

ISSN 0972-8546

PLANT SCIENCE RESEARCH



The Orissa Botanical Society

Volume 32 • December, 2010

PLANT SCIENCE RESEARCH

Official Publication of
THE ORISSA BOTANICAL SOCIETY

EXECUTIVE COMMITTEE FOR 2010

<i>President</i>	Prof. (Mrs.) B. Behera
<i>Vice President</i>	Prof. A.C. Sahoo
<i>Secretary</i>	Prof. B.B. Mishra
<i>Joint Secretaries</i>	Prof. (Mrs.) S.B. Padhi
	Dr. A.K. Bastia
	Dr. M.K. Das
	Dr. (Mrs.) R. Das

Executive Members

Prof. S.N. Patnaik
Dr. J.K. Roy
Prof. M.P. Sinha
Prof. R.C. Mohanty
Dr. B.K. Mishra
Prof. M. Kar
Dr. J.R. Mishra
Prof. H.K. Patra
Prof. P.K. Chand
Prof. S.P. Adhikary
Dr. (Mrs.) S.L. Sahoo
Dr. P.K. Mohapatra
Dr. R.C. Dash
Dr. K.B. Satapathy
Dr. P.K. Jena
Dr. S.K. Pradhan
Dr. A.K. Biswal

Editorial Board

Prof. S.N. Patnaik, *Chief Editor*
Prof. P.K. Chand, *Managing Editor*
Prof. G. Subba Rangaiah
Prof. Jyoti Kumar
Prof. (Mrs.) B. Behera
Dr. A.K. Dubey
Dr. M.K. Mishra
Prof. A.K. Sahoo
Dr. P.K. Mohapatra
Dr. A.K. Mukherjee
Dr. (Mrs.) S. Nayak
Dr. C.C. Rath

Plant Science Research is the official publication of the Orissa Botanical Society and is published once a year. Subscription rate for non-members and institutions is Rs. 100/- in India and \$10/- for overseas per annum. Life members of the Society get the journal free of cost. All communications regarding business matters should be addressed to the Managing Editor, *Plant Science Research*, P.G. Department of Botany, Utkal University, Bhubaneswar - 751004, India.

Physiological, genetic and molecular basis of submergence tolerance in rice: A review

B.P. Das^{1*}, M.K. Kar², P. Dash³ and A.T. Roy⁴

¹S.C.S Autonomous College, Puri-752001, ²Central Rice Research Institute, Cuttack-753006,

³Siksha O Anusandhan University, Bhubaneswar-751030, ⁴A-19, Phase-I, Krishna Gate, Bhubaneswar-751030

(Received : 17 November, 2010; Revision received : 10 December, 2010)

Rainfed lowlands constitute highly fragile ecosystem, always prone to flash-floods and submergence. The risk of frequent submergence due to flash floods and waterlogging makes the ecosystem less responsive to better management. Improvement of germplasm as well as better management technologies are needed to enhance and stabilize rice productivity in these areas. Progress in varietal improvement can be enhanced if the physiological and genetic bases of submergence tolerance are well understood. This review focuses on physiological, genetic and molecular bases of submergence tolerance, new genetic resources and potential of DNA marker technology for incorporating submergence tolerance in elite genotypes for high and stable productivity in this fragile ecology.

Key words: Rice, submergence tolerance, crop improvement, marker assisted selection

Submergence is a serious and important physical constraint in rainfed lowland rice ecologies. It occurs due to flash floods or excessive rain causing inundation without effective drainage in the low lying areas. In India almost 12 million hectares of rainfed lowland rice area is flood prone and out of this about six million hectares are submerged almost every year, where plants are completely submerged for 1-2 weeks, resulting in partial or even complete crop failure. The risk of frequent submergence due to flash floods and waterlogging makes the ecosystem less responsive to better management. In Eastern India, submergence is identified as the third most important of 42 biotic and abiotic stresses that limit rice production and is surpassed only by drought at anthesis and by weeds (Widawsky and O'Toole, 1990). Flooding is also widespread in other South and Southeast Asian countries such as Bangladesh, Thailand, Vietnam, Myanmar and Indonesia. Suitable germplasm as well as management technologies are therefore needed to enhance and stabilize rice productivity in these areas. The present article focuses on the physiological, genetic and molecular basis of submergence tolerance that is necessary for high and stable productivity in submergence prone areas.

