. The antidiabetic activity of fruit, leaf and seed (Kamalakkanan and Prince, 2005; Sachdewa *et al.*, 2001; Upadhyaya *et al.*, 2004; Kesari *et al.*, 2006;

Kamalakkanan *et al.*, 2003; Kamalakkanan and Prince, 2003b; Kar *et al.*, 2003 ) of this plant have also been well established.

*A. marmelos* is propagated through conventional method by seeds. Seeds have short viability and are prone to insect attack. Vegetative propagation through root suckers is slow, difficult and cumbersome (Anonymous, 2003). Studies conducted on seed propagation have shown that the germination percentage of seed is very low under natural and controlled conditions (Raghu, 2006). Due to indiscriminate cutting of the plants and continuous human interference, the plant is disappearing from the wild sources and the species is reported to be vulnerable in the Western Ghats of Kerala, Tamil Nadu and Karnataka states of India (Ravikumar and Ved, 2000). Now it is observed that the plant is going to face the same situation in Orissa and other states of Eastern India. Root being the major medicinally useful part, destructive harvesting poses a serious threat to the sustenance of the tree. To fulfill the increasing demand of this plant, its large-scale propagation is essential and plant tissue culture technique acts as an alternative viable method for the improvement and multiplication of this medicinally important plant. Though *in vitro* propagation of *A. marmelos* by nodal explant culture (Raghu *et al.*, 2007;

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Pati *et al.*, 2008; Ajithkumar and Seeni 1998) and by stem callus as well as node culture (Varghese *et al.*, 1993) has been achieved, the regulation of growth chemicals in order to reduce the time span was necessary.

In the present work, a novel protocol has been developed to propagate *A. marmelos* through tissue culture method in order to ensure abundant supply of this plant material for the preparation of herbal medicine.

### Material and Methods

Shoots of a 15-20 years old *A. marmelos* plant (Fig. 1) with 4-5 nodes were collected from the medicinal plant garden of P. G. Dept. of Botany, Utkal University, Bhubaneswar, India. First the leaves were excised and

the shoots were cut into single node pieces which were used as explants for our experiment. The single node stem pieces were first gently washed under running tap water for 30 minutes. After that, they were treated with 5% teepol for 5 minutes and again were rinsed under running tap water for 30 minutes. Then the explants were taken inside a laminar flow cabinet and were surface sterilized with 0.1%  $\text{HgCl}_2$  for 5 minutes followed by rinse with sterilized distilled water for three to four times to remove traces of  $\text{HgCl}_2$ . The single nodal explants were inoculated on Murashige and Skoog's (1962) basal medium (MS medium) supplemented with cytokinins, 6-benzyl aminopurine (BAP) and Kinetin (Kn) either taken alone or along with auxins  $\alpha$ -naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), 2,4-dichlorophenoxy

Table-1  
Effect of Phytohormones on multiple shoot induction from nodal explants of *A. marmelos*

| Concentration of Phytohormones in mg/l |     |       |     |     | Percentage of Response * | Mean ( $\pm$ SE) * |
|----------------------------------------|-----|-------|-----|-----|--------------------------|--------------------|
| BAP                                    | Kn  | 2,4 D | IAA | NAA |                          |                    |
| 0.5                                    |     |       |     |     | 93                       | $9.0 \pm 0.29$     |
| 0.5                                    |     |       | 0.5 |     | 83                       | $2.33 \pm 0.15$    |
| 1.0                                    |     |       | 0.5 |     | 93                       | $3.37 \pm 0.22$    |
| 1.5                                    |     |       | 0.5 |     | 83                       | $3.17 \pm 0.12$    |
| 2.5                                    |     |       | 1.0 |     | 66                       | $2.97 \pm 0.07$    |
|                                        | 0.5 |       |     |     | 66                       | $1.3 \pm 0.12$     |
|                                        | 0.5 |       | 0.5 |     | 60                       | $1.8 \pm 0.12$     |
|                                        | 1.0 |       | 0.5 |     | 76                       | $3.2 \pm 0.1$      |
|                                        | 1.5 |       | 0.5 |     | 76                       | $2.03 \pm 0.09$    |
| 1.0                                    |     | 0.5   |     |     | 80                       | $2.37 \pm 0.03$    |
| 1.5                                    |     | 0.5   |     |     | 86                       | $2.53 \pm 0.12$    |
| 2.0                                    |     | 0.5   |     |     | 80                       | $1.87 \pm 0.09$    |
| 2.5                                    |     | 1.0   |     |     | 56                       | $1.7 \pm 0.07$     |
|                                        | 1.0 | 0.5   |     |     | 73                       | $1.8 \pm 0.03$     |
|                                        | 1.5 | 0.5   |     |     | 63                       | $1.5 \pm 0.06$     |
|                                        | 2.0 | 0.5   |     |     | 60                       | $1.6 \pm 0.03$     |
| 0.5                                    |     |       |     | 0.5 | 63                       | $2.1 \pm 0.1$      |
| 1.0                                    |     |       |     | 0.5 | 70                       | $2.3 \pm 0.06$     |
| 1.5                                    |     |       |     | 0.5 | 73                       | $2.6 \pm 0.12$     |
| 2.5                                    |     |       |     | 1.0 | 60                       | $2.5 \pm 0.06$     |
|                                        | 0.5 |       |     | 0.5 | 56                       | $1.4 \pm 0.15$     |
|                                        | 1.0 |       |     | 0.5 | 66                       | $2.26 \pm 0.19$    |
|                                        | 1.5 |       |     | 0.5 | 70                       | $2.43 \pm 0.03$    |

\* Data (Percentage of response and Mean values,  $\pm$  SE) of three independent experiments each with 10 replicates.



acetic acid (2,4-D) in various concentrations (cytokinins – 0.5, 1.0, 1.5, 2.0, 2.5 mg/l and Auxins – 0.5, 1.0 mg/l) and combinations. The medium was solidified with 0.8% agar. The explants were placed on the medium vertically with morphologically root end towards lower side and shoot end towards upper side. The pH of the medium was adjusted to 5.8 before autoclaved at 121°C for 20 minutes. The cultures were maintained in a culture room at 25±2°C with 16 hrs photoperiod. For each treatment, 10 replicates were taken and each experiment was repeated thrice. After four weeks of culture, the percentage of response, mean number of shoots per explants and standard error of mean were calculated and tabulated (Table 1). The photographs of each development were taken in Kodak DX 6490 model from time to time.

### Result

In the present investigation, attempts were made to standardize a protocol for micropropagation of *A. marmelos* (L.) Corr. from mature nodal explants of a 15 to 20 years old tree. Different concentrations of cytokinins (BAP and Kn) alone and along with auxins (NAA, IAA and 2,4-D) were used on MS medium for multiple shoot induction from the explants. Among the various treatments, best response towards multiple shoot regeneration was obtained from MS medium

\* Data ( Percentage of response ) of three independent experiments each with 10 replicates

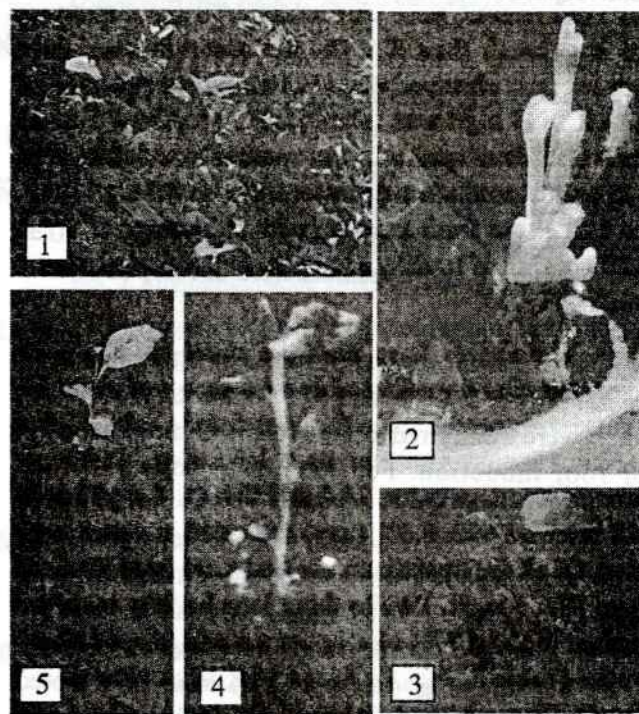


Fig. 1. *A. marmelos* plant, Fig. 2. Bud break response on BAP 0.5mg/l, Fig. 3. Multiple shoot after 4 weeks, Fig. 4. Rooting, Fig. 5. Rooted shoot growing under natural condition after acclimatization.

Table-2

Effect of Auxins on root induction of *A. marmelos*

| Concentration of Auxins (mg/l) |     |     | Percentage of response* | No. of roots per explant | Nature of roots |
|--------------------------------|-----|-----|-------------------------|--------------------------|-----------------|
| NAA                            | IBA | IAA |                         |                          |                 |
| 1.0                            |     |     | 37                      | 1                        | Small and thin  |
| 2.0                            |     |     | 43                      | 1                        | Small and thin  |
| 3.0                            |     |     | 57                      | 1 to 2                   | Long and thin   |
| 4.0                            |     |     | 47                      | 1 to 2                   | Long and thin   |
|                                | 1.0 |     | 53                      | 1 to 2                   | Small and thin  |
|                                | 2.0 |     | 63                      | 1 to 2                   | Long and thin   |
|                                | 3.0 |     | 77                      | 2 to 3                   | Long and thick  |
|                                | 4.0 |     | 70                      | 2 to 3                   | Long and thick  |
|                                |     | 1.0 | 50                      | 1                        | Small and thin  |
|                                |     | 2.0 | 53                      | 1 to 2                   | Small and thick |
|                                |     | 3.0 | 60                      | 1 to 2                   | Long and thick  |
|                                |     | 4.0 | 40                      | 1 to 2                   | Long and thick  |

\* Data ( Percentage of response ) of three independent experiments each with 10 replicates



supplemented with 0.5 mg/l BAP only (Table 1). Bud break response was maximum in BAP 0.5mg/l and in all the media tested, BAP was found to be more effective for bud break than Kn. On the contrary very poor response was observed when MS medium supplemented with Kn 0.5 mg/l was taken (Table 1). About 8 to 11 shoot buds developed, after one week of inoculation, (Fig. – 2) on MS medium supplemented with 0.5 mg/l BAP. On transfer to fresh medium with an average of  $9.0 \pm 0.29$  number of healthy shoots were developed after 4 weeks (Fig. 3).

The shoots from multiple shoot clumps were separated after 4 weeks and *in vitro* shoots of about 2–3 cms. were transferred to fresh medium in culture tubes for root induction. For root initiation, different concentrations of NAA, IAA and IBA (1–4 mg/l) on  $\frac{1}{2}$  MS medium with 0.5% (w/v) activated charcoal were used. Of all the treatments, the best result was obtained from IBA 3 mg/l. Two to three long healthy roots per explant in about 77% of cultures were developed after 6 weeks (Table 2, Fig. 4). About 90% of *in vitro* rooted shoots (Fig. 5) were successfully acclimatized and established in the natural condition.

## Discussion

In the present study, best response towards multiple shoot regeneration from mature nodal explant of *A. marmelos* was obtained from MS medium supplemented with 0.5 mg/l BAP and in all the media tested, BAP was found to be more effective for bud break than Kn. Superior role of BAP over Kn in tissue culture has been established by earlier workers (Benjamin *et al.*, 1987; Arulpragasam and Latiff, 1986 and Rout *et al.*, 2008). About 8 to 11 shoot buds developed, after one week of inoculation, on MS medium supplemented with 0.5 mg/l BAP. An average of  $9.0 \pm 0.29$  number of healthy shoots were developed on the same medium after 4 weeks. Raghu *et al.* (2007) obtained best response using MS+BA 0.5 mg/l after 45 days of culture with an average of  $6.2 \pm 0.4$  shoots per explant which increased at every sub culture. However, many other earlier workers reported different concentration and combinations of phytohormones required for axillary bud multiplication of *A. marmelos*. Varghese *et al.* (1993) suggested a combination of Kn and NAA gave best response. Many other workers (Ajithkumar and Seeni, 1998; Pati *et al.*, 2008) suggested

a combination of BAP and IAA was required for best response. In our present study, it was found that a combination of cytokinin and auxin reduced the number of shoots per explant, which support the findings of Raghu *et al.* (2007). Lower concentration of BAP only was sufficient for shoot multiplication in nodal explant culture of this plant. This finding is in contrast to that of Varghese *et al.* (1993), Ajithkumar and Seeni (1998) and Pati *et al.* (2008) who suggested combination of cytokinin and auxin was essential for shoot multiplication. The use of comparatively lower concentration of growth regulator also minimizes the risk of producing genetically altered individuals (Edson *et al.*, 1996).

*In vitro* shoots (ca. 2–3 cm) were transferred to fresh medium in culture tubes for root induction. In our present study, the best result was obtained on  $\frac{1}{2}$  MS medium with 0.5% (w/v) activated charcoal and IBA 3 mg/l. Two to three long healthy roots per explant in about 77% of cultures were developed after 6 weeks. Raghu *et al.* (2007) reported that one or two roots were developed in less than 30% of cultures in  $\frac{1}{4}$  strength MS medium without any growth regulators and with full strength MS medium supplemented with IBA 10 mg/l after 10 weeks of culture. Varghese *et al.* (1993) reported rooting (25%) in MS basal medium augmented with 2 mg/l or 4 mg/l IAA. Ajithkumar and Seeni (1998) reported rooting in  $\frac{1}{2}$  MS medium with lower concentration of IAA (70%) and higher concentration of IBA (90%) in this plant. Pati *et al.* (2008) reported IAA is best for rooting. Puhan (2006) suggested  $\frac{1}{2}$  MS medium with 0.5% (w/v) activated charcoal and IBA (2.5 mg/l) or IBA (2.5 mg/l) + IAA (2 mg/l) gave better result where one root developed from the base with little callus in 31 and 30 percentages of cultures, respectively. During the present study, the concept of using of lower concentration of IBA proved to be successful. In our present work it was observed that IBA 3 mg/l in  $\frac{1}{2}$  MS medium with 0.5% (w/v) activated charcoal is best for root induction in this plant. The number of roots did not affect the subsequent establishment of rooted plants in the field. In our experiment 90% of *in vitro* rooted shoots were successfully acclimatized and established in the natural condition.

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## Spore Liberation in *Wrangelia argus* Mont. (Ceramiales; Rhodophyta) of Visakhapatnam Coast

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The results on the seasonal tetraspore and carpospore shedding in *Wrangelia argus* against exposure to air (desiccation), salinity, light intensity, photoperiod and wavelength are presented. There are two peak shedding periods one between February-March and other in August-September. Tetraspore shedding showed decreasing trend with increase in the duration of exposure of air and maximum output was found under submerged conditions. Peak shedding of tetraspore was observed at 30% salinity and at the light intensity of 25  $\mu\text{E}/\text{m}^2/\text{s}$  at 20L: 4D cycle under normal light.

**Key words:** Spore liberation, Visakhapatnam, *Wrangelia argus*

### Introduction

Detailed ecological studies were not made adequately on the members of Rhodophyceae. Different environmental factors affect the spore shedding mechanism in algae (Rao and Kaliaperumal, 1983; Rao and Subbarangaiah, 1986). The present work has been initiated to know the conditions in which the maximum numbers of spores are liberated in a Rhodophycean alga. In India, information available on the spore liberation in the members of Ceramiales (Sudhakar and Subba Rangaiah, 1997 a and b). For the last 20 years, many changes in the environment of Visakhapatnam coast took place resulting in the changes in the biology and ecology of marine algae of this coast. Therefore, the present study was carried out to know the difference in the spore shedding capacities of previous (Sudhakar and Subba Rangaiah, 1997) and the present study.

### Materials and Methods

*Wrangelia argus* was collected from Visakhapatnam coast where large accessible boulders occur with dense growth of algae and brought to the laboratory immediately after collection in polyethylene bags filled with sea water. The tetrasporic and cystocarpic plants in the samples were selected by observing under a low power microscope. Spore liberation experiments were carried out with

tetrasporophytes and carposporophytes. Plants with well developed tetrasporangia and cystocarps were used for collecting the data on the seasonal spore output and the effects of different factors on spore shedding.

The material selected for the experimental work were rinsed several times with sterile sea water and placed in 10x10 cm Petri-dishes filled with sterile sea water. Spore liberation experiments were started at 18.00 h at room temperature of  $32\pm 2^\circ\text{C}$ . The Petri-dishes were illuminated by fluorescent cool white tube lights at 25  $\mu\text{E}/\text{m}^2/\text{s}$  for about 10 h during day time i.e. from 08.00 to 18.00 h. Tetraspores and carpospores liberated daily (24 h) were counted. To count the spores, the methodology given by Rao (1976) and Subba Rangaiah (1983, 1984) was followed. Fresh weight of the tetrasporic and cystocarpic fronds was taken and the spore output was expressed as tetraspores/gm. fr. wt./day and carpospores/g. fr. wt./day. Five experiments (replicates) were conducted each month from December 2005 to November 2006, to know the daily liberation of tetraspores and carpospores under laboratory conditions and also to study the seasonal changes in spore shedding.

To know the effect of environmental factors on spore output, ten experiments (replicates) for each alga were conducted and the mean values were plotted. To study the effects of exposure to air (desiccation), the tetrasporic plants were blotted to remove the water on

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the surface of the fronds and exposed to air in the laboratory and also in the open air during the day time. At the time of these experiments, the temperature in the laboratory was  $32 \pm 2^\circ\text{C}$  and the relative humidity varied from 65-70 % and that of outside room temperature was  $35 \pm 2^\circ\text{C}$  and the relative humidity 70 to 75 %.

At 15 min intervals the material exposed to air were transferred to Petri-dishes filled with sterile sea water and the spore output was estimated after 24 hours to know the effect of exposure to air (desiccation).

Spore output was estimated at 10%, 20%, 30%, 40% and 50% salinity, maintaining the Petri-dishes at room temperature ( $32 \pm 2^\circ\text{C}$ ) under 10 hour day length at  $25 \mu\text{E}/\text{m}^2/\text{s}$ . Effects of light intensity on spore shedding was investigated at room temperature ( $32 \pm 2^\circ\text{C}$ ) using light intensities of 0, 25, 35, 45 and  $55 \mu\text{E}/\text{m}^2/\text{s}$ . The light intensities were applied only between 10.00h and 18.00 h. For studying photo periodic effect, spore shedding experiments were conducted at 0:24, 4:20, 8:16, 12:12, 16:8, 20:4, 24:0 (light: dark cycle), keeping the Petri-dishes in separate light and dark chambers. These experiments on photoperiod were carried out at room temperature, and at  $25 \mu\text{E}/\text{m}^2/\text{s}$ . To study the effect of different wavelengths of light, the Petri-dishes were covered with yellow, blue, red and green colored thin cellophane papers and exposed at  $25 \mu\text{E}/\text{m}^2/\text{s}$  at room temperature. The spores liberated from the fronds exposed to yellow, blue, red, green and white (control) were counted after 24 h.

## Results

### Daily liberation of spores

The quantity of tetraspores and carpospores liberated continuously for three days was tested and presented in Fig. 1. Maximum number of tetraspores and carpospores were observed on day one (Fig. 1) and slowly the spore shedding of both the types was seen decreasing upto three days. After three days no spore liberation or very little amount of spores was seen liberating.

### Seasonal changes in spore shedding

Mean monthly values of tetraspores and carpospores of *Wrangelia argus* estimated from December 2005 to November 2006 are shown in Fig. 2. Spore liberation was observed in all months of the year in the tetrasporophytes and cystocarpic plants (Fig. 2). The tetraspore and carpospore shedding was maximum

in two periods one between February-March, and other between August-September. Both types of spores liberated on day one and day two showed the same period of peak shedding in two periods. The tetraspore production varied from 1,020 to 60,140 spores/ g. fr. wt./ day and the carpospore output during the above period ranged from 100 to 7900 spores/g. fr. wt./day.

### Effect of environmental factors on spore shedding:

#### (i) Exposure to air (desiccation)

These experiments were designed not only to study the effect of exposure during low tides and the resulted desiccation of plants on tetraspore production but also to understand the spore liberation in shaded condition (inside laboratory) (Fig. 3) and as well as in areas directly exposed to sunlight (outside laboratory) (Fig. 4). Changes observed in the tetraspore output in control (0 minutes of exposure) and at different periods of exposure to air in room temperature ( $32 \pm 2^\circ\text{C}$ ) and relative humidity of 60-65% and outside temperature ( $35 \pm 2^\circ\text{C}$ ) and at a relative humidity of 70-75 % are shown in Fig. 3 and Fig. 4. In the experiments conducted in shade at room temperature, spore shedding was seen upto 45-60 minutes of exposure of the fronds (Fig. 3). In these experiments the tetraspore shedding was seen maximum in controls i.e. in fronds submerged for 24 h and the number of spores liberated decreased with increasing the duration of drying of the plants at room temperature.

As shown in the Fig. 4, spore shedding was decreased rapidly between 15 to 30 min of exposure to air in sun and very low values of spores were obtained from the fronds exposed for 30-45 min due to high temperature and low humidity (outside laboratory). There was no spore shedding beyond 45 min of exposure in the fronds.

#### (ii) Salinity

Effects of salinity on tetraspore shedding was shown in Fig. 5. Spore output varied markedly in different salinities of the seawater tested. There was no shedding of spores beyond 50 % salinity. Spore shedding was seen from 10% to 50% with peak output of tetraspores at 30%. The spores liberated at 10% seem to be not viable, since only cell walls were seen without any content in the cells.



### (iii) Light intensity

Fig. 6 depicts the quantity of tetraspores liberated in dark and at four different photon flux densities ranging from 25 to 55  $\mu\text{E}/\text{m}^2/\text{s}$ . Spore shedding varied in photon flux density ranging from 0 (dark) to 55  $\mu\text{E}/\text{m}^2/\text{s}$ . There was no tetraspore shedding beyond 55  $\mu\text{E}/\text{m}^2/\text{s}$ . The maximum shedding of tetraspores was seen at 25  $\mu\text{E}/\text{m}^2/\text{s}$  and slowly the tetraspore shedding was decreased from 25  $\mu\text{E}/\text{m}^2/\text{s}$  onwards. Considerable number of tetraspores was seen in dark condition also.

### (iv) Photoperiod

Effects of different light and dark cycles on tetraspore shedding at 25  $\mu\text{E}/\text{m}^2/\text{s}$  were shown in Fig. 7. This photon flux density was selected depending on the maximum daily spore output of spores. The tetraspore shedding increased from 0L: 24D cycle and maximum output was seen at 20L: 4D cycle. More detailed experiments on all photon flux densities with different photoperiods should be conducted to know more about the photoperiod on spore shedding.

### (v) Wavelength

Effect of different wavelengths of light on tetraspore release are presented in Fig. 8. In these experiments, the maximum liberation of tetraspores were found to be in control (white light) and the number of tetraspores decreased gradually in yellow, red, blue and green lights.

## Discussion

### Daily liberation of spores

There is a report on spore output in algae of different taxonomical classes given by Narasimha Rao and Subba Rangaiah (2009). This review that the members of Chlorophyceae and Pheophyceae produced large number of reproductive elements than those of Rhodophyceae and within the class, there is a decrease in spore output from the primitive members like *Bangiopsis* and *Porphyra* to the more advanced members such as *Wrangelia argus*, *Centroceras clavulatum* and *Polysiphonia platycarpa*.

In the primitive members spore output ranged between 1.2 to 2.4 million and that in the advanced members the number of spores was less than 0.2 millions. The number of tetraspores released on day one was maximum in *W. argus* (Fig. 1).

A similar trend was reported in *Gracilaria edulis* (Rama Rao and Thomas, 1974), *Gracilaria corticata* (Rao, 1976 and Subba Rangaiah, 1983), *G. textorii* and *Gracilariopsis sjoestedtii* (Subba Rangaiah, 1984, 1985), *Gelidium* spp. (Suto 1950; Rao, 1974, Kaliaperumal and Rao, 1986), *Pterocladia heteroplotos* and *Gelidiopsis variabilis* (Kaliaperumal and Rao 1982, 1985) and in some species of red algae (Boney, 1960), *Hypnea valentiae* (Subba Rangaiah and Rao, 1983), and in *Wrangelia argus*, *Centroceras clavulatum* and *Polysiphonia platycarpa* (Sudhakar and Subba Rangaiah, 1993, 1997a and 1997b). In contrast to these studies others reported maximum release of carpospores in *Gracilaria verrucosa* (Oza and Krishnamurthy, 1968) and *G. corticata* (Mohan Joseph and Krishnamurthy, 1977) on the second or third day.

In *Gelidium robustum*, Guzman and Del pro *et al.*, (1971) reported that cystocarpic plants released more number of spores than the tetrasporic ones. Sudhakar and Subba Rangaiah (1993) in *W. argus* and *C. clavulatum* have reported that the number of tetraspores released are slightly more than the carpospores. But in the present study the number of tetraspores released were slightly less than the carpospores. The development of cystocarps might be more in number than the tetrasporangia or the selected cystocarpic plants may be healthier than tetrasporic plants resulting in the release of spores which is in agreement with the studies made by Vanillakumari and Subba Rangaiah (1997) in *Grateloupia lithophila*.

### Seasonal changes in spore shedding

Two peaks were observed in the liberation of tetraspores and carpospores (Fig. 2). This agree with Rama Rao and Thomas (1974) on *Gracilaria edulis*, Rao (1974) and Subba Rangaiah (1983) on *G. corticata*, Subba Rangaiah and Rao (1983) on *Hypnea valentiae*, Sudhakar and Subba Rangaiah, (1997b) on *Wrangelia argus* and *Centroceras clavulatum*, Subba Rangaiah and Vanillakumari (1997) on *Amphiroa fragilissima*, *Grateloupia lithophila* and *Jania rubens*. These two peak periods of spore shedding in a year coincides with the two peak periods of maximum growth of the above algae.

### Effect of environmental factors on spore shedding

The present study found a decline in spore output when the alga was exposed to air and subjected to



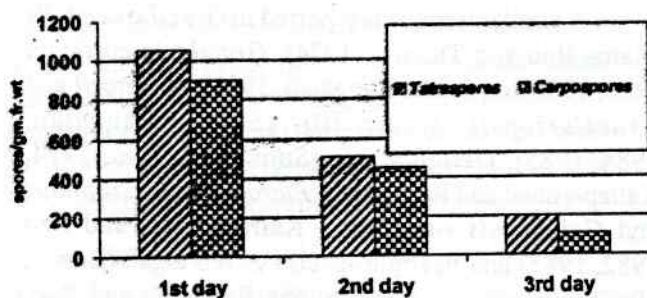


Fig. 1. Daily liberation of spores

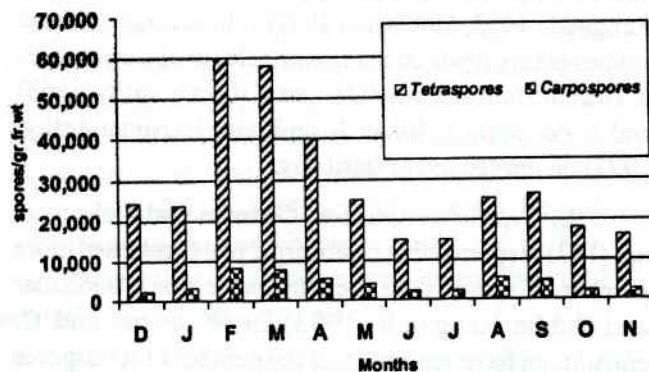


Fig. 2. Seasonal changes in spore shedding

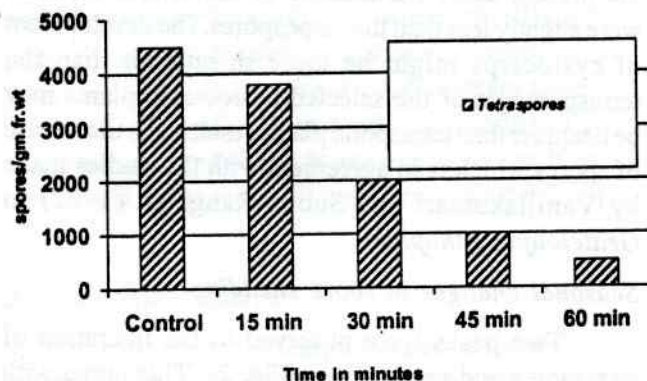


Fig. 3. Exposure to air (inside laboratory)

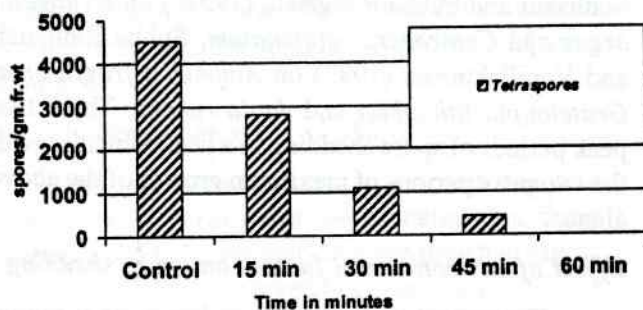


Fig. 4. Exposure to air (outside laboratory)

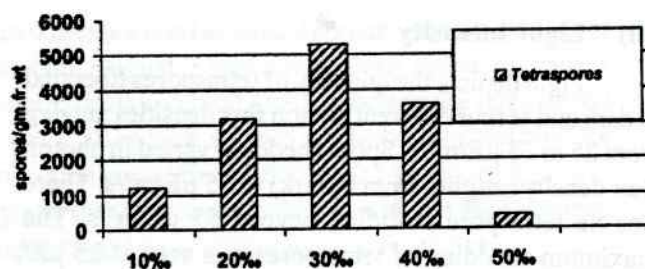


Fig. 5. Effect of salinity

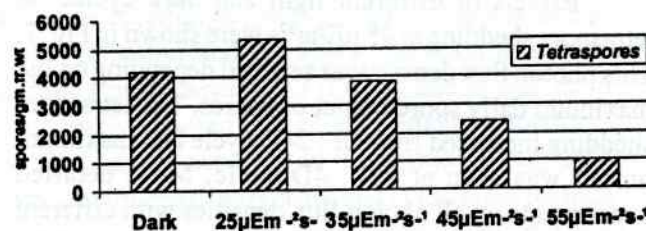


Fig. 6. Effect of light intensity

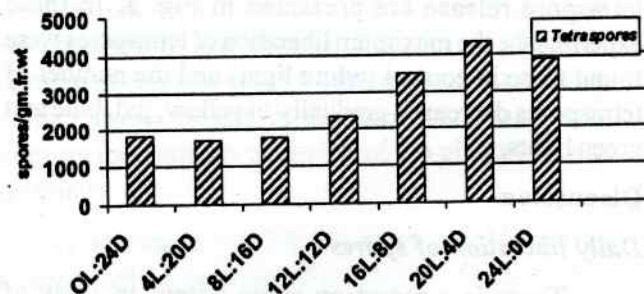


Fig. 7. Effect of photoperiod

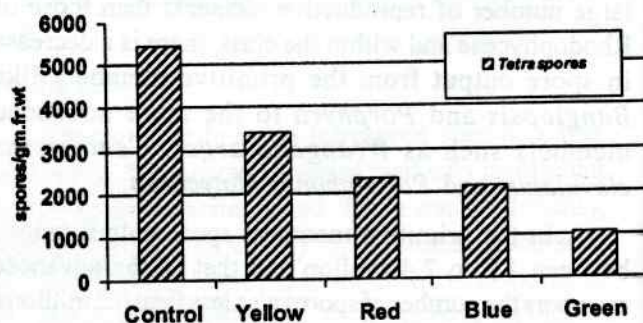


Fig. 8. Effect of wavelength



desiccation (Fig. 3 and Fig. 4). It is, therefore, likely that the exposure of fronds during low tides affects the quantity of spores produced. A similar response was described for Gelidiales (Katada, 1955, Srinivasa Rao, 1971, Rao and Kaliaperumal, 1983), Gigartinales (Rao and Subba Rangaiah, 1986), Ceramiales (Subba Rangaiah, 1985a, Sudhakar and Subba Rangaiah, 1993, 1997a) and Bangiales (Narasimha Rao and Subba Rangaiah, 1991).

In contrast, some studies reported a promotive effect of exposure to air and desiccation of the fronds thereby spore liberation is accelerated in *Ascophyllum nodosum* (Baker, 1910), *Gloiopeltis tenax* and *G. furcata* (Matsui, 1969). From the previous studies made on this aspect showed clearly that plants occurring in the upper-mid littoral region like *Bangiopsis subsimplex*, *Porphyra vietnamensis* could withstand longer periods of desiccation than the infra-littoral species such as the members of Gelidiales, Cryptonemiales, Gigartinales and Ceramiales. These variations are in agreement with the vertical distribution of different species on the shore of Visakhapatnam.

Further, it was observed that the degree of salinity of sea water influences spore release. The optimum salinity range observed for the maximum shedding of tetraspores was 30‰ (Fig. 5). Several studies recorded the influence of the salinity on spore production and release in various algae of the Visakhapatnam coast and reported different optimal ranges (Subba Rangaiah *et al.*, 1975; Rao and Kaliaperumal, 1983; Rao and Subba Rangaiah, 1986; Subba Rangaiah 1985b, 1986, Narasimha Rao and Subba Rangaiah, 1991, Sudhakar and Subba Rangaiah, 1993, 1997a). The annual range of salinity for the inshore waters of Visakhapatnam is 21 to 35‰ (Ganapathi and Satyanarayana Rao, 1962; Rao and Sreeramulu 1964).

This range accommodates the optimal salinity of 30‰ for maximal spore shedding in the present study. However, Baker (1910) and West (1972) suggested that reduced salinity is an important factor causing propagule discharge in *Ascophyllum nodosum* and *Adouinella purpurea*. Matsui (1969) did not observe any significant variation in the liberation of spores between 17-52‰ in *Gloiopeltis furcata* and *G. tenax*. The general trend observed by these workers in different salinities is similar to the present observation.

Tetraspore liberation has been observed under light intensities ranging from 0 (darkness) to 55  $\mu\text{E}/\text{m}^2/\text{s}$  (Fig. 6). A similar trend was observed by Ohno (1971) with gamete liberation in *Monostroma nitidum*, White and Boney (1969) on monospore output in *Acrochaetium endophyticum*, Rao and Kaliaperumal (1983) on *Gelidium pusillum*, *Pterocladia heteroplotos* and *Gelidiopsis variabilis*, Rao and Subba Rangaiah (1986) on *Hypnea valentiae*, Subba Rangaiah (1985b, 1986) on *Agloathamnion cordatum* and *Porphyra vietnamensis*, Naidu (1987) on *Ulva fasciata* and *Enteromorpha compressa*, Narasimha Rao and Subba Rangaiah (1991) on *Ectocarpus mitchellae*, *Bangiopsis subsimplex* and *Porphyra vietnamensis*, Sudhakar and Subba Rangaiah (1993, 1997a) on *W. argus* and *C. clavulatum* and *Polysiphonia platycarpa* and Vanillakumari and Subba Rangaiah (1997) on the members of Cryptonemiales. However temperature may modify the effect of photon flux density in the liberation of reproductive elements which need to be understood.

The effect of photoperiod also plays an important role in spore liberation. While short day condition induced spore liberation in *Porphyra* (Iwasaki 1961, Kurogi and Sato 1962), *Gracilaria corticata*, *G. textorii*, *Gracilariopsis sjoestedtii* and *Hypnea valentiae* (Rao, 1976; Rao and Subba Rangaiah, 1986), *Agloathamnion cordatum* (Subba Rangaiah, 1985b), *Centroceras clavulatum* (Sudhakar, 1992), long day conditions exerted a similar effect in *Porphyra umbilicalis* (Kurogi and Sato, 1962), *Acrochaetium endophyticum* (White and Boney, 1969), *Monostroma nitidum* (Ohno, 1971) and *Gelidiella acerosa* (Rao, 1974), *Wrangelia argus* and *Polysiphonia platycarpa* (Sudhakar and Subba Rangaiah, 1993, 1997a) and on the members of Cryptonemiales (Vanillakumari and Subba Rangaiah, 1997). The long day conditions which favoured maximum shedding of tetraspores in *W. argus* (Fig. 7) coincided with the studies made by Vanillakumari and Subba Rangaiah (1997) on Cryptonemiales viz., *Amphiroa fragilisma*, *Jania rubens* and *Grateloupia lithophila*.

Rao and Kaliaperumal (1983) on *Gelidium pusillum*, *Pterocladia heteroplotos* and *Gelidiopsis variabilis* indicated the peak shedding of spores occurs at low light intensities under long day conditions and at higher intensities under short day conditions. These interacting effects clearly show that the amount of energy



received by plants influences spore release and the periodicity in the discharge of spores may be due to photosynthetic effect as described by Dixon and Richardson (1970).

The quality of light also influenced spore liberation in this alga (Fig. 8). Spore output was maximum in the control (white light). The quantity of spores liberated in yellow light were more compared to other colored lights. The promotive effect of yellow light on spore shedding was also observed by Rao and Subba Rangaiah (1986) in *Gracilaria corticata*, *Gracilariopsis sjoestedtii*, *Hypnea valentiae* and in *Centroceras clavulatum* and *Wrangelia argus* (Sudhakar and Subba Rangaiah, 1993). However, a more detailed study is needed to know the effect of quality of light on the liberation of spores in the members of algae in general and more particularly in red algae.

From the present study and a comparison with the studies made by Sudhakar (1992) and Sudhakar and Subba Rangaiah (1993) revealed that the shedding of spores in different months and the effect of different environmental factors such as exposure to air (desiccation), salinity, light intensity, photoperiod and wavelength was not changed but a drastic reduction in the spore output was observed. For example, in *W. argus* a maximum number of 191,940 tetraspores/g. fr.wt./day and 39,706 carpospores/gm.fr.wt./day were observed earlier (Sudhakar 1992, Sudhakar and Subba Rangaiah, 1993). But in the present study only 1,020 to 60,140 tetraspores/gm.fr.wt./day and 100 to 7900 carpospores/gm.fr. wt. /day was observed in *W. argus*. Similarly the effect of environmental factors on spore shedding was also found to be liberating very less number of spores. If this trend continues, in future there might be no liberation of spores and there will be a threat for the growth of algae in the coast. The drastic changes in the reduction of spores in the coast of Visakhapatnam is due to indiscriminate release of domestic wastes and industrial effluents into the sea, indiscriminate harvesting of seaweeds without knowing the reproductive periodicities of the seaweeds and also due to rise in atmospheric temperature in the coastal areas.

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## Circadian Periodicity in the Spore Shedding of Two Members of Ceramiales (Rhodophyta) of Visakhapatnam Coast

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Circadian periodicity was observed with peak liberation of tetraspores between 1400 and 1800 h in *Wrangelia argus* and between 0200 and 0600 h in *Centroceras clavulatum*. Among the factors studied on circadian periodicity of spore shedding, exposure to air and light intensity had no influence, while salinity influenced the periodicity in the daily liberation of spores. The light and dark periods also played an important role in changing the circadian periodicity of spore shedding in *Wrangelia argus* but not in *Centroceras clavulatum*.

**Key words:** Ceramiales, circadian periodicity, spore shedding

### Introduction

The spore shedding mechanisms in red algae particularly those of economically important algae play an important role in mariculture. The circadian periodicities were little studied in detail whereas the marine algal school of Botany Department of Andhra University have done considerable work to reveal several features in this line.

A better understanding of algal spore ecology is useful both in the basic understanding of ecological studies and in the case of domesticating marine algae for human use.

### Materials and Methods

Materials for the present study namely *Wrangelia argus* and *Centroceras clavulatum* were collected from Visakhapatnam coast where large accessible boulders occur with dense growth of algae and brought to the laboratory immediately after collection in polyethylene bags filled with sea water. The tetrasporic plants present in the samples were selected by observing under a low power microscope. Spore liberation experiments were carried out with tetrasporophytes. The material selected for the experimental work were rinsed several times with sterile sea water and placed in 10 x 10 cm Petri-dishes filled with sterile sea water. Data on the circadian changes in spore shedding were collected by transferring

the material kept in the Petri dishes into another Petridish at four hour intervals i.e. at 2200 h, 0200 h, 0600 h, 1000 h, 1400 h and 1800 h and by counting the spores liberated. To count the spores, the methodology given by Rao (1976) and Subba Rangaiah (1983; 1984) is followed. Ten experiments (replicates) were conducted with each alga to study the circadian periodicities in spore shedding.

Experiments on circadian periodicity of spore shedding were also conducted to know the effects of environmental factors on circadian periodicity by selecting certain periods of exposure to air (0, 15, 30, 45 and 60 minutes), salinities (0%, 10%, 20%, 30% and 40%) and photon flux densities (25, 35, 45 and 55  $\mu\text{E}/\text{m}^2/\text{s}$ ). All these experiments were conducted at room temperature and at a photoperiod of 8 L: 16 D cycle. It has been known for a long time that daily rhythms of activity persist in organisms when the organism concerned is maintained in constant conditions. Indeed, in order to identify such activity as circadian rhythm, continuation of the changes in a rhythmic manner under constant conditions must be demonstrated (Sweeney, 1983). Even today there is some confusion on this point and some processes are erroneously called circadian which have only been measured under light: dark conditions. Therefore, in order to study if there is any circadian rhythm in the periodicity of spore shedding, experiments were conducted in continuous light (24 h L), continuous dark (24 h D), 12 L:

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12 D, 12 D:12 L cycle in the tetrasporic plants of *Wrangelia argus* and *Centroceras clavulatum*. In these experiments the spore shedding was counted after 24 h.

## Results

The quantity of tetraspores liberated at different times of the day varied in these algae (Fig. 1 and Fig. 2). In *Wrangelia argus* (Fig. 1) the tetraspore shedding was not seen between 1800 and 0600 h on day one and less number of spores were observed on day two.

From 0600 h onwards gradual increase in tetraspore shedding was observed and maximum liberation was seen during afternoon i.e. between 1400 h and 1800 h in *Wrangelia argus*. There was no liberation of tetraspores between 1800 h and 2200 h on day one. But, very less number of spores was observed during this period in *Centroceras clavulatum* (Fig. 2). From 2200 h onwards liberation of tetraspores increased and maximum shedding was noticed between 0200 h and 0600h (Fig. 2)

### Factors influencing circadian periodicity

Circadian periodicity curves obtained on the two algae exposed to air for 0 (control), 15, 30, 45 and 60 minutes at room temperature (Fig. 3 and Fig. 4) and 0 (control), 15, 30, 45 and 60 minutes in open air are shown in Fig. 5 and Fig. 6. The circadian periodicity of tetraspores did not vary in *Wrangelia argus* and *Centroceras clavulatum* in control and in the fronds exposed to air for a period of 15 to 60 min inside the laboratory and 15 to 45 min outside the laboratory (Fig. 3 to 6). As observed in the normal periodicity curves (Fig. 1 and Fig. 2) maximum tetraspore shedding was seen between 1400 h and 1800 h in *Wrangelia argus* and between 0200 h and 0600 h in *Centroceras clavulatum*.

In order to show the effect of salinity on the circadian periodicity of tetraspores data collected at five different salinities i.e. 0%, 10%, 20%, 30% and 40% are plotted in Fig. 7 for *Wrangelia argus* and Fig. 8 for *Centroceras clavulatum*.

The circadian periodicity curves varied according to the salinities in both *Wrangelia argus* and *Centroceras clavulatum*. In the case of *Wrangelia argus* the liberation of tetraspores was started within the four hours of the commencement of the experiments and increased slowly and maximum shedding of spores was observed at 0% and 10% between 0200 h and 0600 h

coinciding with the peak period of shedding of *Centroceras clavulatum*. The normal peak period of shedding of spores i.e. between 1400 h and 1800 h was seen at 20% and 30% but whereas at 40% four hours delay in the peak period of shedding of spores was observed i.e. between 1800 h and 2200 h (Fig. 7). In *Centroceras clavulatum* the peak shedding of tetraspores was seen within four and eight hours of the commencement of experiments in between 1800h and 2200 h at 0% and between 2200 h and 0200 h at 10% and the normal peak period of shedding i.e. between 0200 h and 0600 h was seen at 20% and 30%. As observed in *Wrangelia argus* four hours of delay in the peak shedding of spores has been observed at 40% i.e. between 0600 h and 1000 h (Fig. 8). The effect of salinity on the circadian periodicity of spore shedding clearly indicate that the period of maximum shedding of spores was hastened in low salinities i.e. below 20% and delayed in higher salinities i.e. above 30% in both *Wrangelia argus* and *Centroceras clavulatum*. The normal peak period of shedding was seen between 20% and 30%.

Circadian periodicity curves obtained at different light intensities (25, 35, 45 and 55  $\mu\text{E}/\text{m}^2/\text{s}$ ) are shown in Fig. 9 and Fig. 10. In this experiment the daily periodicity of tetraspores was not affected in the four photon flux densities tested.

The normal peak shedding of spores between 1400 h and 1800 h and 0200 h and 0600 h was obtained in *Wrangelia argus* and *Centroceras clavulatum* respectively. And as observed in the total spore output per day Fig. 1 and Fig. 2, the total number of spore shedding in the different photon flux densities varied in the circadian periodicity experiments also. The effects of continuous light (24 L: 0 D) continuous dark (24 D: 0 L), 12 L: 12 D and 12 D:12 L on the circadian periodicity of tetraspore shedding in *Wrangelia argus* and *Centroceras clavulatum* are presented in Fig. 11 and Fig. 12 respectively. In the case of *Wrangelia argus* remarkable variation in the circadian periodicity of tetraspore has been noticed in different light and dark regimes (Fig. 11). In continuous light (Fig. 11a and b) the maximum shedding of tetraspores was observed between 0600 h and 1000 h on day one and day two i.e. the peak period of shedding was observed nearly 8-10 h earlier than normal peak shedding period. In 12 L: 12 D (Fig. 11a and b) the peak period of shedding was similar to the above period on day1 (i.e. 0600 h and 1000 h), but on



day 2 (Fig. 11b) and day 3 (Fig. 11c) the peak period of shedding was seen 2-4 h earlier than the above period.

In the experiments conducted at 12 D:12 L (Fig. 11a and b) the peak period of spore shedding was seen between 1400 h and 1800 h on day 1 and day 2 which is almost similar to the normal peak period of shedding that was observed in the earlier studies (Fig. 1 and Fig. 2) whereas in the experiments conducted in continuous dark (Fig. 11a and b) the normal peak period of shedding was observed on day one i.e. between 1400 h and 1800 h but on day 2 a delay of 2 h was seen i.e. the maximum of tetraspores was seen between (1800 h and 2000 h). These experiments on *Wrangelia argus* clearly indicate that the peak period of shedding of spores can be entrained to required period by changing light and dark periods.

In the case of *Centroceras clavulatum* the peak period of tetraspore shedding does not change in continuous light, 12 L: 12 D and 12 D:12 L (Fig. 12a, b and c.). In these three combinations the peak period of tetraspore shedding was observed between 0200 h and 0600 h corresponding with the normal peak period of shedding as observed in the normal conditions (Fig. 2) but in the experiments conducted in continuous dark (Fig. 12a, b and c) the peak period of shedding of tetraspores was normal i.e. between 0200 h-0600 h (day 1) but a delay of 2-4 h had been observed on day 2 and day 3 respectively i.e. the maximum shedding of tetraspores was observed between 0600 h and 1000 h and 1000 h-1400 h on day 2 and day 3 respectively which indicate that continuous dark conditions delayed the peak period of shedding of tetraspores but the rhythm did not change.

## Discussion

While Matsui (1969) observed that the time of peak liberation of spores in the fronds of *Gloeopeltis* species exposed to air for 2-6 h was accelerated by 10 h, the present study did not notice any change in the time of liberation of spores in both the algae (Fig. 1 and Fig. 2). However the present study agrees with observation of Rao and Subba Rangaiah (1981) in Gigartinales. Rao and Kaliaperumal (1987) reported similarly in some Gelidiales though they found a 4 h of delay in the fronds exposed for 45 min in *Gelidium pusillum*. In contrast to these observations Zechman and Mathieson, (1985)

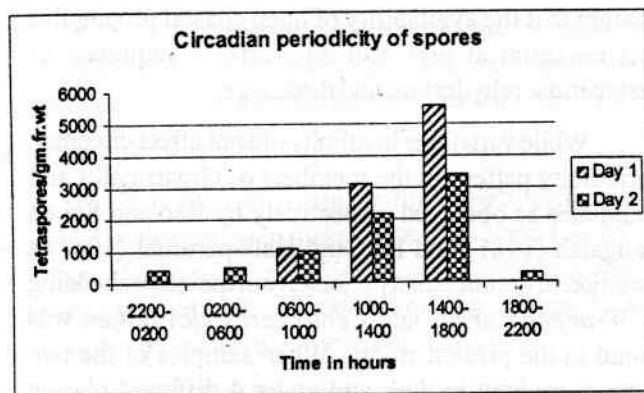
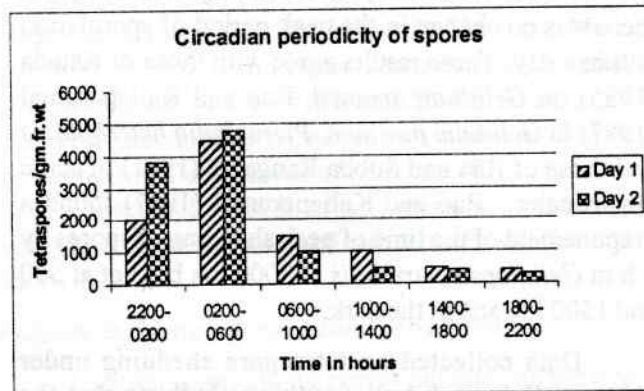
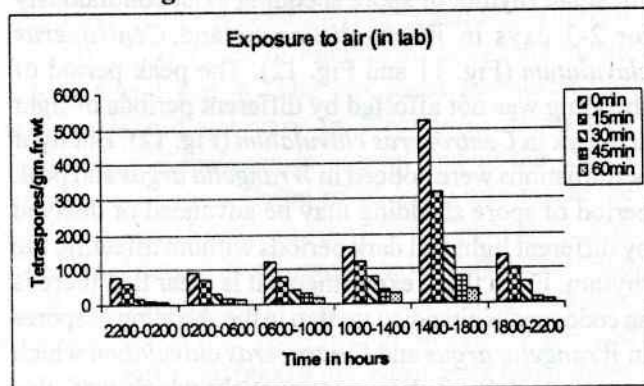
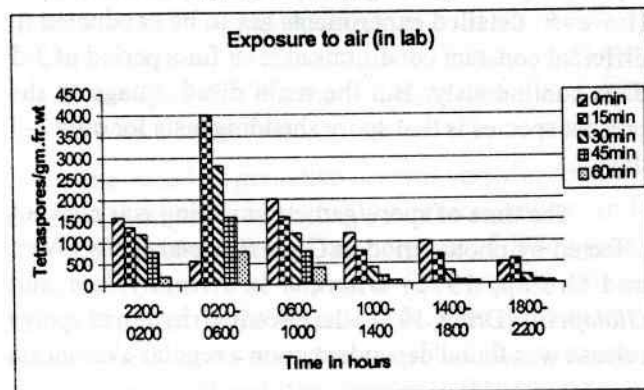
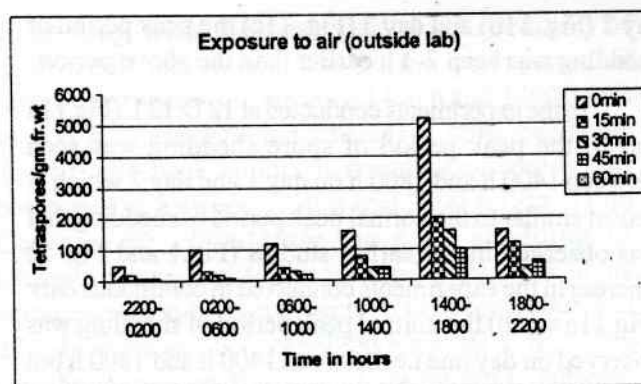
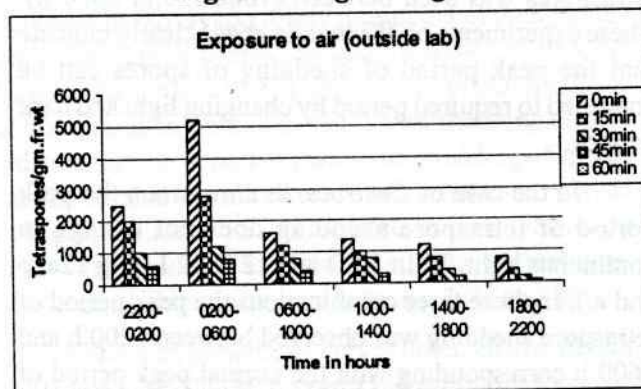
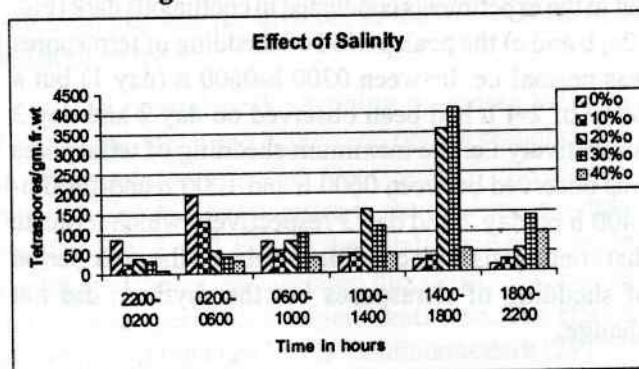
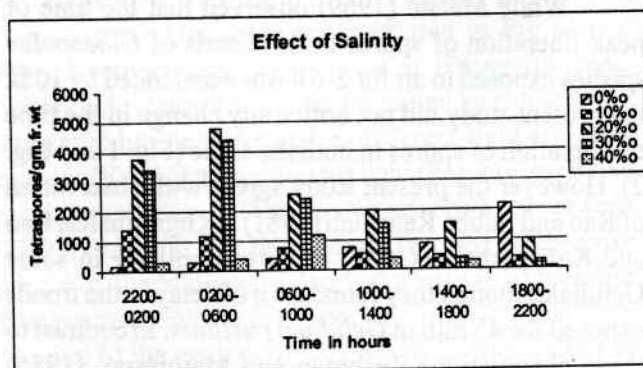
thought that the availability of open coastal propagules was maximal at high tide i.e., after a sequence of desiccation, rehydration and discharge.