Mechanisms of submergence tolerance

Rice is adapted to submergence-prone environments by two strategies. Submergence tolerance to flash floods where a rapid increase in water level causes partial to complete submergence for up to two weeks and shoot elongation by types adapted to deepwater areas (>100 cm) where water stagnates for several months and where survival depends on the shoot remaining in contact with the air. This rapid elongation is disadvantageous in flash flood conditions since lodging usually occurs once floodwater recedes. For deepwater and floating rice, plants have been selected to overcome lodging when the floodwater recedes by further elongation and bending of the upper portions of stems which keeps the panicle upright and off the soil surface (Catling, 1992). Under flash flooding, few characters were identified as playing a key role in submergence tolerance in rice, the most critical are:

- maintenance of high carbohydrate concentration,
- optimum rates of alcoholic fermentation and
- energy conservation by maintaining low elongation growth rates during submergence.

Protective mechanisms as the upregulation of antioxidant system and low synthesis or sensitivity

*Corresponding author; E-mail: bidyutpravadas@rediffmail.com

to ethylene during submergence were also found to be useful.

Stem carbohydrates

Carbohydrate concentration before and during submergence has long been recognized as an important factor in submergence tolerance in rice. A strong positive correlation between carbohydrate concentration prior to submergence and tolerance to submergence is commonly observed. Studies using techniques that alter the concentration of carbohydrates before or during submergence support the significant role carbohydrates plays during submergence. Genetic variability in stem carbohydrate content is present among rice cultivars, but greatly influenced by growth conditions before submergence. Selection of genotypes with high stem carbohydrates and with greater ability to conserve it during flooding could provide an increment of submergence tolerance as our recent studies indicated that the level of carbohydrates remaining after submergence is more critical for survival. Old seedlings tend to have large carbohydrate reserves and therefore good survival during submergence (Adkins *et al.*, 1988; Chaturvedi *et al.*, 1996). Culms of submergence-tolerant plants contained starch even after being submerged for 7 days, whereas the reserves of submergence intolerant rice cultivars were exhausted during the same period (Mallik *et al.*, 1995; Singh *et al.*, 2001).

A critical valuation of submergence-tolerant and intolerant rice cultivars revealed that seedlings of tolerant species have 30-50% more non-structural carbohydrates compared to the susceptible cultivars (Chaturvedi *et al.*, 1995; Setter, *et al.*, 1997; Sarkar, 1998). These non-structural carbohydrates are utilized during submergence to supply the required energy for growth and maintenance metabolism (Sarkar *et al.*, 1996; Huang *et al.*, 2005). Quick regeneration following submergence is a desirable trait under frequent or prolonged flooding, as it can ensure early recovery and production of sufficient biomass for optimum productivity. The old leaves die after flooding, particularly when floodwater is turbid or when flooding is prolonged. Initiation of new leaves and their subsequent growth requires availability of

non-structural carbohydrates. Cultivars that maintained more than 6% of their initial non-structural carbohydrate at the time of re-aeration were found to be capable of developing new leaves rather quickly (Das *et al.*, 2005). Therefore, it is reasonable to conclude that high carbohydrate status after submergence, which is the consequence of its level before submergence and extent of turnover and consumption during submergence, is the key factor that determines the ability of plants to withstand submergence stress.

Alcoholic fermentation (Anaerobic respiration)

Alcoholic fermentation (AF) is one of the major metabolic adaptations that plants assume when they are submerged or faced with lack of oxygen. Under anaerobic conditions aerobic respiration shift to a less efficient anaerobic fermentation pathway to provide energy and sustain plant life. Although the amount of ATP produced by this process is very small (5%) compared to ATP produced through aerobic respiration, it is still vital for survival. The efficiency of this pathway depends on continued supply of substrate (glucose) and the two key enzymes, alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC). Alcoholic fermentation is the key catalytic pathway for recycling NAD to maintain glycolysis and substrate level phosphorylation in the absence of oxygen (Davies, 1980; Ap Rees *et al.*, 1987). Increased alcoholic fermentation is thus one way to alleviate the adverse effect of anoxia on reduced production of energy for growth and maintenance processes during submergence. The importance of alcoholic fermentation in growth and survival of rice under anoxia has been well emphasized (Avadhani *et al.*, 1978; Alpi and Beevers, 1983). Support for the importance of the beneficial effects of augmented rates of alcoholic fermentation under anoxia has been demonstrated by a number of experiments.