While variations in salinity did not affect circadian periodicity pattern in the members of Gigartinales and Gelidiales as observed respectively by Rao and Subba Rangaiah (1981) and Rao and Kaliaperumal (1987), a variation in the circadian periodicity in the spore shedding of *Wrangelia argus* and *Centroceras clavulatum* was found in the present study. When samples of the two algae were kept in dark and under 4 different photon flux densities viz. 25, 35, 45 and 55 (Fig. 9 and Fig. 10) there was no change in the peak period of sporulation within a day. These results agree with those of Katada (1955) on *Gelidium amansii*, Rao and Kaliaperumal (1987) in *Gelidium pusillum*, *Pterocladia heteroplatus* and those of Rao and Subba Rangaiah (1981) in some Gigartinales. Rao and Kaliaperumal (1987) found a preponement of the time of peak shedding of spores by 4 h in *Gelidiopsis variabilis* at 3000 lux but not at 500 and 1500 lux and in the dark.

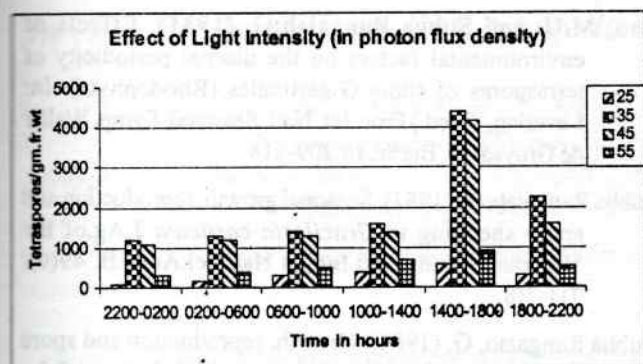
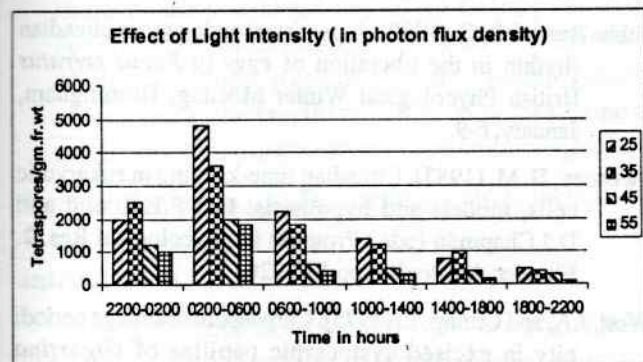
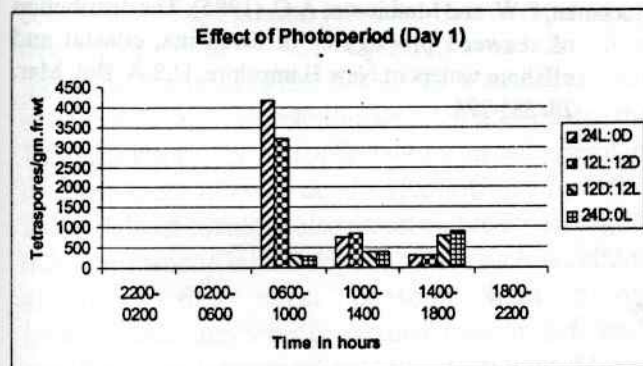
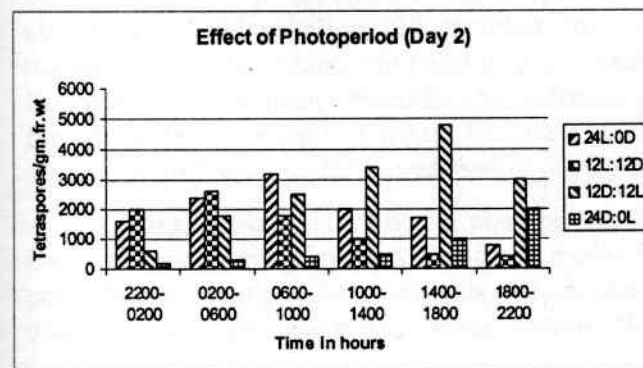
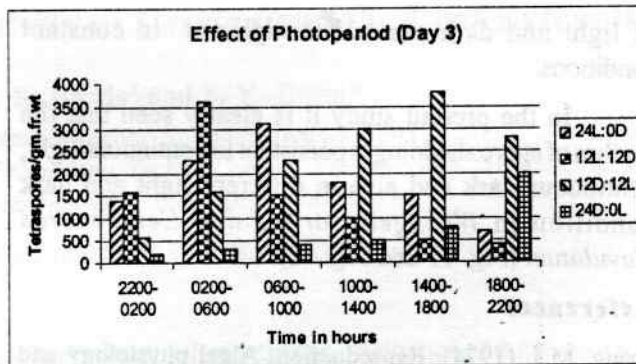
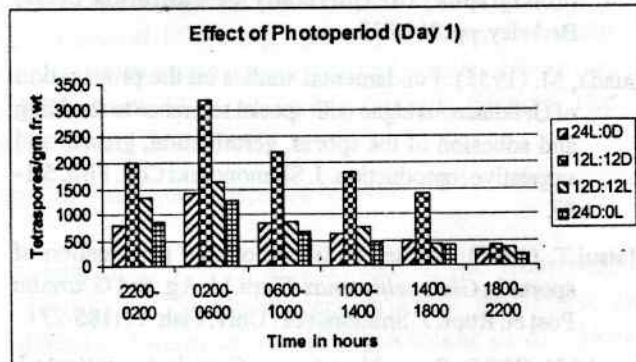
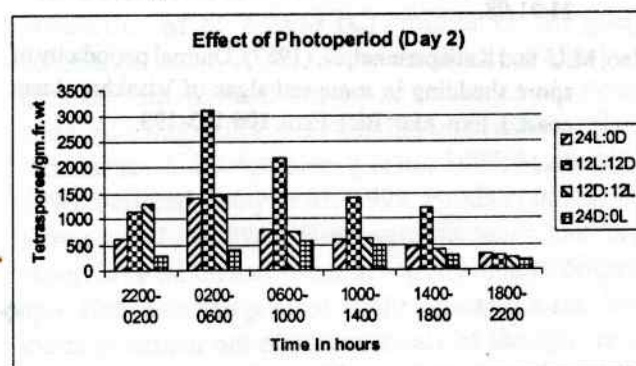
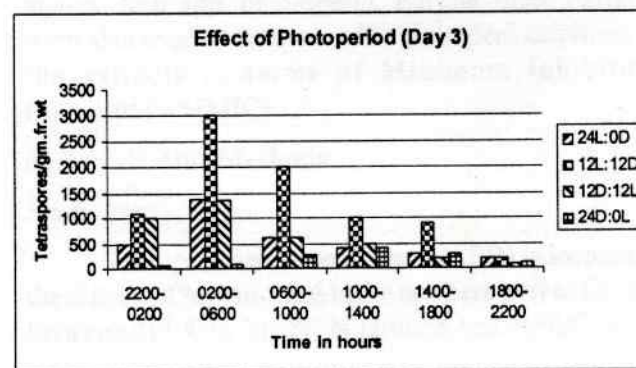
Data collected on tetraspore shedding under different light and dark conditions indicate that the circadian rhythm of spore shedding exist continuously for 2-3 days in *Wrangelia argus* and *Centroceras clavulatum* (Fig. 11 and Fig. 12). The peak period of shedding was not affected by different periods of light and dark in *Centroceras clavulatum* (Fig. 12). But light perturbations were noticed in *Wrangelia argus* and peak period of spore shedding may be advanced or delayed by different light and dark periods without affecting the rhythm. From these experiments it is clear that there is an endogenous circadian rhythm in the shedding of spores in *Wrangelia argus* and *Centroceras clavulatum* which is persistent for 3 days continuously which was also reported by Subba Rangaiah (1992) in *Fucus serratus*. However, detailed experiments are to be conducted in different constant conditions at least for a period of 3-5 days continuously. But the main disadvantage in the present species is that spore shedding lasts for only 2-3 days.

The time of spore/gamete shedding was also not affected by photoperiod in *Gigartina pterocelis* (West and Crump, 1975) whereas in *Nitophyllum* and *Gloiopeltis* (Dring, 1974) the circadian rhythm of spores release was found dependent upon a regular alternation



Fig. 1. *Wrangelia argus*Fig. 2. *Centroceras clavulatum*Fig. 3. *Wrangelia argus*Fig. 4. *Centroceras clavulatum*Fig. 5. *Wrangelia argus*Fig. 6. *Centroceras clavulatum*Fig. 7. *Wrangelia argus*Fig. 8. *Centroceras clavulatum*



Fig. 9. *Wrangelia argus*Fig. 10. *Centroceras clavulatum*Fig. 11a. *Wrangelia argus*Fig. 11b. *Wrangelia argus*Fig. 11c. *Wrangelia argus*Fig. 12a. *Centroceras clavulatum*Fig. 12b. *Centroceras clavulatum*Fig. 12c. *Centroceras clavulatum*



of light and dark periods though not in constant conditions.

In the present study it is clearly seen that the rhythm of spore shedding is persistent in continuous light, continuous dark and also in different light and dark condition in *Wrangelia argus* and *Centroceras clavulatum* (Fig. 11 and Fig. 12)

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## ***In vitro* Evaluation on Anti-*Shigella* Activity of *Cassia fistula* L. Leaf Extracts**

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Four different polar and non-polar solvent extracts viz. (petroleum ether, ethanol, methanol and aqueous extracts) were prepared from *Cassia fistula* L. leaves collected from Similipal Biosphere Reserve, Orissa, India. Antibacterial activity of the all the plant extracts were studied against four *Shigella* spp. viz. *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri* and *Shigella sonnei* following agar cup method and broth dilution methods. Results showed promising antibacterial activity against all the tested strains. However, among the extracts, ethanol showed highest zone of inhibition with 16.5 mm against *Shigella flexneri*. The minimum inhibitory concentration value ranged between 0.156 to 1.25 mg/ml.

**Key words:** *Cassia fistula*, diarrhoea, phytochemicals, *Shigella*, Similipal biosphere reserve

### **Introduction**

*Shigella* infections remain a global public health concern, causing diarrhoea in both the developing and developed regions (Guerrant *et al.*, 1990). Shigellosis is common among children less than five years of age in developing countries and in persons who travel from industrial countries to less developed countries (Juckett *et al.*, 1999). In the developing world, it is estimated that 113 million episodes of shigellosis occur annually, resulting in more than 400,000 deaths (Kotloff *et al.*, 1999). Industrialized countries also report outbreaks of *Shigella* infections among high-risk populations such as children attending day care, persons with human immunodeficiency virus or acquired immunodeficiency syndrome, and inmates of custodial institutions. Most of the *Shigella* species are resistant to quinolones and commonly used first-line antimicrobial drugs such as ciprofloxacin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole (Agtini *et al.*, 2005). Hence, evaluation of plant extracts for alternative source for antibacterial properties would be essential for identification of new drug for the treatment of shigellosis.

*Cassia fistula* L. is a tropical plant belongs to Caesalpiniaceae family is widely used for its medicinal properties. In the Indian literature, this plant has been described to be useful against skin diseases, liver troubles,

tuberculous glands and its use in the treatment of rheumatism, haematemesis, pruritus, leucoderm and diabetes (Alam *et al.*, 1990; Asolkar *et al.*, 1992). Reports are available on hepatoprotective (Bhakta *et al.*, 1999), antifertility (Yadav and Jain, 1999), and antioxidant properties of *C. fistula* (Chaminda *et al.*, 2001; Siddhuraju *et al.*, 2002). Some studies have also been done on antimicrobial activity of *Cassia fistula* flower and seed along with some other Indian medicinal plants (Kumar *et al.*, 2006; Valasraj *et al.*, 1997; Misra *et al.*, 1996; Perumal-Samy *et al.*, 1998; Panda *et al.*, 2009c; Panda *et al.*, 2009e). However, no work has been reported so far on antibacterial activity against *Shigella* spp. Hence in the present study an attempt has been made to screen out phytochemicals of the four crude leaf extracts (petroleum ether, ethanol, methanol and aqueous) and evaluate their antibacterial activity extracts against four spp. of *Shigella*. Further these extracts were also used to determine the biological activities of the extracts in terms of Minimum Inhibitory Concentration (MIC).

### **Materials and Methods**

#### **Study area**

Similipal Biosphere Reserve (SBR) is located in the district of Mayurbhanj in the northern part of Orissa, between 21°08" to 21°27" N latitude and 86°04" to 86°

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15° E longitude a unique habitat of mixed tropical forest and harbors varied flora and fauna (Fig. 1). More than 500 medicinal plants are available in this ecosystem (Saxena and Brahmam, 1989; Pandey and Rout, 2002).

#### **Plant material**

Leaves of *Cassia fistula* L. were collected in the month of April, 2007 from SBR and their identity was confirmed and the voucher specimen was deposited in the Department of Botany, North Orissa University. The shade dried healthy leaves, were powdered separately using mechanical grinder and then were passed through sieve (40 mesh size) so that uniform powder is maintained.

#### **Preparation of extracts**

Leaves of *Cassia fistula* (100 gm of each) powdered plant material was taken in four separate 500 ml conical flasks and soaked with 200 ml of each solvent (petroleum ether, ethanol, methanol and distilled water) at room temperature for 48 h. The extract was filtered through Buchner funnel using Whatman no-1 filter paper. The filtrate was evaporated to dryness under reduced pressure and the concentrated extracts were freeze dried to remove the solvent at -2 °C till further use (Panda *et al.*, 2009 c,d).

#### **Phytochemical analysis**

Qualitative phytochemical analysis was carried out using method described by Trease and Evans, 1989. Each extract was screened for presence of alkaloids (using Mayer's, Wagner's, Hager's and Dragendorff's reagents); flavonoids (NaCl and HCl); carbohydrates (using Molisch's reagent); glycosides (using Keller-Killiani and Borntrager's reagents); protein and amino acids (using Biuret, Xanthoproteic, Ninhydrin and Millon's reagent); tannin and phenolic compounds (FeCl<sub>3</sub> and Gelatin); triterpenoid (thionylchloride solution); steroid and sterols (using Liebermann Burchard and Salkowski's reagents); fat and fixed oil with alcoholic KOH reagents.

#### **Bacteria and growth media**

The antibacterial activity was tested against four species of *Shigella* spp. viz. *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri*, and *Shigella sonnei* (all from RMRC, Bhubaneswar, India). Test organisms were maintained on nutrient agar slants (Hi-media, Mumbai, India).

#### **Agar cup method**

The agar cup method (Barry, 1980) was used to study the antibacterial activity of the extracts as described by Panda *et al.* 2009 a. Mueller-Hinton Agar (MHA) (Hi-Media, Mumbai, India) was used as bacteriological medium. MHA plates were prepared by pouring molten media into sterile petri plates. The plates were allowed to solidify for 5 min. Wells were prepared in seeded agar plates. 0.1% inoculums suspension was swabbed uniformly and the inoculums were allowed to dry for 5 min. The extracts were diluted in 100% DMSO. A total of 6 mm diameter wells were punched into the agar and filled with the 50 µl (20 mg/ml in DMSO) extracts, 20 µl DMSO (negative control) and 5 µl of standard antibiotic (Ciprofloxacin at concentration 10 µg/ml) were used as a positive control. The plates were incubated at 37 °C for 24 h. After the incubation period formation of zones around the wells, confirms the antibacterial activity of the respective extracts. The same procedure was followed for each strain and extract. Each experiment was carried out in triplicates. The mean  $\pm$  SD of the inhibition zone was taken for evaluating the antibacterial activity of the extracts.

#### **Determination of minimum inhibitory concentration (MIC)**

In the present experiment, extracts which showed positive result were further evaluated for determination of MIC. A broth micro-dilution technique was adopted using 96 well micro-titer plates and tetrazolium salt, 2,3,5-Triphenyltetrazolium Chloride (TTC) was carried out to determine the MIC following the methods as described by (Panda *et al.*, 2009 c). Selected extracts were serially diluted in the 96-well plate with overnight culture of microorganisms (0.5 McFarland) grown at 37 °C to obtain final concentration of extracts ranged from 0.2 mg/ml to 5.0 mg/ml. The micro-plate was sealed and incubated at 37 °C at 130 rpm and observed for growth of the microorganism.

#### **Results and Discussion**

Phytochemical screening of four extracts (petroleum ether, ethanol, methanol and aqueous) of leaves has been summarized in Table 1. Percentage yield of different extracts were in the order ethanol > aqueous > methanol > petroleum ether. Evaluation of phytochemicals such as alkaloids, flavonoids, carbohydrates, glycosides, proteins and amino acids,



steroids, saponin and triterpenoid revealed the presence of most of constituents in polar extracts such as ethanol, methanol and aqueous extracts compared to non polar extract (petroleum ether). However, flavonoid, proteins and amino acid, tannin and phenols were found to be universally occurring in all the extracts (Table 1). The result of antibacterial screening (Table 2) of *C. fistula* indicates that all four extracts viz. petroleum ether, ethanol, methanol and aqueous extracts have antibacterial

activity against all tested *Shigella* species. However highest zone of inhibition was shown by the ethanol extract followed by aqueous, methanol and petroleum ether extracts. *Shigella flexneri* was found to be more sensitive followed by *S. sonnei* and other species. Petroleum ether extracts showed less activity than methanol and aqueous extracts possibly because active substances were soluble in polar solvents than compared to non-polar solvents such as petroleum ether extract.

Table-1  
Screening of phytochemicals of *Cassia fistula* leaf.

| Solvent         | Color & physical appearance | % of yield | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|-----------------|-----------------------------|------------|---|---|---|---|---|---|---|---|---|
| Petroleum ether | Brownish, hard              | 2.45       | - | - | - | + | - | + | - | + | - |
| Ethanol         | Blackish, sticky            | 15.86      | + | - | + | + | + | + | + | + | - |
| Methanol        | Greenish black, sticky      | 4.34       | + | + | + | + | + | + | + | + | - |
| Aqueous         | Greenish black, sticky      | 6.82       | + | + | + | + | - | + | - | - | - |

(+) Present, (-) Absent, 1-Alkaloids, 2-Carbohydrates, 3-Protein & amino acids, 4-Tannin & phenols, 5-Glycosides, 6-Flavonoids, 7-Saponins, 8-Triterpenoids, 9-Gums & mucilage.

Table-2  
Screening of *Cassia fistula* leaf by agar cup method

| Name of the bacteria        | Zone of inhibition in mm |           |           |           |           |
|-----------------------------|--------------------------|-----------|-----------|-----------|-----------|
|                             | PE                       | EtOH      | MeOH      | AQ        | A         |
| <i>Shigella boydii</i>      | 13.7±1.15                | 14.7±1.52 | 12.0±1.73 | 11.6±2.51 | 18.0±1.0  |
| <i>Shigella dysenteriae</i> | 13.0±1.0                 | 14.3±1.15 | 12.3±1.52 | 13.6±0.57 | 16.3±1.05 |
| <i>Shigella flexneri</i>    | 13.3±0.6                 | 16.5±0.57 | 14.0±1.73 | 14.0±1.00 | 23.6±1.10 |
| <i>Shigella sonnei</i>      | 12.3±0.6                 | 15.0±1.0  | 14.3±0.57 | 12.3±1.52 | 22.6±1.10 |

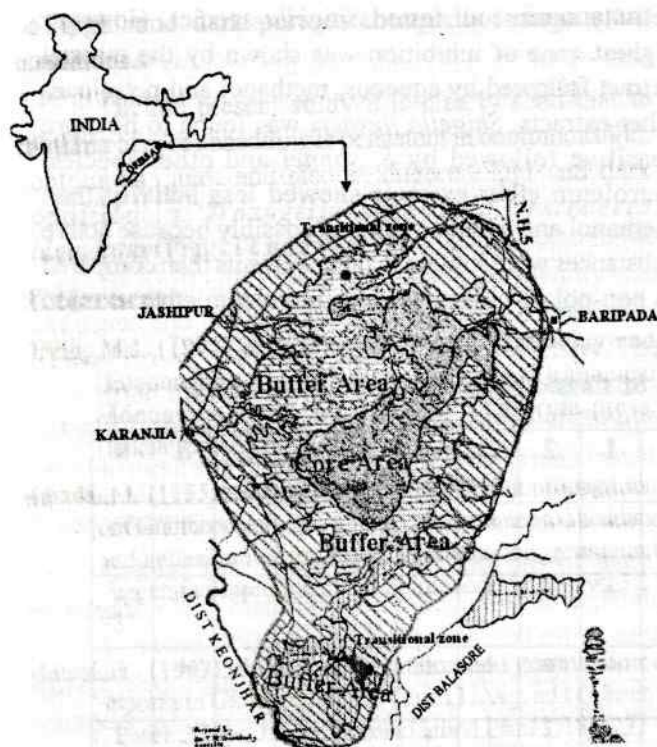
PE-Petroleum ether, EtOH-Ethanol, MeOH-Methanol, AQ-Aqueous, A-Antibiotic (Ciprofloxacin), Mean ± S.D.

Table-3  
Determination of MIC of *Cassia fistula* leaf

| Name of the bacteria        | Minimum inhibitory concentration in mg/ml |       |       |       |               |
|-----------------------------|-------------------------------------------|-------|-------|-------|---------------|
|                             | PE                                        | EtOH  | MeOH  | AQ    | Ciprofloxacin |
| <i>Shigella boydii</i>      | 0.625                                     | 0.625 | 1.25  | 1.25  | 0.001         |
| <i>Shigella dysenteriae</i> | 0.625                                     | 0.625 | 1.25  | 0.625 | 0.002         |
| <i>Shigella flexneri</i>    | 0.625                                     | 0.156 | 0.625 | 0.625 | 0.002         |
| <i>Shigella sonnei</i>      | 0.625                                     | 0.312 | 0.625 | 1.25  | 0.002         |

PE-Petroleum ether, EtOH-Ethanol, MeOH-Methanol, AQ-Aqueous, A-Antibiotic (Ciprofloxacin)





**Fig. 1. Map of Similipal biosphere reserve located in the northern part of Orissa**

The result of MIC showed that 6% of total extracts were active in a concentration of 0.156 mg/ml, 13% in a concentration of 0.312 mg/ml, 75% in a concentration of 0.625 mg/ml, and 100% inhibition in a concentration of 1.25 mg/ml (Table 3).

The phytochemical constituents such as alkaloid, flavonoid, tannin and phenolic compound have been reported to be important compounds in many other medicinal plants (Barnabas and Nagarajan, 1988; Burapedjo and Buncho, 1995; Panda *et al.*, 2009b). In the present investigation ethanol extracts of *C. fistula* leaves possess those compounds which might showed antibacterial activity. Agtini *et al.* (2005) found an overall incidence of shigellosis of 4 out of 1000 individuals per year world wide. According to the authors shigellosis was most common among children and the elderly, with the highest incidence at 32 out of 1000 per year in those 1 to 2 years of age. Out of their total isolated strains, 72% were *S. flexneri*, 23% were *S. sonnei*, 2% were *S. dysenteriae* and 3% were *S. boydii*. The most frequently encountered species of *Shigella* was *S. flexneri*.

Similipal is rich in medicinal plant resources. Looking into the vast area of nearly 2750 sq Km of forest and its rich plant diversity, more studies are required to explore the potential uses of these medicinal plants for ailment of various diseases which could serve as a basis for their pharmaceutical applications. Knowledge on the biological activity and chemical constituents of medicinal plants are desirable not only for the discovery of new therapeutic agents, but also to give scientific basis for the folk medicines.

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## Probiotic Effect of *Lactobacillus* species on *Escherichia coli* and *Candida albicans*

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Today probiotics are available in a variety of food products and supplements, and have got wide applications in the control of cholesterol, cancers, allergies, etc. *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* were found to inhibit the growth of both *Escherichia coli* and *Candida albicans*. *L. acidophilus* formed an inhibition zone diameter of about  $13 \pm 1.25$  mm against *E. coli* and  $13.67 \pm 0.72$  mm against *C. albicans* in disc diffusion method, whereas the inhibition zone diameter were  $42 \pm 1.89$  mm and  $35.33 \pm 1.66$  mm in hole plate method against *E. coli* and *C. albicans* respectively. Similarly *L. bulgaricus* formed  $11.67 \pm 0.98$  mm inhibition zone against *E. coli* and  $14.33 \pm 0.72$  mm against *C. albicans* in disc diffusion method and  $39 \pm 1.7$  mm and  $31.33 \pm 1.52$  mm inhibition zones, in hole plate method against *E. coli* and *C. albicans* respectively. The results suggest that *L. acidophilus* and *L. bulgaricus* inhibit the growth of both *E. coli* and *C. albicans*. The inhibition zone diameter obtained with standard antibacterial agent 'ampicillin' and antifungal agent 'nystatin' were less than the inhibition zone obtained with *Lactobacillus* spp. and the hole plate method was more effective than disc diffusion method. The antimicrobial effect of both the *Lactobacillus* spp. may be due to the production of lactic acid.

**Key words:** *Escherichia coli*, *Candida albicans*, *Lactobacillus* spp., probiotic effect

### Introduction

The first recorded probiotic was fermented milk for human consumption. After that, probiotics became popular with animal nutrition. The role of fermented milk in human diet was known even in 'Vedic' times. But, the scientific interest in this area boosted after the publication of the book entitled "The Prolongation of Life" by Ellie Metchinkoff in 1908. He suggested that people should consume fermented milk containing *Lactobacilli* to prolong their lives. Accelerated aging is because of autointoxication (chronic toxemia), which is due to the toxins produced by gut microflora. Bulgarian peasants who were subjected to the experiments on longevity had consumed large quantities of sour milk. The pathological reaction might be removed and life expectancy could be enhanced by implanting lactic acid bacteria from Bulgarian yoghurt. Since then, researchers started investigations relating to the role of lactic acid bacteria in human and animal health.

Probiotics have been used as growth promoters, for lactose intolerance, antitumour and

anticholestrolaemic effects (Manisha and Prajapati, 2001). Probiotics have been extensively studied under *in vitro* and *in vivo* conditions. The main fields of research with respect to probiotics are heart diseases, allergic reaction, cancer, diarrhoea, etc. The use of probiotics resulting in alleviation of lactose intolerance due to increased concentration of  $\beta$ -galactosidase in the small intestine, relief from constipation by increased bowel function, antitumour activities due to inhibition of tumour cells, destruction of carcinogens, etc. have been well documented. Intestinal infections caused by *Escherichia coli*, *Campylobacter fetus* subsp. *jejuni*, *Clostridium perfringens*, *C. albicans* and *C. botulinum* were reduced in the presence of *Lactobacillus* supplements. The *Lactobacillus* has shown promising results and *Bifidobacterium longum* has been successfully used to reduce the after-effects of antibiotic therapy (Suvama and Boby, 2005).

A good probiotic agent needs to be non-pathogenic, nontoxic, resistant to gastric acid, adhere to gut epithelial tissue and produce antimicrobial substances. It should

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persist, albeit for short periods in the gastro-intestinal tract influencing metabolic activities like cholesterol assimilation, lactose activity and vitamin production. The survival of probiotic organisms in the gut depends on the colonization factors that they possess, organelles which enable them to resist the antimicrobial mechanisms that operate in the gut. In addition to the antimicrobial mechanisms, they need to avoid the effects of peristalsis, which tend to flush out bacteria with food. This can be achieved either by immobilizing themselves or by growing at a much faster rate than the rate of removal by peristalsis. The probiotic strain needs to be resistant to the bile acid, e.g. *Bifidobacteria* strains proved significantly less acid-resistant than the *Lactobacillus* strains, when exposed to human gastric juice (Pathmakanthan *et al.*, 2000).

*Escherichia coli* causes foodborne illness and infection often leads to bloody diarrhoea, and occasionally to kidney failure to surgical wounds, especially in young children and elderly people. Most illness has been associated with eating undercooked, contaminated ground beef, swimming in or drinking contaminated water, and eating contaminated vegetables. Recent years have shown a global increase in the incidence of mycotic infections only. *Candida albicans* can account for upto 75% of the yeasts recovered from various sites of infections. Moreover, the so called non-albicans *Candida* are also implicated with greater frequency as opportunistic pathogens in the compromised hosts (Pfaller, 1996). Rapid and accurate identification of yeast species have thus become important not only for effective management of a disease as these respond differently to antifungals but also to see their behaviour in drug resistance (Umabala and Lakshmi, 2002).

## Materials and Methods

### Isolation and phenotypic characterization

*Escherichia coli* and *Candida albicans* isolated from urine of patients suffering from symptoms of UTI like frequency, urgency, dysuria, pain in abdomen etc. in sterile wide mouth containers. Samples from catheterized patients were collected from the catheter tube (not from the urosac bag) under aseptic condition from patients. The specific organisms were isolated by the conventional methods using bacteriological media (MacConkey agar, and blood agar) as *Candida* is able to grow in such media while carrying out the bacteriological analysis. Then identification up to species level was made using

Chromagars (HiCrome UTI agar and HiCrome Candida agar). All the species identified by this method were reconfirmed by using other conventional methods (Bailey and Scott, 1970).

The curd samples were taken from the OMFED plant, Bhubaneswar. Microorganisms present in the curd were isolated by using MRS agar (deMan, Rogosa and Sharpe) and nutrient agar media. Colonies with typical characteristics were randomly selected from plates and tested for Gram stain and cell morphology before further sugar fermentation and characterization tests. During the test, the cultures were kept in MRS agar slabs at refrigeration temperature.

### Biochemical characterization and presumptive identification

Biochemical tests for *E. coli* like indole, MR-VP, citrate, PPA, urease, glucose, lactose, catalase, oxidase, nitrate, EMB (Eosin methylene blue) and for *C. albicans* like germ tube test (GTT), chlamydospore production on corn-meal agar, sugar tests like glucose, maltose, sucrose were determined.

The carbohydrate tests for *Lactobacillus spp.* were glucose, galactose, lactose, fructose, mannose, dextrin, maltose, trehalose, amygdalin, cellobiose, salicin, sucrose, melibiose, raffinose, glycogen, sorbitol and sterile water were used as positive and negative controls.

### Probiotic effect of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*

The test bacteria *E. coli* and *L. acidophilus* were incubated in 50 ml nutrient broth at appropriate temperature for 24 h. *L. bulgaricus* incubated in 50 ml sorbitol broth which turned purely yellow coloured indicated the presence of *L. bulgaricus* and *C. albicans* broth was prepared in 50 ml sabouraud dextrose broth by incubating 48 h at 35 °C. Approximately  $10^5$  cfu / ml of the respective organisms to be tested for sensitivity.

Probiotic activity was performed by disc diffusion method (Bauer *et al.*, 1966) and hole plate method. Nutrient agar and sabouraud dextrose agar plates were prepared under sterile conditions. These plates were kept for one hour in the oven at 37 °C for drying before inoculation of culture. Nutrient agar plates were inoculated with 0.1 ml of 24 h broth culture of *E. coli* and sabouraud dextrose agar plates were inoculated with



0.1 ml of 48 h broth culture of *C. albicans*. Small Whatman's filter paper No.1 ( diameter 6 mm ) were prepared by dipping in broth cultures of *L. acidophilus* and *L. bulgaricus*. The discs were placed in triplate forms in nutrient agar and sabouraud dextrose agar plates and were allowed to incubate at 37 °C for 24 h. Standard antibiotic discs of ampicillin (10 mcg/disc) and nystatin ( 0.5 mcg /disc ) for *E. coli* and *C. albicans* respectively, were used for comparison. Like disc diffusion method, hole plate method was conducted . A hole was made by the help of cork borer (10 mm). 1ml of broth cultures of *L. acidophilus* and *L. bulgaricus* were added dropwise in the hole in respective plates and were incubated at 37 °C for 24 h. Zones of inhibition were measured at the end of the incubation period.

### Statistical analysis

The experiment for the antimicrobial activity was conducted and analyzed as a factorial experiment with three replications in a completely randomized design. The results were presented as the means with SEM (Standard error of the mean).

### Results

*E. coli* was the most common isolate. HiCrome UTI agar allowed a definite identification of *E. coli* without the need for further biochemical tests. Also the colony colour of *E. coli* was clearer and more distinct than that on MacConkey agar. Further identification tests had to be done for definite speciation. Certain identification tests like the catalase, oxidase and indole test can be done directly from the colonies on HiCrome

UTI agar modified. Apart from HiCrome UTI agar the present study has also used HiCrome Candida agar to speciate the *Candida* isolates. The traditional identification system GTT need the yeasts to be in correct nutrient state, presence of an inducer (serum or chemical formulation mimicking serum biochemically) elevated temperature and natural pH. Although this is a rapid test, problems arise with false negatives due to heavy inoculum and increased incubation. The media used in the present study contains chromogenic substrates that react with enzymes secreted by organisms producing colonies with various pigmentation. The enzymes are species specific so as per the manufacture's instruction, *E. coli* was identified by purple coloured colonies and *C. albicans* was identified by light green coloured colonies.

Confirmation tests for *E. coli* were methyl-red positive and ferment glucose and lactose, they gave negative reactions in the Voges-Proskauer, phenylalanine deaminase and gelatine - hydrolysis tests, they are generally urease negative, they do not produce H<sub>2</sub>S in triple-sugar (TSI) agar. They produced metallic green colonies on eosin methylene blue (EMB) agar ( Table 1). *Candida albicans* was confirmed by germ tube test and sugar fermentation test (Table 2).

*Lactobacillus spp.* were gram negative rods obtained from curd samples and they were grown in various carbohydrate amended media in which it was seen that *L. acidophilus* and *L. bulgaricus* both ferment glucose, galactose, lactose, fructose, mannose. *L. acidophilus* can able to ferment dextrin, maltose,

Table-1  
Speciation tests used in the identification of *Escherichia coli*.

| Types of tests                    | Results                    |
|-----------------------------------|----------------------------|
| HiCrome UTI agar                  | Small purple colour colony |
| Glucose                           | AG                         |
| Lactose                           | +                          |
| Catalase                          | +                          |
| Oxidase                           | -                          |
| Nitrate                           | +                          |
| Indole                            | +                          |
| MR                                | +                          |
| VP                                | -                          |
| Citrate                           | -                          |
| PPA                               | -                          |
| Urease                            | -                          |
| Eosin methylene blue ( EMB ) agar | Metallic green colony      |

AG – Acid with gas



**Table-2**  
**Speciation tests used in the identification of *Candida albicans*.**

| Types of tests           | Results                   |
|--------------------------|---------------------------|
| HiCrome Candida agar     | Light green colour colony |
| Germ tube production     | +                         |
| Chlamydospore production | +                         |
| Glucose                  | AG                        |
| Maltose                  | AG                        |
| Sucrose                  | -                         |

AG – Acid with gas

**Table - 3**  
**Speciation tests used in the identification of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*.**

| Carbohydrate | <i>L. acidophilus</i> | <i>L. bulgaricus</i> |
|--------------|-----------------------|----------------------|
| Glucose      | +                     | +                    |
| Galactose    | +                     | +                    |
| Lactose      | +                     | +                    |
| Fructose     | +                     | +                    |
| Mannose      | +                     | +                    |
| Dextrin      | +                     | -                    |
| Maltose      | +                     | -                    |
| Trehalose    | +                     | -                    |
| Amygdalin    | +                     | -                    |
| Cellobiose   | +                     | -                    |
| Salicin      | +                     | -                    |
| Sucrose      | +                     | -                    |
| Melibiose    | +                     | -                    |
| Raffinose    | +                     | -                    |
| Glycogen     | -                     | -                    |
| Sorbitol     | -                     | +                    |

**Table-4**  
**Probiotic effect of *Lactobacillus* spp. against *Escherichia coli* based on disc diffusion and hole plate methods ( diameter in mm).**

| Method                                                    | Standard antibiotic<br>Ampicillin (10 mcg/disc) | <i>L. acidophilus</i> | <i>L. bulgaricus</i> |
|-----------------------------------------------------------|-------------------------------------------------|-----------------------|----------------------|
| Disc diffusion<br>method(including disc<br>diameter 6 mm) | 16.33 ± 1.66                                    | 13 ± 1.25             | 11.67 ± 0.98         |
| Hole plate<br>method(including hole<br>diameter 10 mm)    | 24 ± 1.7                                        | 42 ± 1.89             | 39 ± 1.7             |

**Table-5**  
**Probiotic effect of *Lactobacillus* spp. against *Candida albicans* based on disc diffusion and hole plate methods (diameter in mm).**

| Method                                                    | Standard antibiotic<br>Nystatin(0.5 mcg/disc) | <i>L. acidophilus</i> | <i>L. bulgaricus</i> |
|-----------------------------------------------------------|-----------------------------------------------|-----------------------|----------------------|
| Disc diffusion<br>method(including disc<br>diameter 6 mm) | 16 ± 0.82                                     | 13.67 ± 0.72          | 14.33 ± 0.72         |
| Hole plate<br>method(including hole<br>diameter 10 mm)    | 21 ± 0.94                                     | 35.33 ± 1.66          | 31.33 ± 1.52         |



trehalose, amygdalin, cellobiose, salicin, sucrose, melibiose, raffinose but not glycogen and sorbitol whereas *L. bulgaricus* can ferment sorbitol but not other carbohydrates (Table 3).

*L. acidophilus* and *L. bulgaricus* were found to inhibit the growth of both *E. coli* and *C. albicans*. *L. acidophilus* initially formed an inhibition zone  $13 \pm 1.25$  mm against *E. coli* and  $13.67 \pm 0.72$  mm against *C. albicans* in disc diffusion method and  $42 \pm 1.89$  mm where as  $35.33 \pm 1.66$  mm in hole plate method respectively. Similarly *L. bulgaricus* formed an inhibition zone  $11.67 \pm 0.98$  mm against *E. coli* and  $14.33 \pm 0.72$  mm against *C. albicans* in disc diffusion method and  $39 \pm 1.7$  mm and  $31.33 \pm 1.52$  mm inhibition zone in hole plate method against *E. coli* and *C. albicans*. The data obtained through the determination of probiotic activity, from the association of standard antibiotic (ampicillin and nystatin) are presented in Table 4 and Table 5.

## Discussion

The results in the present study suggest that *L. acidophilus* and *L. bulgaricus* inhibit the growth of both *E. coli* and *C. albicans*. The inhibition zone diameter obtained with standard antibacterial agent ampicillin and antifungal agent nystatin were less than the inhibition zone obtained with *Lactobacillus spp.* Again the hole plate method was more effective than disc diffusion method. The antimicrobial effect of both the *Lactobacillus spp.* were may be due to the production of lactic acid. Attributes of lactic acid bacteria such as anti-microbial agent production and competition with potential pathogens in the gut provided the impetus for investigating a role for probiotics in diarrhoea. Acute gastroenteritis diarrhoea in infancy is normally due to rotavirus and a number of trials have shown a reduction in the duration. Majamaa *et al.* (1995) showed that the incidence and duration of incidence of diarrhoea in infants was reduced in response to probiotic. Thus, both a therapeutic and preventive effect of probiotics has been demonstrated. The precise mechanism is not known, but evidence points to stimulation of the immune system. Hilton (1992) described the effective use of *L. acidophilus* in the treatment of *Candida* vaginitis. In addition, *Lactobacillus spp.* produce various antimicrobial compounds, which can be classified as low-molecular mass compounds such as hydrogen peroxide, carbon dioxide, diacetyl (2, 3-butanedione),

uncharacterized compounds and high molecular mass compounds like bacteriocin (Piard and Desmazeaud, 1992).

Patients are often prescribed broad-spectrum antibiotics to treat an infection, but diarrhoea can be an unwelcome gastrointestinal side effect. Eight clinical trials have shown that the incidence of diarrhoea is lower in response to probiotics (D'Souza *et al.*, 2002). Mixtures of concentrated probiotics have been shown to decrease the number of patients relapsing with pouchitis compared to placebo, over 12- and 18-month periods (Gionchetti *et al.*, 2000).

Today probiotics are available in a variety of food products and supplements. In the United States, food products containing probiotics are almost exclusively dairy products – fluid milk and yoghurt – due to the historical association of lactic acid bacteria with fermented milk. Probiotics are gaining importance because of the innumerable benefits, e.g. treating lactose intolerance, hypercholesterol problem, cardiac diseases and managing cardiac problems like atherosclerosis and arteriosclerosis. With the current focus on disease prevention and the quest for optimal health at all ages, the probiotics market potential is enormous. Health professionals are in an ideal position to help and guide their clients toward appropriate prophylactic and therapeutic uses of probiotics that deliver the desired beneficial health effects. There are many probiotic products at the market place and most have supporting evidence behind the advertized health claims. New legislation governing the labelling of probiotics, such as indicating the species, strain and number of bacteria present is likely to come into force in the near future. Probiotics should not be considered a panacea for health, but can be incorporated into a balanced and varied diet to maximize good health.

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## Antibacterial Assessment of Essential Oils of Three *Ocimum* spp. against Food Borne Pathogens

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Investigations were carried out to assess the efficiency of antibacterial potential of essential oils of three species *Ocimum* (*O. basilicum*, *O. sanctum*, *O. gratissimum*) on a comparative scale against eleven pathogens of both Gram negative and Gram positive origin. The minimum inhibitory concentration of the oils varied from 0.48 µl/ml to > 250 µl/ml (the maximum concentration tested). The activities of the oils were found to be bactericidal in action. The antibacterial activity of the oils was observed even after heating the oil at high temperature (100 °C for 1h) and autoclaving, indicates the presence of heat stable active components. The oils showed higher activity potential when autoclaved as compared to normal untreated oils. Most remarkable experience was found with *O. gratissimum* that showed 81.81 % of antibacterial activity. In this investigation we have reported the antibacterial activity of essential oils of *Ocimum* spp. against common food borne pathogens at very low concentrations, is suggestive of their possible use in food preservation.

**Key words:** Antibacterial activity, essential oils, food preservation, minimum inhibitory concentration, *Ocimum*, zone of inhibition.

### Introduction

The use of plants and plant products as medicines is as old as the beginning of human civilization (Rastogi and Mehrotra, 2002). Antimicrobials of plant origin have enormous therapeutic potential which are found to be effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are associated with synthetic antimicrobials. This shift from synthetic to natural antimicrobials is due to the increase in multiple drug resistance among pathogens with adverse effects on the host as hypersensitivity, immune-suppression and allergic reactions. The beneficial medicinal effects of plant materials typically result from the combination of secondary products present in the plant that are capable of producing definite physiological action on host body. Out of many plant groups the genus *Ocimum* of Lamiaceae family is believed to have species with long history as culinary herbs, sacred plants and ethnomedicinal potentiality. Its species are believed to be a rich house of various biologically active constituents (Dubey *et al.*, 2000; Hakkim *et al.*, 2008). The aim of the present work is to study the comparative antibacterial activities of essential oils of the three *Ocimum* species (*O. sanctum*, *O.*

*basilicum*, *O. gratissimum*). The issues addressed in this investigation are i) establishment of antibacterial activity of essential oils of *Ocimum* spp; ii) determination of Minimum Inhibitory Concentration (MICs) of the oils; iii) determination of phenol co-efficient values of the oils and iv) comparison of antibacterial activity of the oils with standard antibiotics.

### Materials and methods

#### Test Pathogens

Eleven pathogenic bacteria used in this study were procured from National Institute of Cholera and Enteric Diseases (NICED), Kolkata, viz. *Bacillus subtilis*, *Escherichia coli* 0157: H7, Enteropathogenic *E. coli*, Enterotoxigenic *E. coli*, *Shigella boydii*, *Shigella dysenteriae*, *Shigella sonnei*, *Staphylococcus aureus*, *Vibrio cholerae* (0139), *Vibrio cholerae* (Ogawa) and *Vibrio cholerae* (Inaba).

#### Essential oils

The essential oils investigated in this study were hydrosteam distilled of seeds of the three respective *Ocimum* species and were obtained from the Indian Institute of Chemical Technology (IICT), Hyderabad. A cocktail mixture of the three essential oils (1:1:1) was

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also prepared and used in this investigation to study the antibacterial activities.

### Media and Chemicals

Nutrient broth, Luria broth, Agar-Agar (bacteriological grade), Tween-20 were procured from Hi-Media, Ltd., Mumbai, India and all the media were prepared according to manufacturer's instruction. The nutrient broth was supplemented with 0.75 % Tween-20 to facilitate the miscibility of the oils. Agar-Agar at (1.5-2% W/V) was added to the medium in order to obtain solid nutrient agar medium whenever necessary. Antibiotic discs [Ampicillin (10µg), Nalidixic acid (30µg), Tetracyclin (30 µg), Polymyxin-B(300U), Chloramphenicol (30 µg), Trimethoprim (10 µg), Ciprofloxacin (30 µg), Norfloxacin (10 µg), Sulphadiazine (300 µg), Bacitracin (30 U)] were procured from Hi-Media Ltd., and used in this study.

### Methods

Preliminary screening of oils for their antibacterial properties was done by disc diffusion method described by Bauer *et al.* (1966), with slight modifications Rath *et al.* (1999). Minimum Inhibitory Concentration (MIC) of the oils was determined by tube dilution technique described by Rath *et al.* (1999, 2002). MIC of the oils was determined in 6 selected bacteria against which the oils showed higher degree of toxicity during preliminary screening. Toxicity (bactericidal or bacteriostatic) nature of oils was determined by subculturing one loop of the sample from the MIC dilution tubes onto nutrient agar plates (Rath *et al.* 2005). The effect of temperature and pressure on the antibacterial activity was also studied by boiling and autoclaving the oils through disc diffusion method of Rath *et al.*, (2005, 2007). The antibiogram pattern of the pathogens was also studied following the method of Bauer *et al.* (1966). Phenol-coefficient of the three oils and the cocktail mixture was studied following the method of Rath *et al.* (2008) in order to compare and establish the antibacterial potentiality of the oils.

### Results

The preliminary screening by disc diffusion method (Table 1) implied that *O. gratissimum* essential oil was active against all the test pathogens representing 100% antibacterial activity even at oil concentration of 2.5µl/disc, whereas, *O. sanctum* oil showed activity against 81.81% of the test pathogens. But a degree of variability was recorded in terms of zones of inhibition as well as in

susceptibility of the pathogens. Maximum degree of zone size was recorded against *Shigella sonnei* (i.e. 22.5mm). Least activity was observed against *E. coli* 0157:H7. Both *Bacillus subtilis* and Enterotoxigenic *E. coli* were resistant to *O. sanctum* essential oil. In contrast Enterotoxigenic *E. coli*, *Shigella sonnei*, and *Vibrio cholerae* (Inaba) showed resistance to *O. basilicum* oil. During susceptibility testing it was recorded that *Shigella sonnei* was susceptible to *O. sanctum* and *O. gratissimum* oil, and *Shigella boydii* was susceptible to all the three oils.

The Minimum Inhibitory Concentrations (MIC) was determined only for six test pathogens that showed higher degree of susceptibility during preliminary screening by disc diffusion method. The MIC values (Table 2) of the oils ranged between 0.48 to > 250µl/ml (the maximum concentration tested). The MIC values of the oils corroborate with our previous findings i.e. oils with higher zones of inhibition represented lower MIC values. Interestingly, *B. subtilis* that showed resistance towards *O. sanctum* oil showed lower MIC value of 0.97µl/ml. It could be attributable to poor diffusibility of the oil on agar plates during disc diffusion method. In this investigation it was observed that *O. sanctum* and *O. gratissimum* oils showed MIC value 0.48µl/ml against *S. aureus* and *V. cholerae* (Inaba) whereas, both *O. basilicum* and *O. gratissimum* oils showed same MIC values (0.48µl/ml) against *V. cholerae* O139. But, *O. sanctum* and *O. basilicum* oils represented MIC value of 0.97µl/ml against *B. subtilis* and *E. coli* O157:H7. The activity of all the essential oils were observed to be bactericidal, as no growth was observed when subcultured on to nutrient agar plates from the MIC dilution tubes. Bactericidal activity of different essential oils have been reported by several workers (Pattnaik *et al.* 1995; Gupta *et al.* 2004; Rath *et al.* 1999, 2001, 2002, 2005, 2008).