Stem elongation

The mechanisms of plant adaptation to excessive flooding depend on the water regime. In deepwater areas with >100 cm water depth for 2-3 months, cultivars with sufficient capacity for internode elongation maintain their foliage above the water

surface to sustain leaf photosynthesis and oxygen transport, leading to better survival. In contrast to deepwater rice, rainfed lowland rice is subjected to flash floods (>50 cm water depth), ranging from 1 to 2 weeks, where elongation growth is not sufficient for leaves to regain contact with the air. Furthermore, when water levels recede the elongated growth would lead to serious lodging.

Survival of rainfed lowland rice cultivars is negatively correlated with underwater elongation growth by leaves during complete submergence and varies with seedling age. Survival was least in young seedlings and improved with seedling age. Setter and Laureles (1996) using five rice cultivars, also showed a negative correlation ($r=-0.81$) between survival and shoot elongation growth. This negative relationship between submergence tolerance and elongation during submergence was confirmed using IRRI gene bank database on 903 cultivars (Setter and Laureles, 1996). Rapid leaf elongation during submergence competes with maintenance respiration for carbon sources leaving less assimilates available to support maintenance required for survival during submergence (Greenway and Setter, 1996; Setter *et al.*, 1997; Singh *et al.*, 2001). Plants actively growing during submergence are more susceptible than dormant or slowly growing plants (Vartapetian and Jackson, 1997).

Attempts to manipulate leaf elongation during submergence by the application of GA₃, paclobutrazol (PB, an inhibitor of kaurene oxidation in the gibberellin biosynthesis pathway) and cycocel (CCC) have further confirmed the hypothesis that minimum underwater elongation is associated with increased survival (Setter and Laureles, 1996). Foliar spray of gibberellin 24 h prior to submergence increased underwater elongation during submergence by 6% in FR13A and 24% in IR42 in comparison to control and decreased survival by 53 and 47% in FR13A and IR42, respectively. Treatments with PB and CCC, reduced elongation during submergence and improved survival; they also showed an improved relationship between elongation growth and survival ($r=0.88$ for PB and 0.92 for CCC) relative to control ($r=0.97$). GA₃ worsened this correlation. These results are consistent with the hypothesis that elongation

growth competes for carbon used for maintenance processes for survival, and therefore that reduced elongation growth increases survival during complete submergence of rice.

Rice plants that exhibit only limited elongation during submergence often show tolerance to complete flooding. Some researchers have suggested that the ideal response to submergence is submergence tolerance (survival under water) together with some elongating ability. This ideotype is suitable only if the water level increases and then (i) stays at that level, (ii) recedes only partly, or (iii) recedes but then rises again and stays for longer duration. However, reduced elongation under flash-flood conditions (complete submergence for 1-2 weeks) is vital for survival because elongating plants would tend to lodge as soon as the water level recedes.

Anaerobic proteins (ANPs)

Plant reaction to oxygen deprivation is expressed as the repression of most aerobic protein synthesis and induction of a number of so called ANPs. Submergence and/or anoxia-tolerant and intolerant species may differ in the number and the level of production of these ANPs. Most studies carried out on the expression of ANPs under hypoxia or anoxia emphasizes the importance of energy metabolism, since the majority are enzymes of glycolysis and fermentation. These proteins fall mainly in three functional groups: (i) enzymes mobilizing sucrose (sucrose synthase) or starch hydrolysis (α -amylase), (ii) main glycolytic enzymes (glucose phosphate isomerase, fructose-1, 6-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase) and (iii) enzymes of alcoholic fermentation (PDC and ADH). Enzymes such as adenylate kinase, cytosolic pyruvate orthophosphate dikinase (PPDK) (Moons *et al.*, 1998), sucrose synthase (Ricard *et al.*, 1991) and α -amylase (Perata *et al.*, 1993) are also known to be stimulated in submerged rice seedlings in response to O₂ depletion.

Hormonal regulation

Submergence-induced shoot elongation and reorientation of leaves are initiated by high concentrations of ethylene (Ku *et al.*, 1970; Voesenek *et al.*, 1993). In completely submerged plants, gas