An experiment was designed to study the effect of temperature and/or pressure on the antibacterial activity of the oils. Oils were heated in a boiling water bath (100°C for 1hr) and were also autoclaved (separately) and tested for their antibacterial activities by disc diffusion method (loading MIC values of oils on discs). The findings are presented in Table-3. *O. sanctum* oil lost its activity on heating against *S. dysenteriae*, whereas, an increased activity was recorded against *V. cholerae* strains. Decrease in zone



**Table-1**  
**Preliminary screening of oils for antibacterial activity by Disc Diffusion Method**

| Test Organisms                  | Zones of inhibition* |          |          |                     |           |          |                       |          |          |
|---------------------------------|----------------------|----------|----------|---------------------|-----------|----------|-----------------------|----------|----------|
|                                 | <i>O. sanctum</i>    |          |          | <i>O. basilicum</i> |           |          | <i>O. gratissimum</i> |          |          |
|                                 | 2.5µl                | 5µl      | 10µl     | 2.5µl               | 5µl       | 10µl     | 2.5µl                 | 5µl      | 10µl     |
| <i>Bacillus subtilis</i>        | R                    | R        | R        | 12.0±1.0            | 16.0±1.0  | 18.0±0.0 | 14.0±1.5              | 17.0±2.5 | 31.0±3.0 |
| <i>Escherichia coli</i> 0157:H7 | 12.0±1.0             | 13±0.0   | 15±1.0   | 12.0±0.0            | 13.0±1.0  | 15.0±1.0 | 14.0±0.0              | 16.0±0.0 | 18.0±0.0 |
| <i>Enteropathogenic E.coli</i>  | 13.0±1.0             | 15±0.0   | 15.5±0.5 | 13.0±0.0            | 15.0±10.5 | 17.0±2.0 | 12.0±0.1              | 14.0±1.0 | 16.0±1.5 |
| <i>Enterotoxigenic E.coli</i>   | R                    | R        | R        | R                   | R         | R        | 11.0±1.5              | 14.0±1.0 | 17.0±1.0 |
| <i>Shigella boydii</i>          | 12.0±0.0             | 15.0±2.0 | 14.0±0.0 | 11.0±0.1            | 13.0±0.0  | 15.0±2.5 | 20.0±0.0              | 25.0±0.0 | >30      |
| <i>Shigella dysenteriae</i>     | 13.0±1.5             | 13.0±2.0 | 15.0±1.0 | 12.0±1.0            | 14.0±1.0  | 16.0±0.0 | 11.0±0.0              | 14.0±1.5 | 17.0±2.5 |
| <i>Shigella sonnei</i>          | 10.0±0.0             | 22.0±1.0 | 19.0±1.0 | R                   | R         | R        | 14.0±2.0              | 16.0±1.5 | 19.0±1.0 |
| <i>Staphylococcus aureus</i>    | 13.0±0.5             | 13.0±1.5 | 17.0±6.0 | 13.0±1.5            | 14.0±1.0  | 13.0±1.0 | 13.0±0.5              | 15.0±0.5 | 18.0±0   |
| <i>Vibrio cholerae</i> (0139)   | 12.0±1.5             | 12.0±1.0 | 15.0±0.1 | 13.0±1.0            | 15.0±1.0  | 18.0±0.5 | 12.0±0.0              | 14.0±0.5 | 17.0±1.0 |
| <i>Vibrio cholerae</i> (Ogawa)  | 13.0±0.5             | 15.0±1.0 | 16.0±0.0 | 14.0±1.0            | 26.0±0.5  | 30.0±1.0 | 15.0±1.5              | 17.0±1.0 | 21±1.0   |
| <i>Vibrio cholerae</i> (Inaba)  | 13.0±2.0             | 14.0±1.0 | 15.0±0.0 | R                   | R         | R        | 11.0±1.5              | 13.0±0.5 | 16.0±0.0 |

\* Values represent mean zone sizes (in mm) of three experiments; R – Resistant

**Table -2**  
**Minimum Inhibitory Concentration (MIC\*) of the oils (µl/ml)**

| Test organisms             | Essential oils    |                     |                       |
|----------------------------|-------------------|---------------------|-----------------------|
|                            | <i>O. sanctum</i> | <i>O. basilicum</i> | <i>O. gratissimum</i> |
| <i>B. subtilis</i>         | 0.97              | 15.62               | 125                   |
| <i>E. coli</i> 0157:H7     | 125               | 0.97                | >250                  |
| <i>S. dysenteriae</i>      | >250              | 31.25               | >250                  |
| <i>S. aureus</i>           | 15.62             | 15.62               | 0.48                  |
| <i>V. cholerae</i> (Inaba) | 0.48              | 15.62               | >250                  |
| <i>V. cholerae</i> (0139)  | >250              | 0.48                | 0.48                  |

\* MIC was determined by tube dilution method, \*\*Maximum concentration tested

sizes were also reported against *B. subtilis* and *E. coli* in comparison to untreated oils. However, the zones were found to be increased for the autoclaved oil of *O. basilicum*, *O. gratissimum* than the normal untreated ones, while the zone size of inhibition decreases at 100°C

in comparison to the untreated oils against *B. subtilis*. Specific results were found for the *S. aureus* and *Vibrio cholerae* 0139 where complete inhibition was observed for the autoclaved oils of the three essential oils and their cocktail mixture on heating. *Vibrio cholerae* 0139



Table- 3  
Antibacterial activity heat treated and autoclaved *Ocimum* essentials oils

| Test organisms                | Zones of inhibition * |       |      |                     |       |      |                  |   |
|-------------------------------|-----------------------|-------|------|---------------------|-------|------|------------------|---|
|                               | <i>O. sanctum</i>     |       |      | <i>O. basilicum</i> |       |      | <i>O. gratis</i> |   |
|                               | Untreated             | 100°C | Auto | Untreated           | 100°C | Auto | Untreated        | 1 |
| <i>B. subtilis</i>            | 15                    | 11    | 10   | 18                  | 13    | >30  | >30              |   |
| <i>E. coli</i> (0157:H7)      | 26                    | 15    | 13   | 20                  | R     | R    | 18               |   |
| <i>S. dysenteriae</i>         | 13                    | R     | R    | 15                  | 15    | 17   | 18               |   |
| <i>S. aureus</i>              | Ci                    | 18    | Ci   | Ci                  | 20    | Ci   | Ci               |   |
| <i>Vibrio cholerae</i> (0139) | Ci                    | Ci    | Ci   | 13                  | 10    | 22   | 11               |   |

\* Oils were applied on discs at MIC level after heat treatment (Boiling water bath at 100°C for 1hr and autoclaving-auto); R- Resistance; Ci-Complete inhibition of the pathogens

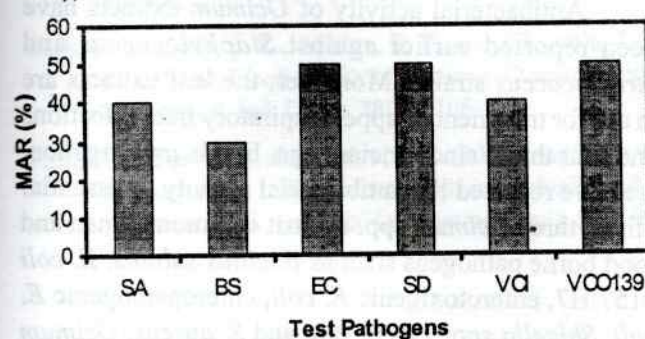


Fig. 1. Multiple antibiotic resistance (MAR) index of the test pathogens

SA: *S. aureus*; BS: *B. subtilis*; EC: *E. coli* 0157: H7; SD: *S. dysenteriae*; VCI: *V. cholerae* (Inaba); VCO139: *V. cholerae* O139

showed complete inhibition at MIC values of untreated, 100°C and autoclaved oils of *O. sanctum* while, the zone size of inhibition increases from untreated to 100°C to autoclaved oils of *O. basilicum* & *O. gratissimum*. *V. cholerae* (Inaba) showed decreased zone sizes in comparison i.e. highest with normal and lowest with autoclaved oils of *O. basilicum*, *O. gratissimum* and the reverse was found in case for *O. sanctum*. Gupta *et al.* (2004), also reported the persistence antibacterial activity of carrot (*Daucus carota*) and celery (*Apium graveolens*) seed essential oils against both Gm+ve and Gm-ve pathogens after heat treatment and autoclaving, as reported in our investigation. Similar results were also reported by Rath *et al.* (2005) while studying the antibacterial activity of lime (*Citrus limonum*) and Juniper (*Juniperus communis*) oils against 32 strains of methicillin resistant *Staphylococcus aureus*.

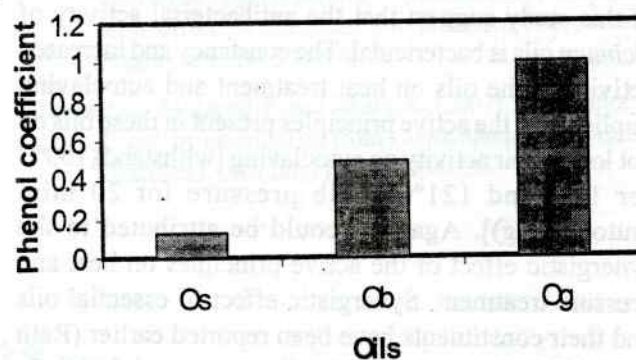


Fig. 2. Phenol Co-efficient value of the oils  
Os : *Ocimum sanctum*; Ob: *Ocimum basilicum*;  
Og: *Ocimum gratissimum*

From the antibiogram pattern it was reported that all the pathogens were susceptible to chloramphenicol, ciprofloxacin, norfloxacin, tetracycline and polymyxin, whereas, all the pathogens were resistant to bactracin, ampicillin, trimethoprim and sulfadiazin. The Multiple Antibiotic Resistant (MAR) index ranged from 40-50% (Fig. 1), which is of great concern in the sector of drug resistance. The phenol co-efficient value of the oils and the cocktail mixture ranged between 0.125-1.0 (Fig. 2). *O. gratissimum* oil showed highest phenol co-efficient i.e. 1.0, *O. basilicum* oil 0.5 and the least phenol co-efficient was recorded with *O. sanctum* oil. These findings corroborate with our earlier observations in this investigation during preliminary screening by Disc Diffusion Method and Minimum Inhibitory Concentration determination. The phenol coefficient values observed to be statistically significant ( $p < 0.05$ ).



## Discussion

The results of this study suggest that the seed essential oils of *O. sanctum*, *O. basilicum* and *O. gratissimum* possess antibacterial activities. Bacterial susceptibility towards the oils was observed at 2.5 µl/disc, but higher concentrations showed larger zones of inhibition when tested by agar plate technique. The results were highly significant for all the treatments, MICs and phenol coefficient values, and the activities are well comparable with standard antibiotics. In general there seemed to be an over all agreement between size of inhibition zones and MICs i. e. larger zones of inhibition correlated with lower MIC values. Further, the results of this study suggest that the antibacterial activity of *Ocimum* oils is bactericidal. The constancy and increased activity of the oils on heat treatment and autoclaving implies that the active principles present in these oils do not lose their activity on autoclaving [withstand (100°C for 1 hr) and 121°C, 15 lb. pressure for 20 min, (autoclaving)]. Again, it could be attributed to the synergistic effect of the active principles on heat and pressure treatment. Synergistic effect of essential oils and their constituents have been reported earlier (Rath *et al.* 2002; Gupta *et al.* 2004; Senatore *et al.* 2004). In certain instances decrease in activity could be attributable to the loss of specific active components of the oils on heating and autoclaving. Rath *et al.* (2001) reported reduction in antibacterial activity with heat treated turmeric leaf and rhizome oil against *E. coli*.

From the antibiogram studies it is well observed that the test pathogens revealed a high degree of multiple antibiotic resistance index (MAR%) which is of great concern, for development and spreading of drug resistance among pathogens. But, it is pertinent to mention that the antibacterial activity in terms of zones of inhibition produced by these oils against the test pathogens is comparable with the zones of sensitivity produced by standard antibiotics used in this investigation. In certain cases, lower zones of inhibition produced by the oils in comparison to antibiotics could be attributable to the crudeness or impurities of the active ingredients present in these oils. Susceptibility of the strains to polymyxin-B, further, indicates that, the possible mode of action of the oil may be due to inhibition of cell membrane synthesis, specifically inhibiting the membrane proteins. Shenhaji *et al.* (2007) observed the antibacterial activity of essential oil from *Cinnamum zeylanicum* against *E. coli*

0157:H7 is through outer membrane disintegration and increasing the permeability to ATP through cytoplasmic membrane. Similarly, Rath *et al.* (2005) also reported the anti staphylococcal activity of Juniper and Lime essential oils against methicillin resistant *Staphylococcus aureus* (MRSA) through inhibition of cell membrane synthesis that corroborates with the findings observed in this investigation. The antibacterial activity of essential oils through membrane inhibition could be attributable to the hydrophobicity of essential oils that enables them to make partitions in the membrane, rendering permeability and leading to leakage of cell contents resulting in death of microbial cells.

Antibacterial activity of *Ocimum* extracts have been reported earlier against *Staphylococcus* and *Streptococcus* strains. Moreover, the leaf extracts are in use for treatment of upper respiratory tract infections and soar throat since ancient age. In this investigation, we have reported the antibacterial activity of essential oils of three *Ocimum* spp. against common human and food borne pathogens such as *Bacillus subtilis*, *E. coli* 0157:H7, enterotoxigenic *E. coli*, enteropathogenic *E. coli*, *Shigella* spp, *Vibrio* spp. and *S. aureus*. *Ocimum* essential oils being edible, having antibacterial properties at low concentrations, and possessing antioxidant properties (Hakkim *et al.* 2008) they hold a promise as to expensive, harmful chemical preservatives against the aforesaid pathogens. Besides their possible use in food industries as natural food preservatives can be well predictable. Of course, further studies are highly necessary especially to examine the toxicity of these oils, if any, in order to recommend an appropriate formulation for this purpose.

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## Structure, Composition and Diversity of Tree Species in Tropical Deciduous Forests of Keonjhar District, Orissa

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The floristic and structural analysis of tree diversity of Keonjhar district, Orissa, India was carried out based on phytosociological studies. One hundred and fifty four woody species belonging to 43 families were recorded, of which 151 were tree species with = 15 cm girth at breast height. The forest is of tropical deciduous type with the dominance of *Shorea robusta* (Dipterocarpaceae). The Shannon-Weiner index ( $H'$ ) of 5.12 infer high species diversity, however, only a few species had more number of individuals as compared to the other species. *Shorea robusta* is wide niched which shows Importance Value Index of 71.76 followed by *Terminalia alata* (19.13), *Syzygium cumini* (12.01), *Anogeissus latifolia* (11.55). Stem density and species richness have consistently decreased with increasing girth class of tree species. The highest number of species are encountered in the low gbh classes (30-60 cm).

**Key words:** Deciduous forest, Keonjhar District, phytosociological studies, tree species.

### Introduction

Vegetation ecology includes the investigation of species composition and sociological interaction of species in communities (Mueller-Dombois and Ellenberg, 1974). The structural property of a community is the quantitative relationship in between the species growing around. The quantitative study of vegetation is called 'phytosociology' and its principal aim is to describe the vegetation, explain or predict its pattern and classify it in a meaningful way (Ilorkar and Khatri, 2003). It indicates species diversity which determines the distribution of individuals among the species in a particular habitat. With increase in human activity in and around forest ecosystems, biodiversity in terms of numbers of species may decline (Swaine and Hall 1983). A sound understanding of the richness of species is necessary for appropriate conservation and restoration of the biological diversity.

Tropical deciduous forests are enriched with economically important species. Vegetation composition, diversity of species and their habitats are well understood for other tropical forest types compared to dry deciduous forests. Deciduous forests are among the most exploited and endangered ecosystems of the biosphere (Janzen, 1988).

In Eastern Ghats of India, few quantitative phytodiversity inventories are available from the forests of Eastern Ghats of Tamil Nadu (Kadavul and Parthasarathy, 1999a,b; Jayakumar *et al.*, 2002; Natarajan *et al.*, 2004). These kinds of studies are poorly made in the State of Orissa, which covers the northern part of Eastern Ghats (Mishra, 2002; Reddy *et al.*, 2007).

Hence, the present study was undertaken to determine the structure and floristic composition in tropical forests of Eastern Ghats of Orissa, India with special reference to Keonjhar district.

### Study area

The district of Keonjhar lies between 21° 01' to 22° 10' N latitude and 85° 10' to 86° 22' E longitude. East of Greenwich, bounded by Singhbhum district of Jharkhand and Mayurbhanj district of Orissa on the North, both Mayurbhanj and Balasore districts of Orissa on East, Jajpur and Dhenkanal districts on the south and Anugul and Sundargarh district of Orissa on the West.

The area of district is 8303 sq.km, with 150 kilometers length of North-South while the average width of East-West is 66 kilometers. The average elevation in its central part is about 500 m. The Baitarani river takes its rise in the hilly north-western division. The rocks of

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older metamorphics comprising of schistose and gneissose rocks are confined to champua area. These rocks were later introduced by ultra basics and Singhbhum-Kendujhar granites. Important minerals available in huge quantity in the district are Iron-ore, Manganese and Chromites (<http://keonjhar.net>).

The climate of Keonjhar is characterized by hot summer, high humidity and well distributed rainfall during the monsoon season. The mean average annual rainfall (5 yrs.) of the district is 113.15 cm. The hot season commences by the beginning of March when temperature begins to rise rapidly. May is the hottest month when the mean daily maximum temperature is about 38.2 °C, and the mean daily minimum temperature is 24.6 °C. The relative humidity is generally high i.e. about 75% in the south-west monsoon and post monsoon months. In other months, the afternoons are comparatively drier. Mean relative humidity is maximum in September (87%) and minimum in April (54%) during 2005-06 (Behuria, 1986).

## Results

### Floristics

During the present study as many as 154 woody elements under 103 genera belonging to 43 families have been reported. Majority of families are represented by one or two species only. Among 43 families, the most dominating family is Rubiaceae which contains 20 species followed by Euphorbiaceae (15 species); Fabaceae (10 species); Moraceae (9 species); Caesalpiniaceae (8 species); Mimosaceae and Flacourtiaceae (7 species).

The predominant forest type of the Keonjhar district is northern tropical moist deciduous forest (Champion and Seth, 1968). Total 210 sample plots (8.1 hectare) represented by 154 woody species, which contains 151 tree species. Species accumulation curve was flattened after 160 sample plots indicate the adequate sampling to cover tree diversity of Keonjhar district (Fig.1).

The predominant tree species are *Shorea robusta*, *Terminalia alata*, *Syzygium cumini*, *Anogeissus latifolia*, *Schleichera oleosa*, *Xylia xylocarpa*, *Madhuca indica* etc. The top twenty predominant species with their IVI values are given in Table 1. While top most 20 tree species represents 73.57% of individuals.

Representation of 154 species and the high proportion of few species in the study area can directly

be attributed to the favourable climatic and edaphic conditions to particular species (Table 1). Species rarity (those represented by <2 individuals) of 21.43% was obtained in the present study area. It is lower as compared to Similipal of 34% rarity (Reddy *et al.*, 2007). The Shannon-Weiner index ( $H'$ ) is 5.12, with Simpson's value 0.34. These values infer that tropical forests in Keonjhar district are species diverse systems.

### Forest structure

With respect to IVI both in tropical moist deciduous and tropical dry deciduous type of vegetation. Dipterocarpaceae is represented by a single species i.e. *Shorea robusta*, which is not a species rich family. The high density, frequency and basal area of *Shorea robusta* contributes to a higher IVI. Thus superceding the species rich family Rubiaceae and Euphorbiaceae in tropical mixed deciduous forest comprising both moist deciduous, dry deciduous and semi-evergreen types of vegetation.

Among the tree species relative density is highest in *Shorea robusta* (30.21) followed by *Terminalia alata* (6.66), *Anogeissus latifolia* (4.31), *Xylia xylocarpa* (4.10), *Syzygium cumini* (3.35), etc. The lowest relative density is observed in *Miliusa tomentosa* (0.01), *Vitex leucoxylon* (0.01), *Acacia pennata* (0.01), *Chionanthus intermedius* (0.01) etc.

Relative dominance is highest in *Shorea robusta* (33.71) followed by *Terminalia arjuna* (7.92), *Terminalia alata* (6.20), *Madhuca indica* (5.49), *Syzygium cumini* (4.33) etc. Lowest relative dominance is observed in *Miliusa tomentosa*, *Vitex leucoxylon*, *Acacia pennata*, *Chionanthus intermedius* etc.

Important value index (IVI) is highest in *Shorea robusta* (71.76) followed by *Terminalia alata* (19.13), *Syzygium cumini* (12.01), *Anogeissus latifolia* (11.55), *Terminalia arjuna* (11.21), etc. Lowest IVI is observed in *Miliusa tomentosa* (0.07), *Vitex leucoxylon* (0.07), *Acacia pennata* (0.07), *Chionanthus intermedius* (0.07) etc.

Due to difference in microclimatic conditions like riparian habitats, specific elements like *Terminalia arjuna*, *Vitex leucoxylon*, *Trema orientalis*, *Homonoia riparia* are noticed.

Mean stem density was 551 per ha trees. In the case of Similipal the mean stem density was 568 per ha



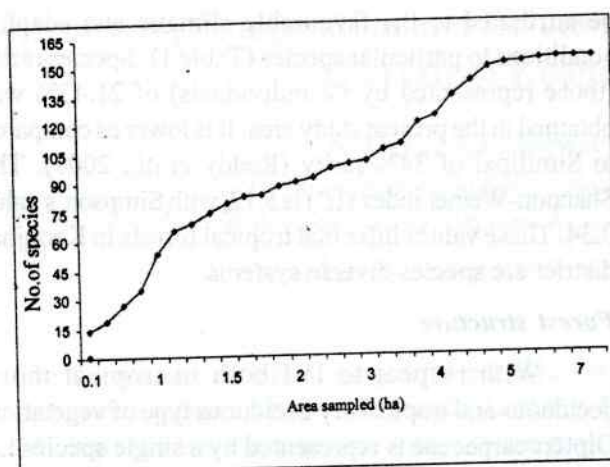


Fig. 1. Species Area (species accumulation) curve for Forests of Keonjhar district.

minority of species dominate the majority of the available resources. The highest GBH was measured in the case of *Bombax ceiba* (580 cm) followed by *Shorea robusta* (550 cm), *Artocarpus lacucha* (541 cm), *Mangifera indica* (538 cm) and *Syzygium cumini* (510 cm).

The mean tree height is 13 m, with a height range from 3 to 30 m. The tallest individual trees were *Bombax ceiba* (30 m), *Shorea robusta* (30 m), *Artocarpus lacucha* (28 m), *Mangifera indica* (27 m) and *Syzygium cumini* (26 m). Considerable number of Tree species show tendency towards shorter and medium stature (22.41% of individuals are with less than 5 m height) due to edaphic conditions.

Table-1  
IVI of the twenty most important species of Keonjhar district.

| Sl. No. | Species                         | Relative Density | Relative Frequency | Relative Dominance | IVI   |
|---------|---------------------------------|------------------|--------------------|--------------------|-------|
| 1       | <i>Shorea robusta</i>           | 30.21            | 7.84               | 33.71              | 71.76 |
| 2       | <i>Terminalia alata</i>         | 6.66             | 6.26               | 6.20               | 19.13 |
| 3       | <i>Syzygium cumini</i>          | 3.35             | 4.33               | 4.33               | 12.01 |
| 4       | <i>Anogeissus latifolia</i>     | 4.31             | 3.62               | 3.63               | 11.55 |
| 5       | <i>Terminalia arjuna</i>        | 2.22             | 1.07               | 7.92               | 11.21 |
| 6       | <i>Schleichera oleosa</i>       | 2.32             | 2.90               | 5.47               | 10.69 |
| 7       | <i>Xylia xylocarpa</i>          | 4.10             | 3.16               | 1.91               | 9.16  |
| 8       | <i>Madhuca indica</i>           | 2.62             | 3.36               | 3.04               | 9.02  |
| 9       | <i>Mangifera indica</i>         | 1.46             | 1.83               | 5.49               | 8.78  |
| 10      | <i>Buchanania lanzan</i>        | 3.23             | 3.87               | 1.42               | 8.52  |
| 11      | <i>Diospyros melanoxylon</i>    | 2.86             | 3.77               | 1.68               | 8.31  |
| 12      | <i>Lagerstroemia parviflora</i> | 1.46             | 2.44               | 0.66               | 4.56  |
| 13      | <i>Bridelia airy-shawii</i>     | 1.44             | 1.78               | 1.33               | 4.56  |
| 14      | <i>Boswellia serrata</i>        | 1.25             | 1.63               | 1.50               | 4.38  |
| 15      | <i>Lannea coromandelica</i>     | 1.31             | 1.78               | 0.74               | 3.83  |
| 16      | <i>Bombax ceiba</i>             | 0.40             | 0.97               | 2.08               | 3.45  |
| 17      | <i>Protium serratum</i>         | 1.06             | 1.07               | 1.18               | 3.31  |
| 18      | <i>Cleistanthus collinus</i>    | 1.44             | 1.43               | 0.31               | 3.18  |
| 19      | <i>Morinda pubescens</i>        | 1.01             | 1.83               | 0.29               | 3.14  |
| 20      | <i>Diospyros montana</i>        | 0.85             | 1.58               | 0.68               | 3.11  |

for trees. The mean stand density of 551 stems per ha is well within the range of 276 - 905 stems per ha reported for trees  $\geq 30$  cm gbh in the tropics (Reddy *et al.*, 2007).

The mean basal area was 44.3 m<sup>2</sup>/ha. The distribution of the basal area across gbh interval classes reveals the dominance of small stemmed individuals in the plot (table 2). The mean diameter of top 20 dominant tree species covers 83.5% of ground cover. It means

In terms of the overall ecological dominance within plots, the high importance value species (IVI value of 71.76) is *Shorea robusta*. It is commonly found (wide niched) throughout the forests.

The basal area and vertical structure of a forest is difficult to summarise as these rely heavily upon the climate and topographic conditions. Tree heights are heavily influenced by the abundance of samplings,



**Table-2**  
Population density of tree species across Girth Class intervals

| Sl. No. | GBH class (cm)     | Number of Species | % of Species | Total Individuals | % of Individuals |
|---------|--------------------|-------------------|--------------|-------------------|------------------|
| 1       | <30                | 98                | 63.64        | 975               | 21.29            |
| 2       | 30-60              | 119               | 77.27        | 1657              | 36.19            |
| 3       | 61-90              | 79                | 51.30        | 1101              | 24.04            |
| 4       | 91-120             | 52                | 33.77        | 394               | 8.60             |
| 5       | 121-150            | 48                | 31.17        | 223               | 4.87             |
| 6       | 151-180            | 29                | 18.83        | 100               | 2.18             |
| 7       | 181-210            | 12                | 7.79         | 74                | 1.62             |
| 8       | >210               | 7                 | 4.55         | 55                | 1.20             |
|         | <b>Grand total</b> | <b>154</b>        | <b>100</b>   | <b>4579</b>       | <b>100</b>       |

**Table-3**  
Height class wise proportion of tree individuals

| Sl. No. | Height class (Meters) | Number of Species | % of Species | Total Individuals | % of Individuals |
|---------|-----------------------|-------------------|--------------|-------------------|------------------|
| 1       | <5m                   | 109               | 70.78        | 1026              | 22.41            |
| 2       | 5-10                  | 122               | 79.22        | 1629              | 35.58            |
| 3       | 10-15                 | 104               | 67.53        | 1074              | 23.45            |
| 4       | 15-20                 | 74                | 48.05        | 516               | 11.27            |
| 5       | >20                   | 59                | 38.31        | 334               | 7.29             |
|         | <b>Grand Total</b>    | <b>154</b>        | <b>100</b>   | <b>4579</b>       | <b>100</b>       |

richness of nutrients and anthropogenic pressure (since forest fires are recurrent).

Stem density and species richness have consistently decreased with increasing girth class of tree species from 30-60 cm girth category. The highest number of species are encountered in the low gbh classes (30-60 cm) (Table 3). Girth class having 10-30 cm gbh contributed to about 21.29% of species richness.

Girth class frequency showed reverse J-shaped population structure of trees exhibited in all the three

study sites and are in conformity with other forest stands in Eastern Ghats such as Shervarayan hills (Kaduvul and Parthasarathy, 1999a) and Kalrayan hills (Kaduvul and Parthasarathy, 1999b).