exchange between intercellular spaces and the aerial atmosphere is severely impeded. Physical entrapment of high concentrations of ethylene in plant tissues which then acts in concert with gibberellin, promotes elongation (Musgrave *et al.*, 1972; Voesenek *et al.*, 1993). Inhibited gas exchange may influence O_2 and CO_2 concentrations also, which are likely to influence the regulation of ethylene biosynthesis. In addition to promoting elongation, high ethylene induces leaf chlorosis during submergence, but not at high CO_2 concentration. Submergence-intolerant cultivars usually showed increased levels of leaf chlorosis followed by seedling death during submergence. Ethylene produced during submergence trigger leaf senescence that reduces seedling survival. Ethylene is accumulated in rice plants during submergence because gas diffusion is 10^4 -fold slower in solution than in air (Armstrong, 1979). Application of an ethylene biosynthesis inhibitor, 1-methyl cyclopropene led to the reduction in elongation growth and better survival (Ella *et al.*, 2003). Plants also maintained higher carbohydrate levels and as a result survival percentage increased. The accumulated ethylene during submergence adversely affected antioxidant mechanism in intolerant rice cultivars, especially after de-submergence (Kawano *et al.*, 2002) and directly affected chlorophyll degradation (Ella *et al.*, 2003). GA_3 application to plants 48 h before submergence resulted in more elongation during submergence, with consequent reduction in plant survival. In contrast, when GA_3 biosynthetic inhibitor paclobutrazol was applied, shoot elongation under submergence was reduced resulting in higher survival percentage (Das *et al.*, 2005; Setter and Laureles, 1996). Furthermore, dwarf mutants exhibiting little or no capacity for GA_3 biosynthesis showed submergence tolerance at par with the highly tolerant landrace FR 13A, when plants of the same mass or carbohydrate content were used (Setter and Laureles, 1996). Pretreatment with exogenous ABA-reduced underwater elongation and improved survival of intolerant plants but there was little effect on elongation or survival of tolerant lines. This suggests that endogenous levels of ABA may be higher in submergence-tolerant lines. There may be an interplay between gibberellins, ABA and ethylene (Jackson and Ram, 2003).

Genetic and molecular basis of submergence tolerance

The knowledge of the genetic and molecular basis of submergence tolerance is important in initiating a sound breeding programme adapted to adverse conditions in the environment. Increasing the tolerance of rice varieties to submergence would clearly reduce the annual yield losses due to flooding (Suprihanto and Coffman, 1981). Therefore, tolerance to submergence is a highly desirable varietal characteristic for flood prone areas (Sinha and Saran, 1998). Research results suggested both simple and quantitative inheritance for submergence tolerance. Additive and non-additive gene effects are important in the inheritance of submergence tolerance. The contribution of non-additive gene effects to the inheritance of submergence tolerance is greater than that of the additive effect (Suprihanto and Coffman, 1981). Sinha and Saran (1998) reported that both additive and dominance effects are important for determining the inheritance of submergence tolerance. Ray *et al.* (1991) observed the importance of additive gene effects in the inheritance of submergence tolerance which also agrees with the result of Mohanty *et al.* (1982) and Haque *et al.* (1989). The additive effects could facilitate fixation of desirable combination of genes (Ray *et al.*, 1991). At least three dominant genes are involved in the control of submergence tolerance. Two have duplicate gene action and the third is complementary to the either of the first two (Suprihanto and Coffman, 1981). Rice genotype with considerable levels of submergence tolerance and high yield can be tailored by genetic manipulation of FR 13A and Janaki with high yielding varieties (Sinha and Ali, 1993). Submergence tolerance in FR 13A and FR 43 B has been reported to be a dominant character with atleast one gene or partially dominant trait under the control of more than one gene, not exceeding function of complementary nature (Mohanty *et al.*, 1982).

Sarkar *et al.* (2006) reported that in Eastern India, sometimes early rains cause water stagnation in the field just after sowing which results in poor crop establishment. They have identified certain landraces like 'Panikekoa' and 'T 1471', which can establish relatively well under water. They also observed that in most cases, rice crop is submerged more than once

during a single season. This repeated stress normally is more devastating even to the previously identified tolerant lines, though the extent of damage is dependent on the duration of each cycle. This necessitates search for cultivars that can tolerate such conditions, particularly those with capacity for faster post-submergence regeneration. This holds true for most of the submergence avoiding types, which are capable of extending their leaf tips above the water surface and thus avoid complete submergence. 'Sabita', a well-known submergence-avoiding variety, showed only 35% survival when submerged twice with a gap of ten days between, whereas 'Matia', a local landrace had more than 80% survival under similar conditions, suggesting that genetic variability in tolerance to repeated flooding may also exist in rice. Most of the existing rice cultivars are seriously damaged if they are completely submerged for more than three days. However, a few tolerant landraces viz. 'FR 13A', 'FR 43B', 'Goda Heenati', 'Kurkaruppan' and 'Thavalu' can withstand complete submergence for 10-14 days. However, these tolerant landraces have poor combining ability and agronomic characters and possess the same locus that controls most of the submergence-tolerance phenotype (*Sub1*) (Xu and Mackill, 1996; Mallik, 2000). Recently, they have identified some new rice landraces with reasonably higher levels of tolerance to submergence but with better agronomic traits than the landraces identified before. These lines are 'Atiranga', 'Khoda', 'Khudara', 'Kusuma' and 'Kalaputia'. Among these new submergence-tolerant genotypes, 'Atiranga' was found to have higher levels of tolerance compared to other tolerant types, including the traditionally most tolerant check, 'FR 13A'. This line has higher regeneration capacity and survival rate even when submerged for long period (18 days). Identification of such lines suggested the possibility of identifying new genes or alleles for higher levels of tolerance.

Concerted efforts made by CRRI, Cuttack and other Rice Research Stations in eastern India on varietal improvement for these adverse situations in flood-prone areas under the ICAR-IRRI collaborative Rainfed Lowland Shuttle Breeding Programme, led to the development of a number of improved lines for submergence tolerance. Some lines like 'CR 2003-

13', 'CR 2006-7', etc. are found to have submergence tolerance at par with 'FR 13A'. A number of improved lines developed/identified under this programme namely 'Kishori', 'Satyam', 'OR 1206-25-1 (Jagabandhu)', 'OR 1234-12-1 (Upahar)', 'CN 1035-61 (Bhudev)', 'CRLC 899 (Varshadhan)', 'TTB 238-3-38-3 (Prafulla)', 'NDR 8002', 'CR 2003-2', 'CR 2003-3', 'CR 978-8-2', etc., have been released in different states of eastern India or are in the advanced stages of testing in national coordinated trials. Besides, a set of advanced lines, namely 'IR 40931-26-3-3-5', 'IR 49830-7-1-2-1', etc. with strong submergence tolerance and desirable agronomic features developed at IRRI are being used in breeding programmes to incorporate submergence tolerance into some of the popular high-yielding lowland varieties using conventional as well as molecular breeding approaches.

Submergence tolerance is reported to be controlled by a single major quantitative trait locus (QTL) on chromosome 9, along with a number of minor QTLs (Xu and Mackill, 1996; Nandi *et al.*, 1997; Toojinda *et al.*, 2003). All these studies have used the landrace FR 13A, which is one of the most submergence-tolerant donor varieties. The major QTL, named *Sub1*, with a LOD score of 36 and an R^2 value of 69% (Xu and Mackill, 1996), provides tolerance to complete submergence for up to 2 weeks. Sequencing the *Sub1* region in an 'FR 13A'-derived line revealed the presence of three genes encoding putative ethylene responsive factors (ERF), *Sub1A*, *Sub1B* and *Sub1C*. *Sub1A* was subsequently identified as the major determinant of submergence tolerance (Xu *et al.*, 2006). The cloning of the major QTL *Sub1* has provided an excellent opportunity not only to gain a better understanding of the molecular mechanisms and unravel the pathways underlying submergence tolerance, but also to design gene-based or tightly linked markers for more precise genotyping. Previous studies have reported the development of submergence tolerant varieties by introducing the *Sub1* locus using marker-aided selection (Siangliw *et al.*, 2003; Toojinda *et al.*, 2005). However, these efforts did not take advantage of the benefits of marker-aided selection in precisely and rapidly transferring the locus. More recently this gene has

been successfully introgressed through marker-assisted backcrossing (MAB) into a popular high-yielding variety from India, Swarna, within a 2-year time frame (Neeraja *et al.*, 2007). 'Swarna-Sub1', the first example of a submergence-tolerant mega variety, was evaluated in submergence-prone areas of India and Bangladesh. Under non-submerged control conditions no significant differences in agronomic performance, grain yield and grain quality between Swarna and Swarna-Sub1 were observed indicating complete restoration of the Swarna background in Swarna-Sub1 (Sarkar *et al.*, 2006; Neeraja *et al.*, 2007), but 'Swarna-Sub1' showed a 2-fold or higher yield advantage over 'Swarna' after submergence for 10 d or more during the vegetative stage (Singh *et al.*, 2009).