### Discussion

The growing demand of increasing population has created various disturbances in the existing forests resulting in the loss of biodiversity and soil erosion. Various threats to loss of biodiversity are fragmentation of habitat, loss of habitats, climate change, soil erosion,



biotic interference at various magnitude are immensely affecting the forest ecosystem. Shifting cultivation practiced by some tribes of Keonjhar is one of the major anthropogenic pressure on biodiversity.

The study was aimed at gathering information in relation to structure and function of tropical forests of Keonjhar. It was based on the quantitative information of woody elements chiefly 151 trees and 3 woody climbers with special reference to density, frequency, abundance, distribution pattern and important value index.

From the analytical point of view, it is observed that tree species richness is lower as compared to species recorded in Similipal biosphere reserve (Reddy *et al.*, 2007). It may be due to anthropogenic pressure in forests of Keonjhar and/ or low rainfall.

The tree species with higher IVI values indicate their good regenerating capability, more adaptability and wide ecological amplitude. In the present study *Shorea robusta* shows wide adaptability and good regenerating ability in different ecological conditions.

It is imperative to study the existing forest in relation to structure and function of forest ecosystem. Phytosociological study helps to understand the ecological significance of species. The present study will serve as a primary input towards monitoring and sustaining the plant diversity of tropical forests in the State of Orissa.

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## Assessment of Floristic Diversity in the Harishankar Protected Forest Area of Gandhamardan Hill Ranges Using Shannon- Wiener Index

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Survey and documentation of the floristic wealth of a region is a prerequisite for planning proper utilization of its potential plant resources on one hand and to conserve the depleting genetic resources on the other. Population structure and dispersion pattern of all trees, seedlings, shrubs, climber, twinner and herbs were surveyed in the *in situ* preserved forest plot at Harishankar of Gandhamardan Hill ranges in varying altitude( 365.45 m to 561.441 m) and slope( 10% to 85%) gradients from January to June, 2008. In total 58 plant taxa were documented in which 40 tree, 8 shrub, 2 climber, 1 twinner and 7 herb species were present. Besides 35 seedling species were found during the study. The probability of occurrence of highest and lowest number of species of tree, seedling, shrub, climber, twinner and herb at a time in a sample quadrat are 21, 13, 3, 3, 1 & 3 and 7, 1, 1, 1, 1 & 1 respectively. Phytosociological characteristics of all tree species have been analysed. Frequency ranges from 3.33 to 93.33, density from 0.03 to 5.1, dominance from 0.00000031523 to 0.00005006423, abundance from 1.0 to 5.46 and IVI value from 0.616658676 to 34.49630147 amongst different species. Shannon-Wiener diversity index ( $H'$ ) varies between 0.00552614008 (*Ficus benghalensis*) and 0.108048339 (*Shorea robusta*) where as 1.428292243 represents the whole tree community. Higher is this value, greater is the species richness. The present study provides information on species richness and diversity for sustainability of this biodiversity hub of Orissa in the wake of indiscriminate bioresource exploitation and forest fire.

**Key Words:** Floristic diversity, Gandhamardan hill ranges, Shannon-Wiener index

### Introduction

Study of plant species diversity is an important aspect of forest ecology. It reflects the status and contribution of various species in structure and function of ecosystem. Floristic diversity studies particularly in and around protected area serve as base line information and would be useful in predicting the future course of succession and habitat conditions and also indicates the level of disturbance. Study of plant species composition and diversity has been widely accomplished in order to perform conservation, effective management and logical exploitation of forests (Lovett *et al.*, 2000; Andel, 2001; Chiarucci *et al.*, 2001; Nebel *et al.*, 2001; Parthasarathy, 2001; Aubert *et al.*, 2003 and Huang *et al.*, 2003).

Quantitative descriptions of diversity have been of two kinds, one dealing with Alpha, Beta and Gamma diversities which describes the local diversity, species turnover and the size of species pool in large region

respectively (Whittaker, 1972) and the other dealing with the different type of diversity indices i.e. Shannon-Wiener index, Simpson's index etc which attempt to summarise the information on the number of species together with that of their abundance in a single number (Pielou, 1975; Ludwig and Reynolds, 1988).

The study of the forest of Gandhamardan hill ranges has attracted the attention of many botanists, ecologists and plant explorers because of its unique geography, biodiversity and phytosociological aspects. A number of floristic works have been carried out by many plant explorers like Mooney (1950), Haines (1921; 1925), Panigrahi *et al.* (1964), Misra (1990, 1998, 2004), Misra and Das (1998), Saxena and Brahmam (1996) in the forest of Gandhamardan hill ranges which is the homeland of many economically important species. The present study is focused on status of vegetation

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experiencing different degrees of disturbance as well as phytosociological aspects of the tree community.

### Materials and Methods

**Study area:** Gandhamardan Hill ranges lies between 20° 42' to 21° 00' N latitude and 82° 41' to 83° 05' E longitude in the North-West of Bolangir and South-West of Baragarh district, Orissa, India. The hill ranges contain an undulating mountain system ranging from 320 m to 1220 m height from mean sea level (MSL) which extends over several miles in north-east and south-west direction and receives annual rainfall ranging from 750mm to 1600mm.

**Field sampling:** The field study period extended over the months of January- June 2008. The size and number of quadrates were determined by species area curve method. The numbers of quadrates were determined by plotting number of quadrate against the number of species (Mishra, 1968). Total 30 sample quadrates of 20m×20m for trees and 5m×5m for shrubs, climbers, twinners and herbs were taken inside the 100ha protected forest area covering its whole geography. All the plots were recorded for trees greater than or equal to 15cm GBH (Marimon *et al.*, 2002 and Mishra *et al.*, 2005).

### Data analysis

The data recorded was quantitatively analysed for frequency, density and dominance and the relative values of these parameters were calculated and summed up for Importance Value Index (IVI) following the recent methods (Curtis, 1959 and Mishra, 1968). Abundance to Frequency ratio (A/F) of each tree species was determined to get distribution pattern of various species as indicates, regular (0.025), random (0.025-0.050) and contiguous (0.050) distribution (Curtis and Cotton, 1956). Species were identified following the standard procedure by the help of regional and national flora (Saxena and Brahmam, 1996 and Hooker, 1872-97) and Herbarium of IMMT, Bhubaneswar was consulted for reconfirmation. Measurement of species diversity and richness was done using parametric and non parametric methods (Krebs, 1999; Magurran, 2004). Non parametric methods are extensively used to quantify species diversity since these methods have no need for statistical distributions, in other words, they are free distribution and their calculations are simply conducted (Brockway, 1998; Aubert *et al.*, 2003) Beta diversity was calculated

by formula given by Whittaker (1975). Shannon-Wiener index has been used to measure species diversity and compared with Simpson's value.

Importance Value Index (IVI) = Relative Frequency + Relative density + Relative dominance

Beta(β) diversity =  $S_c/S$

Where ;  $S_c$  is total number of species occurring in a set of sample counting each species only once whether or not it occur more than once, and  $S$  is average number of species per individual sample.

Shannon-Wiener index ( $H'$ ) =  $-\sum P_i \log P_i$ , where  $P_i = n_i / N$

Simpson's index =  $1 - [\sum (n_i/N)^2]$

Where:

$n_i$  = Importance value of species

$N$  = Sum of importance values of all the species.

### Results and Discussion

Vegetation profile differs from site to site along stream lines, valleys and altitudes. The forest type falls under Tropical Deciduous (Champion and Seth, 1968). All fifty eight plant species belonged to 55 genera and 32 families. Forty tree species fall in to 37 genera and 22 families, eight shrubs fall into 8 genera and 8 families, seven herbs fall into 7 genera and 6 families, two climbers fall into 2 genera and 2 families and one twinner fall into 1 genera as well as one family. Quantitative assessment of forest vegetation is essential due to various human activities, the most significant being exploitation of natural resources and tree felling. This had resulted in the depletion of habitat as well as population of plants. Among all forty tree species found (Table 1) in the studied site ten most frequent ones in descending order according to their abundance are *Anogeissus latifolia*, *Diospyros melanoxylon*, *Pterocarpus marsupium*, *Buchanania lanzan*, *Cleistanthus collinus*, *Schleichera oleosa*, *Madhuca indica*, *Desmodium oojeinensis*, *Lagerstroemia parviflora*, *Dalbergia paniculata* and *Terminalia alata*. These ten species are the indicator series of the forest. Top ten species in descending order of density value are *Diospyros melanoxylon*, *Anogeissus latifolia*, *Cleistanthus collinus*, *Buchanania lanzan*, *Schleichera oleosa*, *Madhuca indica*, *Pterocarpus marsupium*, *Desmodium oojeinensis*, *Lagerstroemia parviflora* and



**Table-1**  
**Phytosociological attributes and distribution pattern of tree species.**

| Tree species                                                       | IVI   | A/F    | Distribution |
|--------------------------------------------------------------------|-------|--------|--------------|
| <i>Acacia lenticularis</i> Buch.-Ham.ex Benth.                     | 2.44  | 0.0750 | C            |
| <i>Aegle marmelos</i> (L.) Corr.                                   | 4.88  | 0.1219 | C            |
| <i>Anogeissus latifolia</i> (Roxb. ex DC.) Wall. ex Guill. & Perr. | 24.01 | 0.0550 | C            |
| <i>Bombax ceiba</i> L.                                             | 3.33  | 0.1501 | C            |
| <i>Bridellia retusa</i> (L.) Spreng.                               | 7.01  | 0.0512 | C            |
| <i>Buchanania lanzan</i> Spreng.                                   | 11.77 | 0.0346 | Ra           |
| <i>Careya arborea</i> Roxb.                                        | 2.77  | 0.0600 | C            |
| <i>Casearia elliptica</i> Willd.                                   | 3.16  | 0.0672 | C            |
| <i>Casearia graveolens</i> Dalz.                                   | 2.13  | 0.2252 | C            |
| <i>Cassia fistula</i> L.                                           | 3.61  | 0.0857 | C            |
| <i>Chloroxylon swietenia</i> DC.                                   | 1.04  | 0.1501 | C            |
| <i>Cleistanthus collinus</i> (Roxb.) Benth.ex Hook .f.             | 18.57 | 0.0762 | C            |
| <i>Dalbergia latifolia</i> Roxb.                                   | 0.76  | 0.3003 | Ra           |
| <i>Dalbergia paniculata</i> Roxb.                                  | 8.67  | 0.0281 | Ra           |
| <i>Desmodium oojeinensis</i> (Roxb.) Ohashi                        | 10.16 | 0.0382 | Ra           |
| <i>Diospyros melanoxylon</i> Roxb.                                 | 24.31 | 0.0585 | C            |
| <i>Eriolaena hookeriana</i> Wight & Arn.                           | 1.81  | 0.3003 | Ra           |
| <i>Ficus benghalensis</i> L.                                       | 0.61  | 0.3003 | Ra           |
| <i>Flacoutia indica</i> (Burm.f.) Merr.                            | 1.89  | 0.1501 | C            |
| <i>Gardenia latifolia</i> Ait.                                     | 3.01  | 0.0937 | C            |
| <i>Haldinia cordifolia</i> (Roxb.) Ridsdak                         | 13.55 | 0.0332 | Ra           |
| <i>Lagerstroemia parviflora</i> Roxb.                              | 8.99  | 0.0331 | Ra           |
| <i>Lannea coromandelica</i> (Houtt.) Merr.                         | 7.20  | 0.0428 | Ra           |
| <i>Madhuca indica</i> Gmel.                                        | 12.55 | 0.0378 | Ra           |
| <i>Mitragyna parvifolia</i> (Roxb.) Korth.                         | 6.08  | 0.0406 | Ra           |
| <i>Morinda pubescens</i> Sm.                                       | 5.76  | 0.0444 | Ra           |
| <i>Phyllanthus emblica</i> L.                                      | 2.09  | 0.0203 | Re           |
| <i>Pterocarpus marsupium</i> Roxb.                                 | 15.50 | 0.0340 | Ra           |
| <i>Schleichera oleosa</i> (Lour.) Oken                             | 12.75 | 0.0340 | Ra           |
| <i>Schrebera swietenoides</i> Roxb.                                | 7.50  | 0.3003 | Ra           |
| <i>Semecarpus anacardium</i> L.f.                                  | 5.84  | 0.0352 | Ra           |
| <i>Shorea robusta</i> Gaertn.f.                                    | 34.49 | 0.0937 | C            |
| <i>Sterculia urens</i> Roxb.                                       | 0.94  | 0.3003 | Ra           |
| <i>Stereospermum chelonoides</i> (L.f.) DC.                        | 4.13  | 0.0672 | C            |
| <i>Syzygium cumini</i> (L.) Skeels                                 | 2.30  | 0.3003 | Ra           |
| <i>Tamilnadia uliginosa</i> (Retz.) Tirveng & Sastre               | 0.66  | 0.3003 | Ra           |
| <i>Terminalia alata</i> Heyne ex Roth                              | 10.92 | 0.0328 | Ra           |
| <i>Terminalia bellirica</i> (Gaertn.) Roxb.                        | 3.78  | 0.0580 | C            |
| <i>Wrightia arborea</i> (Dennst.) Mabb.                            | 2.10  | 0.0600 | C            |
| <i>Ziziphus xylopyrus</i> (Reitz.) Willd.                          | 6.57  | 0.0351 | Ra           |

Note: Re: Regular; Ra: Random; C: Contiguous



**Table-2**  
**Tree population structure across girth class intervals**

| GBH Class(cm) | No of species | % of species | No of individuals | % of individuals |
|---------------|---------------|--------------|-------------------|------------------|
| 15-25         | 32            | 80           | 377               | 38.58            |
| 25-35         | 31            | 77.5         | 172               | 17.60            |
| 35-45         | 29            | 72.5         | 92                | 9.41             |
| 45-55         | 17            | 42.5         | 43                | 4.40             |
| 55-65         | 15            | 37.5         | 29                | 2.96             |
| 65-75         | 6             | 15           | 13                | 1.33             |
| 75-85         | 12            | 30           | 15                | 1.53             |
| 85-95         | 12            | 30           | 19                | 1.94             |
| 95-105        | 10            | 25           | 18                | 1.84             |
| 105-115       | 7             | 17.5         | 12                | 1.22             |
| 115-125       | 9             | 22.5         | 24                | 2.45             |
| 125-135       | 12            | 30           | 22                | 2.25             |
| 135-145       | 9             | 22.5         | 16                | 1.63             |
| 145-155       | 10            | 25           | 28                | 2.86             |
| 155-165       | 8             | 20           | 19                | 1.94             |
| 165-175       | 6             | 15           | 11                | 1.12             |
| 175-185       | 3             | 7.5          | 6                 | 0.61             |
| 185-195       | 2             | 5            | 2                 | 0.20             |
| 195-205       | 6             | 15           | 14                | 1.43             |
| 205-215       | 4             | 10           | 9                 | 0.92             |
| 215-225       | 4             | 10           | 9                 | 0.92             |
| 225-235       | 5             | 12.5         | 9                 | 0.92             |
| 235-245       | 2             | 5            | 3                 | 0.30             |
| >245          | 7             | 17.5         | 15                | 1.53             |

*Terminalia alata*. The top ten dominant tree species in descending order are *Shorea robusta*, *Schrebera swietenoides*, *Haldinia cordifolia*, *Pterocarpus marsupium*, *Terminalia alata*, *Madhuca indica*, *Mitragyna parviflora*, *Bombax ceiba*, *Bridellia retusa* and *Anogeissus latifolia*. Ten top species according to their abundance values in descending order are *Cleistanthus collinus*, *Diospyros melanoxylon*, *Anogeissus latifolia*, *Aegle marmelos*, *Buchanania lanzan*, *Schleichera oleosa*, *Madhuca indica*, *Desmodium oojenensis*, *Cassia fistula* and *Lagerstroemia parviflora*. Top ten species according to IVI value in descending order are *Shorea robusta*, *Diospyros melanoxylon*, *Anogeissus latifolia*, *Cleistanthus collinus*, *Pterocarpus marsupium*, *Haldinia cordifolia*, *Schleichera oleosa*, *Madhuca indica*, *Buchanania lanzan* and *Terminalia alata*.

Out of forty tree species, twenty two species are randomly, only one species (*Phyllanthus emblica*) is regularly and seventeen species are contiguously distributed (Table 1). The prevalent random distribution of tree species indicates growing discontinuity and isolation of the different forest patches. Population structure in the girth classes depicts good regeneration but with increase of girth class the tree population declines (Table 2 and Fig. 1). This confirms to the biotic as well as abiotic interference in the growing stage of seedlings and saplings. One of the major constraints of regeneration in this forest is identified to be rab burning, in which all the vegetation on the forest floor is burnt. Rab burning is just before the onset of the monsoon, where by most of the viable seeds of tree species on the forest floor and young saplings are destroyed. The forest fire also removes humus from the forest floor, which



Table-3

List of shrubs, herbs, climbers and twinner found.

|          |                                                            |
|----------|------------------------------------------------------------|
| Shrubs   | 1. <i>Acacia torta</i> (Roxb.) Craib                       |
|          | 2. <i>Combretum roxburghii</i> Spreng.                     |
|          | 3. <i>Cryptolepis buchananii</i> Roem. & Schult.           |
|          | 4. <i>Dendrocalamus strictus</i> (Roxb.) Nees              |
|          | 5. <i>Grewia hirsuta</i> Vahl.                             |
|          | 6. <i>Helicteres isora</i> L.                              |
|          | 7. <i>Indigofera cassioides</i> Rottl. ex DC.              |
|          | 8. <i>Ziziphus oenoplia</i> (L.) Mill.                     |
| Herbs    | 1. <i>Andrographis paniculata</i> (Burm. f.) Wall. ex Nees |
|          | 2. <i>Asparagus racemosus</i> Willd.                       |
|          | 3. <i>Byttneria herbacea</i> Roxb.                         |
|          | 4. <i>Curculigo orchoides</i> Gaertn.                      |
|          | 5. <i>Desmodium gangeticum</i> (L.) DC.                    |
|          | 6. <i>Guizotia abyssinica</i> (L. f.) Cass.                |
|          | 7. <i>Lepidagathis fasciculata</i> (Retz.) Nees.           |
| Climbers | 1. <i>Bauhinia vahlii</i> Wight & Arn.                     |
|          | 2. <i>Butea parviflora</i> Roxb.                           |
| Twinner  | 1. <i>Dioscorea alata</i> L.                               |

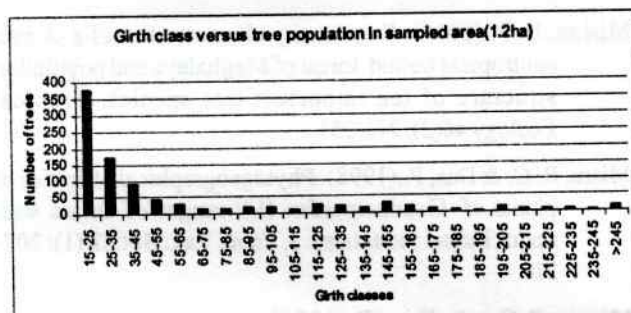


Fig. 1. Relationship between girth class and tree population in the protected forest area.

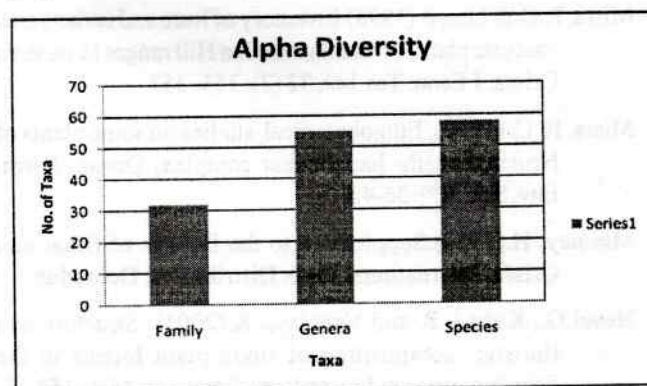
Fig. 2. Alpha ( $\alpha$ ) diversity of studied forest area.

Table-4

Alpha diversity of various vegetation.

| Total no of plant species found ( $\alpha$ diversity) |           |        |      |         |        |
|-------------------------------------------------------|-----------|--------|------|---------|--------|
| Tree                                                  | Seedlings | Shrubs | Herb | Climber | Twiner |
| 40                                                    | 35        | 8      | 7    | 2       | 1      |

Table-5

Beta diversity values

| Values of Beta ( $\beta$ ) diversity |           |        |       |        |         |
|--------------------------------------|-----------|--------|-------|--------|---------|
| Tree                                 | Seedlings | Shrubs | Herbs | Twiner | Climber |
| 3.06                                 | 6.52      | 7.54   | 13.20 | 5      | 2.73    |

severely hampers regeneration and makes the soil almost sterile.

Besides tree species, all shrub, herb, climber and twinner species are documented (Table 3). Total no of families, genera and species ( $\alpha$  diversity) recorded from the studied site are 32, 55 and 58 respectively (Fig. 2 and Table 4). Beta diversity is often been used to indicate habitat variation with regarded to species composition (Whittaker, 1977). The disturbed community can have as much more Beta diversity than an undisturbed community because of finer division of habitat. Within present study the values of Beta diversity for trees, seedlings, shrubs, herbs, climbers and twinner are given in Table 5. The higher value of Beta diversity in case of herbs indicates that this community is highly disturbed during the conducted study period. Destruction of established plants by disturbance may create recruitment microsites which allows the the community to be invaded by new species leading to increased species richness (Crawley, 1986).

Species richness has been evaluated based on IVI value using Shannon-Wiener and Simpson's diversity indices. This index is simply the information entropy of the distribution, treating species as symbols and their relative population sizes as the probability. The Shannon-Wiener index 1.428292243 of the tree community with Simpson's index of 0.921651916 indicates that tropical dry deciduous forest encompasses species diverse tree community.

Quantitative assessment of the tree community has been conducted for sustainable management of economically important species. The problem of forest fire, tree cutting and lopping, grazing and fuel wood extraction was observed inside the protected forest area which should be checked and must be given priority for



conservation. Higher Beta diversity value of seedlings, shrubs, herbs and climbers indicate that these communities are disturbed and Shannon-Wiener index value shows the tree community to be diversified.

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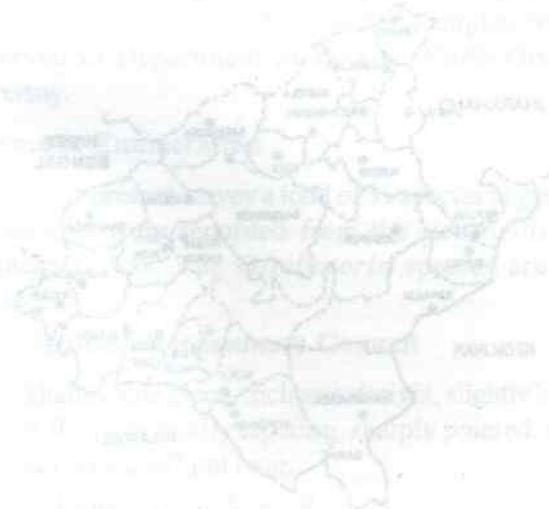


Fig. 1. District map of Odisha showing location of Harishankar protected forest area

1 = Bhubaneswar Block

2 = Cuttack Block

3 = Puri Block

4 = Khurda Block

5 = Balasore Block

6 = Jagatsipura Block

7 = Kendrapada Block

8 = Bargarh Block

9 = Deogarh Block

10 = Nuapada Block



# The Genus *Oscillatoria* Vaucher from Rice Field Soils of Northern Orissa

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An extensive study on abundance of *Oscillatoria* in the rice fields of Northern Orissa especially in Mayurbhanj district was carried out during September 2006 to December 2008. During our field survey altogether 31 species of *Oscillatoria* were recorded. *Oscillatoria chalybea* was the most abundant cyanobacteria followed by *O. subbrevis*, *O. chlorina*, *O. princeps* and *O. animalis* etc. This genus *Oscillatoria* was found to be one of the most abundant non-heterocystus cyanobacteria in the rice field soils of the region.

**Key words:** Abundance, Northern Orissa, *Oscillatoria*, rice fields.

## Introduction

In recent years, cyanobacteria an ubiquitous group of soil microorganisms, have been shown to be agriculturally important particularly in tropical rice fields. The agricultural importance of cyanobacteria in rice cultivation is directly related with their ability to fix nitrogen and other positive effects for plants and soils (Venkataraman *et al.*, 1974; Rai *et al.*, 2000; Whitton & Potts, 2000). Most of the heterocystous species are capable of fixing atmospheric nitrogen (De, 1939; Singh, 1961), however several non-heterocystous cyanobacteria are able to fix atmospheric nitrogen under microaerophilic condition (Fay, 1983). Cyanobacteria play an important role in maintenance and build-up of soil fertility, consequently increasing rice growth and yield as a natural biofertilizer. The paddy field ecosystem provides a favorable environment for growth of cyanobacteria with respect to their requirement for light, water, high temperature and nutrient availability. This could be the reason for more abundant cyanobacterial growth in paddy field soils than in upland soils (Roger and Reynaud, 1982, Kondo and Yasuda 2003).

The abundance of cyanobacteria in rice fields has been reported in numerous papers since Fritsch's accounts (Fritsch, 1907, a & b; Singh, 1961; Pandey, 1965; Tiwari, 1972; Padhy *et al.*, 1992; Goyal, 1995; Sahu *et al.*, 1996; Sen and Gupta 1998; Sen, 2000; Sahu 2000; Mishra and Pabbi, 2004; Board, 2004; Song *et al.*, 2005; Wilson, 2006; Rodriguez *et al.*, 2006; Nayak and Prasanna, 2007; Dey and Bastia, 2008; Digambar Rao *et al.*, 2008; Saadatnia and Riahi, 2009). The present investigation was carried out to survey and identify

different species of Genus *Oscillatoria* from the rice fields of Northern Orissa.

## Material and Methods

The study was conducted in the rice fields of Northern Orissa especially in Mayurbhanj district (Fig.1) during September 2006 to December 2008. The sampling was done randomly from paddy field soils during Kharif and Rabi crops with the help of forceps and needles. The collected samples were put into clean sampling bottles and polythene bags with assigned collection number, date and place of collection in field note book.