Septiningsih *et al.* (2009) reported that the evaluation of the six converted mega varieties under submergence stress showed that all *Sub1* varieties had significantly higher survival rates compared with the original recipient parents. This demonstrated that *Sub1* confers tolerance in different genetic backgrounds. However, there were some differences in the level of tolerance among the *Sub1* lines. Varieties that recovered faster after desubmergence tended to have better vegetative growth. Evaluation of grain quality, yield performance and agronomically important traits of the first three *Sub1* lines (Swarna-Sub1, Samba Mahsuri-Sub1 and IR64-Sub1) grown under control and stress conditions at IRRI was encouraging. The *Sub1* lines were superior under stress and there was no significant difference under non-stress conditions (Singh *et al.*, 2009). Preliminary field trials at different national agricultural research and extension systems (NARES) sites in India and Bangladesh confirmed these results (Sarkar *et al.*, 2006). Evaluation of the six *Sub1* mega varieties under shallow and submerged conditions at the IRRI farm as well as in other countries in flood-prone areas of south and south-east Asia is currently ongoing.

Future prospects

Rice is the only cereal crop that is well adapted to the conditions of waterlogging or partial flooding. Performance of high-yielding varieties can further be augmented if their submergence tolerance is enhanced, which is now becoming more feasible.

Current understanding of the physiological, biochemical, genetic and molecular bases of submergence tolerance has progressed well in recent years, making it possible to design varieties with tolerance to submergence.

So far, only one submergence tolerant landrace ('FR 13A') has been extensively exploited in breeding because of its higher level of tolerance compared with all other lines that were tested before. Recently few more tolerant genotypes distinct from 'FR 13A' were identified. Efforts should be made to characterize these new materials, to study their genetic and physiological bases of tolerance and to discover useful genes and alleles associated with tolerance.

Cultivars are also needed that can withstand repeated flooding and have better regeneration capacity as well as faster growth after flooding to produce sufficient biomass in a shorter period. Maintenance of high levels of stored carbohydrates in the seedlings prior to submergence coupled with minimum shoot elongation and retention of chlorophyll are all desirable traits for submergence tolerance. Biotechnological and genetic approaches to enhance the efficiency of pathways involved in carbohydrate metabolism and regulation of plant hormones to restrict elongation and reduce chlorophyll loss under submergence could help in developing varieties with enhanced tolerance to submergence.

References

- Adkins S.W., Shiraishi, T. and McComb, J.A. (1988). A new glasshouse method for experimental submergence of rice plants. In: Banta, S.J. and Argosino, G.S. (eds.) Proc. 1987 International Deepwater Rice Workshop, IRRI, Los Banos, The Philippines, pp. 311-318.
- Alpi, A. and Beevers, H. (1983). Effect of O₂ concentration on rice seedlings. *Plant Physiol.* 71: 30-34.
- Ap Rees, T., Jenkin, L.E.T., Smith, A.M. and Wilson, P.M. (1987). The metabolism of flood tolerant plants. In: Crawford, R.M.M. (ed.) *Plant Life in Aquatic and Amphibious Habitats*. Blackwell Scientific Publ., Oxford, U.K., pp. 227-238.
- Armstrong, W. (1979). Aeration in higher plants. In: Woolhouse, H.W. (ed.), *Adv. Bot. Res.*, Vol. 7, pp. 225-331.

- Avadhani, P.N., Greenway, H., Lefroy, R. and Prior, L. (1978). Alcoholic fermentation and malate metabolism in rice germinating at low oxygen concentration. *Aust. J. Plant Physiol.* 5 : 15-25.
- Catling, D. (1992). *Rice in Deep Water*. MacMillan Press, London, U.K.
- Chaturvedi, G.S., Mishra, C.H., Singh, O.N., Pandey, C.B., Yadav, V.P., Singh, A.K., Dwivedi, J.L., Singh, B.B. and Singh, R.K. (1995). Physiological basis and screening for tolerance for flash flooding. In: Ingram, K.T. (ed.), *Rainfed Lowland Rice-Agriculture Research for High Risk Environments*. International Rice Research Institute, Manila, Philippines, pp. 79-96.
- Chaturvedi, G.S., Ram, P.C., Singh, A.K., Ram, P., Ingram, K.T., Singh, B.B., Singh, R.K. and Singh, V.P. (1996). Carbohydrate status of rainfed lowland rice in relation to submergence, drought and shade tolerance. In: Singh, V.P., et al. (eds.), *Physiology of Stress Tolerance in Rice*, Proc. International Conf. on Stress Physiology of Rice, Lucknow, India, pp. 103-122.
- Das, K.K., Sarkar, R.K. and Ismail, A.M. (2005). Elongation ability and non-structural carbohydrate levels in relation to submergence tolerance in rice. *Plant Sci.* 168: 131-136.
- Davies, D.D. (1980). Anaerobic metabolism and the production of organic acids. In: Davies, D.D. (ed.) *The Biochemistry of Plants*, Vol. 2. Academic Press, New York, U.S.A. pp. 581-611.
- Ella, E.S., Kawano, N., Yamauchi, Y., Tanaka, K. and Ismail, A.M. (2003). Blocking ethylene perception enhances flooding tolerance in rice seedlings. *Funct. Plant Biol.* 30: 813-819.
- Greenway, H. and Setter, T.L. (1996). Is there anaerobic metabolism in submerged rice plants? A viewpoint. In: Singh, V.P., Singh, R.K., Singh, B.B. and Zeigler, R.S. (eds.) *Physiology of Stress Tolerance in Rice*, Narendra Deva University of Agriculture and Technology, and International Rice Research Institute, Manila, Philippines, pp. 11-30.
- Haque, Q.A., HilleRisLambers, D., Tepora, N.M. and Dela Cruz, Q.D. (1989). Inheritance of submergence tolerance in rice. *Euphytica* 41: 247-251.
- Huang, C., He, W., Guo, J., Chang, X., Su, P. and Zhang, L. (2005). Increased sensitivity to salt stress in an ascorbate-deficient *Arabidopsis* mutant. *J. Exp. Bot.* 56: 3041-3049.
- Jackson, M.B. and Ram, P.C. (2003). Physiological and molecular basis of susceptibility and tolerance of rice plants to complete submergence. *Ann. Bot.* 91:227-241.
- Kawano, N., Ella, E., Ito, O., Yamauchi, Y. and Tanaka, K. (2002). Metabolic changes in rice seedlings with different submergence tolerance after desubmergence. *Env. Exp. Bot.* 47: 95-203.
- Ku, H.S., Suge, H., Rappaport, L. and Pratt, H.K. (1970). Stimulation of rice coleoptile growth by ethylene. *Planta* 90 : 333-339.
- Mallik, S., Kundu, C., Banerji, C., Nayak, D.K., Chatterji, S.D., Nanda, P.K., Ingram, K.T. and Setter, T.L. (1995). Rice germplasm evaluation and improvement for stagnant flooding. In: Ingram, K.T. (ed.) *Rainfed Lowland Rice-Agricultural Research for High Risk Environments*. International Rice Research Institute, Manila, Philippines, pp. 97-109.
- Mallik, S. (2000). Rainfed lowland rice research in India - perspectives and future projections. In: Sharma, R.D., Gahlot, P. and Gahlot, M. (eds.) *Advances in Agricultural Research in India*, International Book Distributors, Dehradun, India Vol. XIII, pp. 1-32.
- Mohanty, H.K., Suprihatno, B., Khush, G.S., Coffman, W.R. and Vergara, B.S. (1982). Inheritance of submergence tolerance in deepwater rice. In: *Proceedings of the 1981 International Deepwater Rice Workshop*. International Rice Research Institute, Los Banos, The Philippines, pp. 121-134.
- Moons, A., Valcke, R. and van Montagu, M. (1998). Low-oxygen stress and water deficit induce cytosolic pyruvate orthophospho dikinase (PPDK) expression in roots of rice, a C₃ plant. *Plant J.* 15 (1): 89-98.
- Musgrave, A., Jackson, M.B. and Ling, E. (1972). Callitriche stem elongation controlled by ethylene and gibberellin. *Nature New Biol.* 238: 93-96.
- Nandi, S., Subudhi, P.K., Senadhira, D., Manigbas, N.L., Sen-Mandi, S. and Huang, N. (1997). Mapping QTLs for submergence tolerance in rice by AFLP and selective genotyping. *Mol. Gen. Genet.* 255: 1-8.
- Neeraja, C., Maghirang-Rodriguez, R., Pamplona, A., Heuer, S., Collard, B. and Septiningsih, E. (2007). A marker-assisted backcross approach for developing submergence-tolerant rice cultivars. *Theor. Appl. Genet.* 115: 767-776.
- Perata, P., Geshi, N., Yamaguchi, J. and Akazawa, T. (1993). Effect of anoxia on the induction of α -amylase in cereal seeds. *Planta* 191: 402-408.