Fig.1. District map of Mayurbhanj showing block wise collection sites ( $S_1$  -  $S_{18}$ )

$S_1$  = Baripada Block

$S_2$  = Suliapada Block

$S_3$  = Betnoti Block

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- S<sub>4</sub> = Khunta Block  
 S<sub>5</sub> = Samakhunta Block  
 S<sub>6</sub> = Udala Block  
 S<sub>7</sub> = Kaptipada Block  
 S<sub>8</sub> = Morada Block  
 S<sub>9</sub> = Bisoi Block  
 S<sub>10</sub> = Kuliana Block  
 S<sub>11</sub> = Rasgobindpur Block  
 S<sub>12</sub> = Bangripasi Block  
 S<sub>13</sub> = Jashipur Block  
 S<sub>14</sub> = Thakurmunda Block  
 S<sub>15</sub> = Karanjia Block  
 S<sub>16</sub> = Sarat Block  
 S<sub>17</sub> = Raruan Block  
 S<sub>18</sub> = Badsahi Block

A part of each collected sample was preserved in 4% formaldehyde solution for microscopic observation and the other part cultured in freshly prepared BG11±N medium (Rippka *et al.*, 1979) incubated at 28±2 °C with illumination at 3000 Lux white continuous light. Temporary slides were prepared for each sample for identification. The species were identified basing on their morphological features like colour of thallus, cell shape and cell size following the monographs of Desikachary (1959), Anand (1989). All collected samples were preserved in Department of Botany, North Orissa University.

### Systematic Enumeration

In the present survey a total of 31 species of genus *Oscillatoria* were recorded from the rice fields of Northern Orissa. The *Oscillatoria* species are as follows.

1. *Oscillatoria acuminata* Gomont  
Thallus blue green, trichome straight, slightly bent at the ends briefly tapering, sharply pointed. 4-5 µm broad, 6-7 µm long.  
Collection site – S<sub>2</sub>, S<sub>6</sub>, S<sub>8</sub>, S<sub>9</sub>, S<sub>11</sub>
2. *Oscillatoria acuta* Bruhl et Biswas  
Trichomes either solitary or a number of them parallel to each other aggregated into bundles of moderate size, 5-6 µm thick.  
Collection site – S<sub>5</sub>, S<sub>6</sub>, S<sub>12</sub>, S<sub>14</sub>, S<sub>18</sub>
3. *Oscillatoria amoena* (Kutz.) Gomont  
Thallus more or less blue green, trichomes straight, slightly constricted at the crosswalls 3.5 – 4.5 µm broad, 2.5 – 3.5 µm long.  
Collection site – S<sub>3</sub>, S<sub>10</sub>, S<sub>15</sub>
4. *Oscillatoria amphibia* Ag. ex Gomont  
Thallus deep blue green, trichome straight or coiled, cells 2-3 time longer than broad, 3.5-4 µm broad, 7-8 µm long.  
Collection site- S<sub>3</sub>, S<sub>4</sub>, S<sub>8</sub>, S<sub>10</sub>, S<sub>12</sub>, S<sub>13</sub>, S<sub>18</sub>
5. *Oscillatoria animalis* Ag. ex Gomont  
Thallus dark, blue green, trichomes straight, not constricted at the cross walls, 3-3.5 µm broad, 2.5 – 3.5 µm long.  
Collection site – S<sub>3</sub>, S<sub>4</sub>, S<sub>8</sub>, S<sub>10</sub>, S<sub>12</sub>, S<sub>13</sub>, S<sub>18</sub>
6. *Oscillatoria boryana* Bory ex Gomont  
Trichome coiled screw like either fully or only at the apices, sometimes straight, cells 6-7 µm broad, 5-5.8 µm long, end cells rounded or more or less pointed.  
Collection site – S<sub>1</sub>, S<sub>6</sub>
7. *Oscillatoria chalybea* (Mertens) Gomont  
Thallus dark blue green, trichome straight or irregularly spirally coiled, 7-8 µm broad, 3-4 µm long.  
Collection site – S<sub>1</sub>, S<sub>2</sub>, S<sub>3</sub>, S<sub>4</sub>, S<sub>6</sub>, S<sub>8</sub>, S<sub>9</sub>, S<sub>10</sub>, S<sub>11</sub>, S<sub>18</sub>
8. *Oscillatoria chlorina* Kutz. ex Gomont  
Thallus very thin, yellowish green, trichomes straight or curved, 3-4 µm broad, 3.5-4 µm long.  
Collection site – S<sub>1</sub>, S<sub>3</sub>, S<sub>4</sub>, S<sub>12</sub>, S<sub>13</sub>, S<sub>17</sub>, S<sub>18</sub>
9. *Oscillatoria claricentrosa* Gardner  
Trichome nearly straight, 2.3 – 2.5 µm broad, 6-7 µm long.  
Collection site – S<sub>3</sub>, S<sub>5</sub>, S<sub>9</sub>
10. *Oscillatoria curviceps* Ag. ex Gomont  
Thallus light or dark blue green, trichome more or less straight, bent at the end or spirally coiled. 10-11 µm broad, 3-4 µm long.  
Collection site – S<sub>5</sub>, S<sub>7</sub>



11. *Oscillatoria earlei* Gardner

Trichome short, straight, curved or bent at the ends, 2-2.5  $\mu\text{m}$  broad, cells quadrate to 2.5 times longer than broad, end cells prominently pointed.

Collection site -  $S_1, S_8$ .

12. *Oscillatoria foreau* Fremy

Trichomes sparse, elongate, bent or suberect, apex gently curved, sub-falsiform, more or less torulose, 3  $\mu\text{m}$  broad, cells  $\frac{1}{2}$  as long as broad.

Collection site -  $S_3, S_{10}, S_{18}$

13. *Oscillatoria formosa* Bory ex Gomont

Thallus blue green, trichome straight, slightly constricted at the crosswalls, 4-4.5  $\mu\text{m}$  broad, 3.5-4  $\mu\text{m}$  long.

Collection site -  $S_6, S_9, S_{16}$ .

14. *Oscillatoria geitleriana* Elenkin

Trichome single, long flexible, blue green 2.5  $\mu\text{m}$  broad, 3-3.5  $\mu\text{m}$  long.

Collection site -  $S_2, S_4$ .

15. *Oscillatoria irrigua* (Kutz.) Gomont

Thallus blackish blue green, trichome light bluish purple, straight. 6-7  $\mu\text{m}$  broad, 4-4.5  $\mu\text{m}$  long.

Collection site -  $S_{10}, S_{17}$ .

16. *Oscillatoria laete-virens* (Crouan) Gomont

Thallus thin, membranous, green, trichome yellowish green, straight, 3-3.5  $\mu\text{m}$  broad, 2.5-3  $\mu\text{m}$  long.

Collection site -  $S_1, S_3, S_4, S_6$

17. *Oscillatoria limosa* Ag. ex Gomont

Thallus dark blue green, trichome straight, brown or olive green not constricted at the cross walls, 13-14  $\mu\text{m}$ , broad 3-4  $\mu\text{m}$  long.

Collection site -  $S_7, S_{10}$

18. *Oscillatoria margaritifera* (Kutz.) Gomont

Trichome olive green, forming blackish thallus, 19-20  $\mu\text{m}$  broad, 4-5  $\mu\text{m}$  long.

Collection site -  $S_2, S_3, S_8$

19. *Oscillatoria okeni* Ag. ex Gomont

Thallus dull bluegreen; trichome straight 6-6.5  $\mu\text{m}$  broad, 3-3.5  $\mu\text{m}$  long, end cells obtuse or subconical not capitate.

Collection site -  $S_1, S_2, S_4, S_6, S_{10}, S_{12}, S_{18}$

20. *Oscillatoria ornata* Kutz. ex Gomont

Thallus dark blue green trichomes spirally coiled at the end, 8-8.5  $\mu\text{m}$  broad, 3-3.5  $\mu\text{m}$  long.

Collection site -  $S_1, S_{13}, S_{15}$

21. *Oscillatoria princeps* Vaucher ex Gomont

Thallus blue-green, more or less brownish mostly straight, 30-35  $\mu\text{m}$  broad, 5-6  $\mu\text{m}$  long.

Collection site -  $S_1, S_2, S_3, S_5, S_8, S_{13}, S_{14}, S_{15}$

22. *Oscillatoria pseudogeminata* G. Schmid

Thallus pale or dirty, trichome coiled 1.5-2  $\mu\text{m}$  broad, 2.5  $\mu\text{m}$  long, end cell rounded.

Collection site -  $S_1, S_8, S_{10}$

23. *Oscillatoria rubescens* DC ex Gomont

Trichome straight, free swimming in bundles, 6-7  $\mu\text{m}$  broad, 2-3  $\mu\text{m}$  long, often granulated at the septa.

Collection site -  $S_2, S_4, S_{10}, S_9, S_{17}$

24. *Oscillatoria splendida* Grev. ex Gomont

Thallus blue green or olive green, trichome straight or curved. 2-3  $\mu\text{m}$  broad, 4-5  $\mu\text{m}$  long.

Collection site -  $S_3, S_6, S_{16}$ .

25. *Oscillatoria subbrevis* Schmidle

Trichome single, 5-6  $\mu\text{m}$  broad, nearly straight 1-2  $\mu\text{m}$  long, end cell rounded.

Collection site -  $S_1, S_2, S_5, S_6, S_7, S_8, S_{11}, S_{12}, S_{18}$

26. *Oscillatoria tanganyikae* G.S. West

Trichome single, free swimming, olive green, straight or nearly straight, end gradually tapering, bent, rounded; 11-12  $\mu\text{m}$  broad, 6-7  $\mu\text{m}$  long.

Collection site -  $S_1, S_4, S_{11}, S_{15}$

27. *Oscillatoria tenuis* Ag. ex Gomont

Thallus thin blue green, slimy, trichome straight, 5-7  $\mu\text{m}$  broad, 3-4  $\mu\text{m}$  long, sometime bent at the end.

Collection site -  $S_8$ .



**28. *Oscillatoria terebriformis* Ag. ex Gomont**

Thallus dull blue, trichomes end bent in a screw like manner, end cells rounded, 4.5 µm broad, 3.5 - 4 µm long.

Collection site – S<sub>1</sub>, S<sub>3</sub>

**29. *Oscillatoria trichoids* Szafer**

Trichome straight, not constricted at the crosswalls, 1-1.5 µm broad, yellow green, 3-4 µm long

Collection site – S<sub>3</sub>, S<sub>4</sub>

**30. *Oscillatoria vizagapatensis* Rao, C.B.**

Thallus blue green, trichomes straight, or bent, pale blue green, uniformly broad except at the extreme apex, 8 - 10 µm broad, 1.5-2.5 µm long.

Collection site – S<sub>1</sub>, S<sub>6</sub>, S<sub>12</sub>, S<sub>12</sub>

**31. *Oscillatoria willei* Gardner em. Drouet**

Trichome pale blue green to grey blue green, bent at the end or screw like, 3-3.5 µm broad, 1.5 µm long.

Collection site – S<sub>8</sub>

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## Isolation and Identification of Chromium Resistant Bacteria from Slag of a Metal and Ferroalloys Factory

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In view of the chromium contamination of the environment from industrial discharges an attempt was made in the present investigation to isolate and characterize bacteria inhabiting chromium containing waste slag from a metal and ferroalloys factory. A total of 18 isolates were found in the chromium containing slag out of which 10 were gram negative rods. The isolates were found resistant to 100 mg/l concentration of hexavalent chromium under laboratory condition.

**Key words:** Chromium (VI) resistance, Gram negative bacteria, metal and ferroalloys factory, slag

### Introduction

Chromium is released into the environment by a large number of industrial operations including chrome plating, petroleum refining, leather tanning, wood preserving, textile manufacturing pulp processing etc. Chromium is released in different oxidation form from such industries out of which trivalent {Cr (III)} and hexavalent {Cr (VI)} forms are the dominant oxidation states of chromium that exist in the environment (Bartlett and James, 1988). The solubility, environmental mobility and toxicity of chromium depends upon its oxidation state. At physiological environmental pH levels, Cr (VI) compounds are more soluble, more environmentally mobile and more toxic than trivalent chromium {Cr (III)}. Chromium (III), an essential micronutrient for many higher organisms, is relatively insoluble in water and 100 times less toxic than Cr (VI) (De Flora *et al.*, 1990; Katz and Salem, 1994).

Slag arising in ferrochromium and stainless steel production is known to contain chromium. Once released to the environment, chromium is subjected to oxidation/reduction and subjected to bioaccumulation by producer organisms. It is then subjected to bio-magnification in different trophic level in a food chain and ultimately reach to human being. Although chromium at low concentration is beneficial to human being, accumulation of high amount of chromium in general and chromium (VI) in particular is toxic to different metabolic activities. In the present

investigation, slag of a metal and ferroalloy company which produce charge chrome (high carbon ferrochrome) has been taken. The slag released and dumped by the side of the factory as heaps contain high amount of chromium. The slag is subjected to aerial dispersal during summer when the coastal wind is very fast. Moreover during rainy season wash out of the slag enter into local crop field as well as river Mahanadi which sink into the ocean bed, at a distance of 80 kms from the factory site. The slag is loaded with different pollutants, the most significant being chromium. Accordingly an attempt was made in the present study to isolate and identify bacteria inhabiting the slag that are resistant to Cr (VI).

### Materials and Methods

#### Collection of slag samples

In the present investigation random collection of slag samples were carried out aseptically from three different places of slag deposits of the ferroalloy factory. From each place, three samples were collected aseptically within a distance of 2 km. The samples were then air dried, grinded manually and culture was made in laboratory condition to find out microbes inhabiting in the slag.

#### Physico-chemical parameters of the slag

The physico-chemical parameters viz., colour, texture, pH, electrical conductivity and total chromium content of the slag were determined. The pH and

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**Table-1**  
**Physico-chemical parameters of the slag sample**

|                         |                         |
|-------------------------|-------------------------|
| Colour                  | Blackish-silver         |
| Texture                 | Solid                   |
| pH                      | 6.89                    |
| Electrical conductivity | 0.022 dsm <sup>-1</sup> |
| Total chromium content  | 9450 mg/kg of slag      |

with the help of atomic absorption spectrophotometer (Perkin Elmer, USA).

#### Microbiological analysis

The slag samples collected were processed to determine the total aerobic heterophilic bacterial load. Isolation of different strains of bacteria were carried out, axenic culture of the strains were made to study their

**Table-2**  
**Enumeration of total bacterial load of slag collected from a metal and ferroalloys factory**

| Slag (gm) | Distilled water (ml) | Number of bacteria present in different dilutions |                  |                  |                  |                  |                  |                  |                  |                  |                  |
|-----------|----------------------|---------------------------------------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|           |                      | S                                                 | 10 <sup>-1</sup> | 10 <sup>-2</sup> | 10 <sup>-3</sup> | 10 <sup>-4</sup> | 10 <sup>-5</sup> | 10 <sup>-6</sup> | 10 <sup>-7</sup> | 10 <sup>-8</sup> | 10 <sup>-9</sup> |
| 1         | 9                    | —                                                 | —                | —                | —                | —                | —                | —                | —                | —                | —                |
| 2         | 8                    | 2                                                 | —                | —                | —                | —                | —                | —                | —                | —                | —                |
| 3         | 7                    | 6                                                 | 2                | —                | —                | —                | —                | —                | —                | —                | —                |
| 4         | 6                    | 161                                               | 22               | 9                | —                | —                | —                | —                | —                | —                | —                |
| 5         | 5                    | 191                                               | 93               | 80               | 76               | 30               | 17               | 12               | 8                | 2                | —                |

S : Sample,    -- : No growth

**Table-3**  
**Response of bacterial isolates from the slag of a metal and ferroalloys factory to Gram stain**

| Sl. No. | Gram reaction     | Isolate                           |
|---------|-------------------|-----------------------------------|
| 1.      | gram -ve rods     | <i>Achromobacter</i> sps.         |
| 2.      | gram -ve rods     | <i>Achromobacter</i> sps.         |
| 3.      | gram -ve rods     | <i>Flavobacterium spiritvorum</i> |
| 4.      | gram -ve rods     | <i>Pseudomonas mallei</i>         |
| 5.      | gram -ve rods     | <i>Enterobacter cloacae</i>       |
| 6.      | gram -ve rods     | <i>Vibrio metschnikovii</i>       |
| 7.      | gram -ve rods     | <i>Listonella anguillarum</i>     |
| 8.      | gram -ve rods     | <i>Achromobacter</i> sps.         |
| 9.      | gram -ve rods     | <i>Pseudomonas mallei</i>         |
| 10.     | gram -ve rods     | <i>Serratia plymuthica</i>        |
| 11.     | (8) gram +ve rods | <i>Bacillus</i> sps.              |

electrical conductivity of the slag sample was determined by using conductivity meter 306 systromics. The total chromium content of the slag sample were estimated

morphological and physiological parameters and to identify through a series of biochemical characteristics, also other features required for their characterization following standard microbiological techniques of Collins and Lyne (1970).

#### Enumeration of aerobic heterotrophic bacteria

The total aerobic heterotrophic bacterial load in the samples were enumerated by serial dilution technique followed by spread plating. Diluents were prepared with sterilized phosphate buffer having pH 7.2 to avoid osmotic shock to the microbes. From each dilution tube 100 µl of samples were used for spread plating on pre-sterilized Nutrient Agar (NA) plates. Three replicates of each dilution were used for spread plating to minimize the error. The NA plates were supplemented with 100 µg Cr VI/ml. All the plates were incubated at 30 °C ± 2 °C for 48 h. Plates having viable colony counts (30-300 colonies per plate) were selected for enumeration. Different amount of powdered slag were taken (1-5 g) at an interval of 1 g and the same was made 10 ml with distil water.



Table-4

Chromium tolerance capacity of the isolates from slag of metal and ferroalloys factory in the medium

|      | mg/l of Cr (VI) (W/V) NA with K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub> |     |     |     |    |    |    |     |     |      | mg/l of Cr (VI) (W/V) NA |     |     |     |     |     |     |     |     |      |
|------|-----------------------------------------------------------------------------|-----|-----|-----|----|----|----|-----|-----|------|--------------------------|-----|-----|-----|-----|-----|-----|-----|-----|------|
|      | 2                                                                           | 5   | 10  | 20  | 30 | 40 | 50 | 100 | 500 | 1000 | 2                        | 5   | 10  | 20  | 30  | 40  | 50  | 100 | 500 | 1000 |
| SA1  | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   | --   |
| SA2  | +++                                                                         | +++ | +++ | +++ | ++ | +  | -- | --  | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | ++  | --  | --  | --   |
| SA3  | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   | --   |
| SA4  | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   | --   |
| SA5  | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | --  | --   |
| SA6  | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   | --   |
| SA7  | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | --  | --   |
| SA8  | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   | --   |
| SA9  | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   | --   |
| SA10 | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | --  | --   |
| SA11 | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | --  | --   |
| SA12 | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   | --   |
| SA13 | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   | --   |
| SA14 | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   | --   |
| SA15 | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   | --   |
| SA16 | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | --  | --   |
| SA17 | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | --  | --   |
| SA18 | +++                                                                         | +++ | +++ | +++ | ++ | ++ | +  | +   | --  | --   | +++                      | +++ | +++ | +++ | +++ | +++ | +++ | +   | --  | --   |

+++ : Luxuriant growth, ++ : Moderate growth, + : Slow growth, - : No growth

The solution was mixed thoroughly in a vortex mixture and was taken for enumeration of microbes.

#### Isolation and maintenance of Cr (VI) resistant bacteria

Selected bacterial colonies showing different morphological features were picked up from the plates and were re-streaked several times on pre-sterilized NA plates supplemented with 100 µg Cr (VI)/ml as K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> for 2 days. The plates were incubated at 30 °C. The Cr (VI) solutions were filter sterilized with a 0.22 µm membrane filter (Millipore). The pure cultures of the isolates were preserved at 4 °C by re-streaking on NA slants supplemented with Cr (VI) for further characterization. These isolates were assigned with strain

number (SA1, SA2, ..., SA18) for easy recognition and laboratory use.

#### Identification of the bacterial isolates

The isolated bacterial strains from the slag sample were identified on the basis of their colony characteristics, gram reaction, different biochemical test, sugar utilization tests and enzyme activities shown by the isolates. The different medias required for different tests were supplemented with 100 µg Cr (VI)/ml as K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>.

#### Screening of bacterial isolates for different concentration of Cr (VI) resistance

The Cr (VI) resistance of bacterial isolates was studied by incubating 100 µl of culture in different tubes



containing 5 ml of NB with 2, 5, 10, 20, 30, 40, 50, 100, 500, 1000 mg/l of Cr (VI) as  $K_2Cr_2O_7$ . After incubating for 24 to 48 h at 30 °C, a loopful of the culture was sub-cultured onto NA plates with Cr (VI) and only NA plates (served as control). The plates were then incubated and the highest concentration of Cr (VI) on which growth was observed, was considered as the maximum Cr (VI) resistance of the isolate.

## Results and Discussion

The physico-chemical conditions of the slag sample were studied and the results have been presented in Table 1. The pH of the slag sample was found to be almost neutral, with an electrical conductivity of 0.022 d/m. The total chromium content of the slag was found to be 9450 mg/kg of slag sample.

The bacterial load was very less (Table 2). A valid count was not found.

A total number of 18 different bacterial isolates were identified (Table 3). Out of 18 isolates, 10 isolates (55.55%) were gram -ve rods, 8 isolates (44.44%) were gram +ve rods. Three of the isolates belonged to the *Achromobacter* group and two of them were *Pseudomonas mallei* strains. Despite the high concentration of Cr in contaminated soils and sediments, occurrence of a substantial quantity of bacterial population has been reported (Bopp *et al.*, 1988; Losi and Frankenberger, 1994).

The bacterial isolates were able to grow on NA supplemented with 100 mg Cr (VI)/l medium, excepting a *Bacillus* spp. which was able to grow with 40 mg Cr (VI)/l (Table 4). None of the isolates were able to grow on 500 mg Cr (VI)/l. Similar results were obtained with *Arthrobacter* spp. and *Bacillus* spp. by Megharaj *et al.*, (2003). Most Cr (VI) - resistant microorganisms are tolerant upto 10 to 1500 mg/l of Cr (VI) (McLean and Beveridge, 2001; Basu *et al.*, 1997; Losi and Frankenberger, 1994; Bopp *et al.*, 1983).

It is evident from the present finding that the slag, dumped as heaps around the factory is loaded with higher

amount of chromium, causing a potential threat to chromium toxicity to the environment. However, the slag also inhabits diverse group of microorganisms. Out of the 18 isolates, 10 were gram negative and the remaining 8 were gram positive rods, having high chromium tolerance, which is indicative that microbes from the slag must be playing some role in oxidation/reduction of chromium in the inhabiting environment.

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## Potential Wild Edible Plants of Malkangiri District, Orissa

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Systematic floristic surveys were conducted on the under-explored Malkangiri district during the period 2007-2009. A total of 84 wild edible plants those supplement the substandard food habit of the local tribals are enumerated. These potential plants necessitate future value addition through their germplasm conservation.

**Key words:** Malkangiri, Orissa, wild edible plants.

Malkangiri, the tribal dominated district of Orissa is well known for its forest wealth and natural resources. The district is considered backward because of its poor economic development and exposure. A bulk of the population is still under the grab of bitter poverty and malnutrition. Traditional shifting cultivation, growing population, physical and geographical barrier and subsequent climate change made their life miserable. To supplement their food, wild plants were given attention apart from traditional cultivation. Literature on plant uses of the district is meager and mainly on therapeutic uses viz. Das and Ramakant (1988), Mishra and Das (1988), Hemadri and Rao (1989-91), Aminuddin and Girach (1991), Prusty and Behera (2007), Prusty (2007), Pattnaik *et al.* (2008). The knowledge of wild edible plants is the result of age old intimacy of the forest dwellers with the plants of their locality. Therefore, an effort has been made to explore and document such indigenous knowledge.

During the year 2007-2009 extensive field trips were undertaken and by developing good amount of rapport with locals and tribals, the data were collected and documented. The local names of the plants and their uses were cross checked consulting the elderly people, herbal healers and village heads. The plant specimens were collected & identified with reference to "The Flora of Orissa" by Saxena and Brahmam (1994-96). The voucher specimens are preserved in the herbarium of RRL-B, Orissa.

During the survey, numbers of plants were collected. A total of 84 plant species including 32 herbs, 7 shrubs, 33 trees and 12 climbers belonging to 43 families and 65 genera are enumerated in this paper (Table 1).

Apart from the traditional crops like *Oryza sativa*, *Eleusine coracana*, *Zea mays*, *Sorghum vulgare* and pulses etc. the local people supplement their requirement with these wild plant species in day to day life. The plants and their parts are used in various ways. Fruits of 34 plants are eaten raw, ripe or cooked, 32 plants are used as leafy vegetables and the rhizomes/seeds/flowers/watery sap of the rests are consumed in different ways. Among the collections, the plants frequently in use belong to the families like Amaranthaceae, Araceae, Arecaceae, Caesalpiniaceae, Dioscoreaceae and Rhamnaceae are followed by Ebenaceae, Rubiaceae, Tiliaceae etc. Local forests are depleting day by day as a result of rapid deforestation for various reasons like smuggling, growing population etc. This act is detrimental for the biodiversity as well as the local inhabitants depending on the forest directly or indirectly. Some of the plants enumerated here may become a source of nutritious foods in future as all the present day crops and other useful plants are developed from their wild relatives in due course of time. So there is an urgent need for the *in situ* conservation of the plants which will be a valued endowment for the future generations.

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## Enumeration

Table: 1

| Name of the plant                                 | Family           | voucher no. | Habit   | Local names  | Locality    | Parts used                                                                                                                      |
|---------------------------------------------------|------------------|-------------|---------|--------------|-------------|---------------------------------------------------------------------------------------------------------------------------------|
| <i>Achyranthes aspera</i> L.                      | Amaranthaceae    | 10345       | Herb    | Apamaranga   | Govindpally | Dried achenes fried & powdered & consumed.                                                                                      |
| * <i>Amaranthus caudatus</i> L.                   | Amaranthaceae    | 10411       | Herb    | Khadasag     | Mudulipada  | Whole plant used as vegetable.                                                                                                  |
| <i>Amaranthus spinosus</i> L.                     | Amaranthaceae    | 10985       | Herb    | Kantakhada   | Chitrakonda | Leaves & stem are used as vegetable.                                                                                            |
| <i>Amaranthus viridis</i> L.                      | Amaranthaceae    | 12170       | Herb    | Bana khada   | Tamasapally | Leaves & stem are used as vegetable.                                                                                            |
| <i>Celosia argentea</i> L.                        | Amaranthaceae    | 10283       | Herb    | Gudgudia     | Balimela    | Young shoots & leaves are cooked & consumed.                                                                                    |
| <i>Buchanania lanzan</i> Spreng.                  | Anacardiaceae    | 10588       | Tree    | Charuphal    | Nalagunthi  | Ripe fruits are eaten.                                                                                                          |
| * <i>Mangifera indica</i> L.                      | Anacardiaceae    | 10642       | Tree    | Amba         | Undragonda  | Fruits are eaten raw and ripe.                                                                                                  |
| <i>Amorphophallus bulbifer</i> (Roxb.) Bl.        | Araceae          | 10312       | Herb    | Olua         | Dasmantpur  | Young leaves & corms are cooked & consumed.                                                                                     |
| <i>Colocassia esculenta</i> (L.) Schott.          | Araceae          | 11262       | Herb    | Saru         | Dasmantpur  | Rhizome, petiole, leaves & young shoots are cooked & consumed.                                                                  |
| <i>Plesmonium margaritifolium</i> (Roxb.) Schott. | Araceae          | 11417       | Herb    | Kharcol      | Koilipari   | Whole herb is cooked and consumed.                                                                                              |
| <i>Therophonum minutum</i> (Willd.) Baill.        | Araceae          | 11243       | Herb    | Sansaru      | Kamalapadar | Leaves are cooked and consumed.                                                                                                 |
| <i>Borassus flabellifer</i> L.                    | Arecaceae        | 12166       | Tree    | Tala         | Tamasapally | Young seedlings, watery sap of male inflorescence (Tadi) & white growing apices are consumed in addition to raw or ripe fruits. |
| * <i>Caryota urens</i> L.                         | Arecaceae        | 11897       | Tree    | Salap        | Nuaguda     | Watery sap (Salapa) tapped through peduncle is consumed as beverages.                                                           |
| <i>Phoenix acaulis</i> Buch.-Ham. ex Roxb.        | Arecaceae        | 10479       | Shrub   | Bana khajuri | Kamalapadar | Young seeds, ripe fruits & white growing apices are consumed.                                                                   |
| <i>Phoenix sylvestre</i> (L.) Roxb.               | Arecaceae        | 12167       | Tree    | Khajuri      | Tamasapally | Young seeds, white growing apex, ripe fruits & stem sap (Tadi) are consumed.                                                    |
| <i>Sphaeranthus indicus</i> L.                    | Asteraceae       | 10380       | Herb    | Mundi        | Kamalapadar | Juvenile leaves are used as vegetable.                                                                                          |
| <i>Careya arborea</i> Roxb.                       | Barringtoniaceae | 10704       | Tree    | Kumam        | Balimela    | Young fruits are used as vegetable.                                                                                             |
| <i>Protium serratum</i> (Wall. ex Colebr.) Eng.   | Burseraceae      | 11252       | Tree    | Andiri       | Kamalapadar | Ripe fruits are eaten.                                                                                                          |
| <i>Bauhinia purpurea</i> L.                       | Caesalpiniaceae  | 10284       | Tree    | Baroda       | Chitrakonda | Young leaves are eaten as vegetable.                                                                                            |
| <i>Cassia fistula</i> L.                          | Caesalpiniaceae  | 10532       | Tree    | Sunari       | Balimela    | Flowers are fried and consumed.                                                                                                 |
| <i>Cassia tora</i> L.                             | Caesalpiniaceae  | 10509       | Herb    | Chakunda     | Chalanguda  | Young leaves are cooked & consumed.                                                                                             |
| <i>Tamarindus indica</i> L.                       | Caesalpiniaceae  | 10528       | Tree    | Tentuli      | Govindpally | Young leaves, flowers, raw & ripe fruits are eaten.                                                                             |
| <i>Cleome monophylla</i> L.                       | Capparaceae      | 11221       | Herb    | Rango soriso | Tamasapally | Young leaves are used as vegetable.                                                                                             |
| <i>Cleome viscosa</i> L.                          | Capparaceae      | 11223       | Herb    | Bana sorisa  | Tamasapally | Young leaves are used as vegetable.                                                                                             |
| <i>Terminalia bellirica</i> (Gaertn.) Roxb.       | Combretaceae     | 10670       | Tree    | Bahada       | Chitrakonda | Mature endosperm is eaten by children.                                                                                          |
| <i>Commelina benghalensis</i> L.                  | Commelinaceae    | 11485       | Herb    | Kanisiri     | Motu        | Young shoots & leaves are fried and consumed.                                                                                   |
| <i>Commelina erecta</i> L.                        | Commelinaceae    | 10429       | Herb    | Kansiri      | Sitakund    | Young shoots & leaves are cooked & consumed.                                                                                    |
| <i>Ipomoea aquatica</i> Forssk.                   | Convolvulaceae   | 11080       | Herb    | Kalama sag   | Satiguda    | Young shoots & leaves are consumed after cooked.                                                                                |
| <i>Luffa aegyptiaca</i> Mill.                     | Cucurbitaceae    | 12165       | Climber | Dundol       | Tamasapally | Young leaves & fruits used as vegetable.                                                                                        |



|                                                   |                |       |         |               |               |                                                                      |
|---------------------------------------------------|----------------|-------|---------|---------------|---------------|----------------------------------------------------------------------|
| <i>Trichosanthes cucumerina</i> L.                | Cucurbitaceae  | 11377 | Climber | Banakotala    | Goiparbat     | Fruits are used as vegetable.                                        |
| <i>Cyperus rotundus</i> L. subsp. <i>Rotundus</i> | Cyperaceae     | 10950 | Herb    | Mutha         | Tamasapally   | Rhizomes are eaten raw or boiled.                                    |
| <i>Dioscorea bulbifera</i> L.                     | Dioscoreaceae  | 10270 | Climber | Pita kanda    | Biralaxmanpur | Rhizomes & young leaves are cooked and consumed                      |
| <i>Dioscorea glabra</i> Roxb.                     | Dioscoreaceae  | 10299 | Climber | Pindalu       | Dasmantpur    | Rhizomes & young leaves are cooked and consumed.                     |
| <i>Dioscorea oppositifolia</i> L.                 | Dioscoreaceae  | 11463 | Climber | Ban kanda     | Phulkelkonda  | Rhizomes & young leaves are cooked and consumed                      |
| <i>Dioscorea puber</i> Bl.                        | Dioscoreaceae  | 11456 | Climber | Bariha kanda  | Phulkelkonda  | Rhizomes are cooked and consumed.                                    |
| <i>Diospyros malabarica</i> (Desr.) Kostel.       | Ebenaceae      | 10692 | Tree    | Kalakendu     | Chitrakonda   | Young seeds are eaten by children.                                   |
| <i>Diospyros melanoxylon</i> Roxb.                | Ebenaceae      | 10501 | Tree    | Kendu         | Chalanguda    | Ripe fruits are eaten by all age group.                              |
| <i>Diospyros sylvatica</i> Roxb.                  | Ebenaceae      | 10272 | Tree    | Mankadake ndu | Biralaxmanpur | Young seeds & ripe fruits are eaten by children.                     |
| <i>Bridelia montana</i> (Roxb.) Willd.            | Euphorbiaceae  | 11362 | Shrub   | Marda         | Goiparbat     | Ripe fruits are eaten by all age group.                              |
| <i>Bridelia retusa</i> (L.) Spreng.               | Euphorbiaceae  | 10378 | Tree    | Kosi          | Udlibed way   | Ripe fruits are eaten.                                               |
| <i>Pongamia pinnata</i> (L.) Pierre.              | Fabaceae       | 11135 | Tree    | Karanja       | Chitapari     | Endosperms are eaten.                                                |
| <i>Pueraria tuberosa</i> (Willd.) DC.             | Fabaceae       | 12196 | Climber | Bhuin kakharu | Balimela      | Tubers are eaten after boiling.                                      |
| <i>Hyptis suaveolens</i> (L.) Poit.               | Lamiaceae      | 10255 | Herb    | Ghodatulas i  | Biralaxmanpur | Ripe seeds are consumed as 'Sagu' by soaking in water.               |
| <i>Asparagus racemosus</i> Willd.                 | Liliaceae      | 10291 | Climber | Satamuli      | Dasmantpur    | Tuberous roots are eaten raw.                                        |
| <i>Abelmoschus moschatus</i> Medic.               | Malvaceae      | 11460 | Herb    | Bana bhendi   | Phulkelkonda  | Young fruits are eaten raw or cooked.                                |
| <i>Pavonia odorata</i> Willd.                     | Malvaceae      | 11480 | Herb    | Kurubeli      | Motu          | Young leaves are used as vegetable.                                  |
| <i>Marsilea minuta</i> L.                         | Marsileaceae   | 11069 | Herb    | Sunsunia      | Satiguda      | Leaves are used as vegetable.                                        |
| <i>Pithecellobium dulce</i> (Roxb.) Benth.        | Mimosaceae     | 12289 | Tree    | Simakoya      | Orkel         | Ripe fruits are eaten.                                               |
| <i>Mollugo pentaphylla</i> L.                     | Molluginaceae  | 10688 | Herb    | Pitasag       | Chitrakonda   | Young plant is cooked and consumed.                                  |
| * <i>Artocarpus heterophyllus</i> Lam.            | Moraceae       | 10978 | Tree    | Panasa        | Chitrakonda   | Raw & Ripe fruits are consumed.                                      |
| <i>Ficus racemosa</i> L.                          | Moraceae       | 10671 | Tree    | Dimiri        | Chitrakonda   | Raw figs are cooked and consumed.                                    |
| <i>Ardisia solanacea</i> Roxb.                    | Myrsinaceae    | 10446 | Shrub   | Kukur koli    | Mudulipara    | Young leaves are cooked & consumed.                                  |
| <i>Syzygium cumini</i> (L.) Skeels.               | Myrtaceae      | 11137 | Tree    | Jamun         | Chitapari     | Ripe fruits are eaten.                                               |
| <i>Syzygium roxburghianum</i> Raizada             | Myrtaceae      | 11139 | Tree    | Jamun         | Chitapari     | Ripe fruits are eaten.                                               |
| <i>Boerhavia diffusa</i> L.                       | Nyctaginaceae  | 10620 | Herb    | Puruni        | Undragonda    | Young leaves & shoots are cooked & consumed.                         |
| <i>Nelumbo nucifera</i> Gaertn.                   | Nymphaeaceae   | 12194 | Herb    | Padma         | Mathili       | Ripe seeds are fried & consumed.                                     |
| <i>Nymphaea pubescens</i> Willd.                  | Nymphaeaceae   | 10512 | Herb    | Kain          | Pangam        | Spongy petiole & ripe seeds are fried and consumed.                  |
| <i>Passiflora foetida</i> L.                      | Passifloraceae | 11063 | Climber | Gandhata mala | Satiguda      | Ripe fruits are eaten by children.                                   |
| <i>Dendrocalamus strictus</i> (Roxb.) Nees.       | Poaceae        | 10572 | Shrub   | Baunsa        | Nalagunthi    | Young sproutings (locally called as "Karodi") are cooked & consumed. |
| <i>Paspalum scrobiculatum</i> L.                  | Poaceae        | 10654 | Herb    | Kodoa         | Balimela      | Grains are eaten as rice in scarcity.                                |
| <i>Portulaca oleracea</i> L.                      | Portulacaceae  | 11082 | Herb    | Badabalba lua | Satiguda      | Whole herb is cooked and consumed.                                   |
| <i>Ziziphus mauritiana</i> Lam.                   | Rhamnaceae     | 10627 | Tree    | Barkoli       | Undragonda    | Ripe fruits are eaten by all age group.                              |
| <i>Ziziphus oenoplia</i> (L.) Mill.               | Rhamnaceae     | 10325 | Shrub   | Kanteikoli    | Dasmantpur    | Ripe fruits are eaten by all age group.                              |



|                                               |               |       |         |              |             |                                                                                                                 |
|-----------------------------------------------|---------------|-------|---------|--------------|-------------|-----------------------------------------------------------------------------------------------------------------|
| <i>Ziziphus rugosa</i> Lam.                   | Rhamnaceae    | 10999 | Tree    | Banakoli     | Chitrakonda | Ripe fruits are eaten.                                                                                          |
| <i>Ziziphus xylopyrus</i> (Retz.) Will.       | Rhamnaceae    | 11079 | Tree    | Mankadkoli   | Satiguda    | Ripe fruits are eaten.                                                                                          |
| <i>Catunaregam spinosa</i> (Thunb.) Tirveng.  | Rubiaceae     | 11171 | Tree    | Mainphal     | Disariguda  | Young fruits are used as vegetable.                                                                             |
| <i>Gardenia resinifera</i> Roth.              | Rubiaceae     | 10684 | Tree    | Kurdu        | Chitrakonda | Ripe fruits are eaten by children.                                                                              |
| <i>Paederia foetida</i> L.                    | Rubiaceae     | 12195 | Climber | Gandhamadali | Tamasapally | Leaves are cooked & consumed.                                                                                   |
| * <i>Aegle marmelos</i> (L.) Corr.            | Rutaceae      | 10960 | Tree    | Bel          | Chitapari   | Fruits are eaten by all age group.                                                                              |
| * <i>Murraya koenigii</i> (L.) Spreng.        | Rutaceae      | 10439 | Shrub   | Bhursanga    | Mudilipada  | Leaves are used as curry leaf.                                                                                  |
| <i>Mimusops elengi</i> L.                     | Sapindaceae   | 12193 | Tree    | Khirkoli     | Balimela    | Ripe fruits are eaten.                                                                                          |
| <i>Schleichera oleosa</i> (Lour.) Oken.       | Sapindaceae   | 11116 | Tree    | Kusum koli   | Chitapari   | Ripe fruits are eaten.                                                                                          |
| <i>Madhuca indica</i> Gmel.                   | Sapotaceae    | 10587 | Tree    | Mahul        | Nalagunthi  | Raw fruit mesocarp is cooked and country liquor is prepared from the flowers; oil from cotyledons are consumed. |
| <i>Manilkara hexandra</i> (Roxb.) Dubard      | Sapotaceae    | 10290 | Tree    | Khirkoli     | Balimela    | Ripe fruits are eaten.                                                                                          |
| <i>Solanum torvum</i> Sw.                     | Solanaceae    | 10340 | Herb    | Denga vegi   | Govindpally | Young fruits are fried & consumed.                                                                              |
| <i>Sterculia urens</i> Roxb.                  | Sterculiaceae | 10597 | Tree    | Genduli      | Kalimela    | Resins & mature seeds are consumed.                                                                             |
| <i>Sterculia villosa</i> Roxb. ex DC.         | Sterculiaceae | 11157 | Tree    | Kodala       | Disariguda  | Resins & mature seeds are consumed.                                                                             |
| <i>Tacca leontopetaloides</i> (L.) Kuntze     | Taccaceae     | 10542 | Herb    | Bag-olua     | Balimela    | Whole herb is cooked & consumed.                                                                                |
| <i>Corchorus aestuans</i> L.                  | Tiliaceae     | 11375 | Herb    | Bano nalita  | Goiparbat   | Leaves are cooked and consumed.                                                                                 |
| <i>Corchorus olitorius</i> L.                 | Tiliaceae     | 11483 | Herb    | Nalita       | Motu        | Leaves are cooked and consumed.                                                                                 |
| <i>Grewia tiliifolia</i> Vahl.                | Tiliaceae     | 11368 | Shrub   | Dhamura      | Goiparbat   | Fruits are eaten by children.                                                                                   |
| <i>Trapa natans</i> L.                        | Trapaceae     | 11752 | Herb    | Panisingada  | Balisagar   | Fruits are eaten by children.                                                                                   |
| <i>Ampelocissus latifolia</i> (Roxb.) Planch. | Vitaceae      | 11250 | Climber | Kanjinoi     | Kamalapadar | Ripe fruits are eaten by children.                                                                              |
| <i>Ampelocissus tomentosa</i> (Roth.) Planch. | Vitaceae      | 10529 | Climber | Kanja lata   | Chitrakonda | Ripe fruits are eaten.                                                                                          |

\* Planted as fruit trees.

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# Floating Associates Influence Growth and Nitrogen Content of *Azolla* Species

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Three species of *Azolla* viz. *Azolla pinnata*, *Azolla filiculoides*, *Azolla mexicana*, five species of other floating plants viz. *Lemna* sp., *Spirodela* sp., *Wolffia* sp., *Pistia* sp. and *Salvinia* sp. in different combinations were examined for their biomass productivity in plastic tubs. Out of eighteen possible species combinations, nine of them showed significant positive value, two showed significant negative values and remaining seven combinations showed non-significant associations. A combination of *A. pinnata* and *Lemna* sp. proved to be the best and closely followed by *A. filiculoides* plus *Lemna* sp. Growth of *Azolla* in mixed culture was found better than the *Azolla* monoculture.

**Key words:** *Azolla*, biomass, floating associates, N content

*Azolla*, a genus of aquatic fern, is commonly observed in temporary ponds and ditches along with other free-floating plants like *Lemna*, *Spirodela* and *Pistia* (Satapathy and Chand, 1984). It is symbiotically associated with a

heterocystous blue-green alga *Anabaena azollae* that fixes atmospheric nitrogen. The potentiality of *Azolla*-*Anabaena* consortium as an organic nitrogen source in agriculture, especially in conjunction with rice culture, had been visualized by several workers from India (Singh, 1977; Singh *et al.*, 1981; Satapathy and Singh, 1985; Satapathy, 1999; Pabby *et al.*, 2003), Vietnam (Tran and Dao, 1973), China (Liu Chung Chu, 1979) and Philippines (Watanabe, 1977). Adaptability of *Azolla* species to varied environmental conditions is a prerequisite for its wide spread use. Monoculture of *Azolla* has been found to be prone to fungal infection (Anonymous, 1976) and insect infestation (Satapathy and Singh, 1987) during its repeated use in a particular area. Use of mixed culture may alleviate this problem. While, a good number of information on the distribution of the fern in the water bodies of India are available (Rao, 1936; Loyal, 1958; Gopal, 1967; Singh, 1977; Singh *et al.*, 1982; Satapathy and Chand, 2004), only a few of these dealt with the influence of its aquatic associates or macrophyte competitors for space and nutrient on its qualitative and quantitative growth. Satapathy and Chand (2004) observed that the fern was commonly growing in temporary ponds, ditches or lowland rice fields of Orissa along with other free-floating plants like *Lemna*, *Wolffia*, *Spirodela* and *Pistia*. In some water-bodies, species of *Eichhornia*, *Salvinia*, *Utricularia*, *Marsilea*, *Commelina*, *Trapa*, *Nymphaea* and *Lemnanthemum*

were also found growing along with *Azolla* at different sectors. *Azolla* appeared with the free-floating vegetation during the end of July, while a thick growth of the fern was found in many temporary ponds of Orissa by the end of October. However, the percentage coverage of the pond, frond-size and N, P content of *Azolla* varied with places of different water pH and phosphate concentration (Satapathy and Chand, 1984, 2004). In this investigation attempts were made to identify possible beneficial associations.

Three *Azolla* species viz. *Azolla pinnata* (Ap), *Azolla filiculoides* (Af), *Azolla mexicana* (Am), and five species of other floating plants viz. *Lemna* sp. (Lm), *Spirodela* sp. (Sp.), *Wolffia* sp. (Wo), *Pistia* sp. (Pi) and *Salvinia* sp. (Sa) were the plant materials used in the experiment. *Azolla* species were brought from the Laboratory of Blue-green Algae of Central Rice Research Institute, Cuttack. Other floating hydrophytes were collected from a temporary pond of Vyasagar municipality (erstwhile Jajpur Road municipality) of Jajpur district of Orissa. All these plants were grown individually in soil extract solution for five days. In eighteen different combinations these hydrophytes were cultured in plastic tubs of size 30 x 45 cm (h x d), each containing 3.5 kg soil with 7 cm standing water. The individual tub was inoculated with 5.0 g of each of the species combinations. Single super phosphate at the rate of 8 kg P<sub>2</sub>O<sub>5</sub> /ha on

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**Table-1**  
Growth of *Azolla* species in the presence of its floating associates

| Treatment                                  | Fresh matter (g/ pot) | Nitrogen (%) | Phosphorus (%) | Organic C (%) | C : N |
|--------------------------------------------|-----------------------|--------------|----------------|---------------|-------|
| T <sub>1</sub> <i>A. pinnata</i> (Ap)      | 25.6                  | 4.18         | 0.29           | 31.22         | 7.47  |
| T <sub>2</sub> <i>A. filiculoides</i> (Af) | 26.0                  | 4.75         | 0.37           | 29.07         | 6.12  |
| T <sub>3</sub> <i>A. mexicana</i> (Am)     | 23.1                  | 4.31         | 0.36           | 29.64         | 6.88  |
| T <sub>4</sub> Ap + Af                     | 30.5                  | 4.2          | 0.30           | 30.0          | 7.14  |
| T <sub>5</sub> Ap + Am                     | 28.1                  | 4.2          | 0.28           | 31.0          | 7.38  |
| T <sub>6</sub> Af + Am                     | 29.0                  | 4.5          | 0.35           | 28.0          | 6.22  |
| T <sub>7</sub> Ap + <i>Lemna</i> sp.       | 33.8                  | 4.3          | 0.31           | 30.0          | 6.98  |
| T <sub>8</sub> Ap + <i>Spirodela</i>       | 25.1                  | 4.0          | 0.28           | 32.0          | 8.0   |
| T <sub>9</sub> Ap + <i>Wolffia</i>         | 32.0                  | 4.2          | 0.30           | 29.9          | 7.12  |
| T <sub>10</sub> Af + <i>Lemna</i>          | 33.5                  | 4.6          | 0.35           | 28.5          | 6.19  |
| T <sub>11</sub> Af + <i>Spirodela</i>      | 26.3                  | 4.5          | 0.35           | 30.0          | 6.67  |
| T <sub>12</sub> Af + <i>Wolffia</i>        | 32.0                  | 4.6          | 0.36           | 28.8          | 6.26  |
| T <sub>13</sub> Am + <i>Lemna</i>          | 30.8                  | 4.4          | 0.36           | 28.9          | 6.57  |
| T <sub>14</sub> Am + <i>Spirodela</i>      | 23.0                  | 4.2          | 0.34           | 30.0          | 7.14  |
| T <sub>15</sub> Am + <i>Wolffia</i>        | 28.7                  | 4.3          | 0.34           | 29.5          | 6.86  |
| T <sub>16</sub> Ap + <i>Pistia</i>         | 25.2                  | 4.0          | 0.31           | 30.8          | 7.7   |
| T <sub>17</sub> Af + <i>Pistia</i>         | 25.8                  | 4.1          | 0.35           | 31.0          | 7.6   |
| T <sub>18</sub> Am + <i>Pistia</i>         | 20.1                  | 2.9          | 0.32           | 32.0          | 11.0  |
| T <sub>19</sub> Ap + <i>Salvinia</i>       | 25.4                  | 4.1          | 0.30           | 30.6          | 7.46  |
| T <sub>20</sub> Af + <i>Salvinia</i>       | 26.2                  | 4.4          | 0.34           | 29.0          | 6.59  |
| T <sub>21</sub> Am + <i>Salvinia</i>       | 20.4                  | 3.1          | 0.32           | 30.1          | 9.71  |
| C.D. at 5 %                                | 2.132                 | 0.334        | 0.0417         | 1.037         | 0.359 |

Inoculum size - 5.0 g fresh blotted frond, Culture period - 10 days

soil weight basis was sprinkled over the plants after mixing with the water. Each treatment was replicated thrice. The growth of *Azolla* species was recorded after ten days of inoculation.

*Azolla* fronds were harvested and kept separately treatment wise for the estimation of their biomass, nitrogen, phosphorus and organic carbon content. The biomass was determined by weighing the blotted fronds with the help of single pan balance (No. 50 T, Salter). *Azolla* samples were oven dried at 70 °C for 48 h, powdered in a Willey mill and analysed for nitrogen and phosphorus by modified microkjeldahl method and amidal blue colouration method respectively (Jackson, 1967). Organic carbon was estimated by Walkley and Black's wet oxidation method (Piper, 1966). All the data were statistically analysed for critical differences (C.D.) up

to 5 % ( $P = 0.05$ ) level of significance (Gomez and Gomez, 1984).

Three species of *Azolla* viz. Ap, Af, Am, five species of other floating plants viz. Lm, Sp., Wo, Pi and Sa in different combinations were examined for their biomass production along with N, P and organic C of the tissue. Out of eighteen possible species combinations, nine of them showed significant positive value (Ap x Af, Ap x Am, Af x Am, Ap x Lm, Ap x Wo, Af x Lm, Af x Wo, Am x Lm, Am x Wo), two showed significant negative values (Am x Pi, Am x Sa) and remaining seven combinations showed non-significant associations (Table 1). This indicates that environment nutrition and biotic factors have a profound role in the spatial distribution of the species. A combination of *A. pinnata*



and *Lemna* sp. proved to be the best, closely followed by *A. filiculoides* plus *Lemna* sp. Growth of *Azolla* in mixed culture was found better than the *Azolla* monoculture (Table 1). The above identified combinations with synergistic effect on the biomass production of *Azolla* might be due to its decreased parasitization and predation and/or increased ecological benefits. The nitrogen and phosphorus content of *Azolla* tissues were also improved in some of the mixed culture treatments (Table 1). This enhanced N and P content of *Azolla* tissues might be due to the increased nutrient status of its aquatic environment consequent upon gradual disappearance and decay of its aquatic associates.

Dual culture of *Azolla* with some of its natural floating macrophytes was found to be beneficial with respect to its biomass production. This finding suggests clues for the adoption of suitable species combinations in the rice field for the optimum fern growth and consequently enhanced crop yield.

Ecological studies on the distribution of *Azolla* in the water bodies as well as rice fields of Orissa indicates that the fern growing with other plant species, including rice, results in some interaction. As reported earlier (Satapathy, 1993, 1995) *Azolla* multiplication is lower in dual culture with rice than in fallow or crop-free rice field. The growth competence of *Azolla* with respect to its associates may be advantageous to the rice crop under specific management practices. Researches carried out at CRRI, Cuttack showed that growing of *Azolla* in dual culture with rice was effective in suppressing (controlling) weeds in the rice field. The overall effect of *Azolla* was to keep the biomass of weeds in rice fields at a significantly lower level than that in the fields with no *Azolla* cover (Satapathy and Singh, 1987).

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