- Ray, P.K.S., HilleRisLambers, D. and Tepora, N.M. (1991). Genetic analysis of submergence tolerance in rice. *Sabao J.* 23(2): 103-110.
- Ricard, B., Rivol, J., Spiteri A. and Pradet, A. (1991). Anaerobic stress induces the transcription and translation of sucrose synthase in rice. *Plant Physiol.* 95: 669-674.
- Sarkar, R.K. (1998). Saccharide content and growth parameters in relation with flooding tolerance in rice. *Biol. Plant.* 40: 597-603.
- Sarkar, R.K., De, R.N., Reddy, J.N. and Ramakrishnayya, G. (1996). Studies on submergence tolerance mechanism in relation to carbohydrate, chlorophyll and specific leaf weight in rice. *J. Plant Physiol.* 149: 623-625.
- Sarkar, R.K., Reddy, J.N., Sharma, S.G. and Ismail, A.M. (2006). Physiological basis of submergence tolerance in rice and implications for crop improvement. *Curr. Sci.* 91: 899-906.
- Septiningsih, E.M., Pamplona, A.M., Sanchez, D.L., Neeraja, C.N., Vergara, G.V., Heuer, S., Ismail, A.M. and Mackill D.J. (2009). Development of submergence-tolerant rice cultivars: the *Sub1* locus and beyond. *Ann. Bot.* 103(2): 151-160.
- Setter, T.L. and Laureles, E.V. (1996). The beneficial effect of reduced elongation growth on submergence tolerance in rice. *J. Exp. Bot.* 47: 1551-1559.
- Setter, T.L., Ellis, M., Laureles, E.V., Ella, E.S., Senadhira, D., Mishra, S.B., Sarkarung, S. and Datta, S. (1997). Physiology and genetics of submergence tolerance in rice. *Ann. Bot.* 79: 67-77.
- Siangliw, M., Toojinda, T., Tragoonrung, S. and Vanavichit, A. (2003). Thai jasmine rice carrying QTLch9 (Sub QTL) is submergence tolerant. *Ann. Bot.* 91: 255-261.
- Singh, H.P., Singh, B.B. and Ram, P.C. (2001). Submergence tolerance of rainfed lowland rice: search for the physiological markers traits. *J. Plant Physiol.* 158: 883-889.
- Singh, S., Mackill, D.J. and Ismail, A.M. (2009). Responses of SUB1 rice introgression lines to submergence in the field: yield and grain quality. *Field Crop Res.* 113: 12-23.
- Sinha, M.M. and Ali, M. (1993). Combining ability for submergence tolerance in rainfed lowland rice. *Oryza* 30: 157-158.
- Sinha, M.M. and Saran, S. (1998). Inheritance of submergence tolerance in lowland rice. *Oryza* 25: 351-354.
- Suprihanto, B. and Coffman, W.R. (1981). Inheritance of submergence tolerance of rice (*Oryza sativa* L.). *Sabao J.* 13: 98-108.
- Toojinda, T., Siangliw, M., Tragoonrung, S. and Vanavichit, A. (2003). Molecular genetics of submergence tolerance in rice: QTL analysis of key traits. *Ann. Bot.* 91: 243-253.
- Toojinda, T., Tragoonrung, S., Vanavichit, A., Siangliw, J.L. and Jantaboon J. (2005). Molecular breeding for rainfed lowland rice in the Mekong region. *Plant Prod. Sci.* 8: 330-333.
- Vartapetian, B.B. and Jackson, M.B. (1997). Plant adaptation to anaerobic stress. *Ann. Bot.* 79 Suppl. A : 3-20.
- Voesenek, L.A.C.J., Banga, M., Their, R.H., Muddle, C.M., Harren, F.J.M., Barendse, G.W.M. and Blom, C.W.P.M. (1993). Submergence-induced ethylene synthesis, entrapment and growth in two plant species with contrasting flooding resistances. *Plant Physiol.* 103: 783-791.
- Widawsky, D.A. and O'Toole, J.C. (1990). Prioritizing the Rice Biotechnology Research Agenda for Eastern India. The Rockefeller Foundation, New York, U.S.A.
- Xu, K. and Mackill, D. J. (1996). A major locus for submergence tolerance mapped on rice chromosome 9. *Mol. Breed.* 2: 219-224.
- Xu, K., Xu, X., Fukao, T., Canlas, P., Maghirang-Rodriguez, R. and Heuer, S. (2006). Sub1A is an ethylene-response-factor-like gene that confers submergence tolerance to rice. *Nature* 442: 705-708.

Micropropagation of pomegranate through *in vitro* culture of meristems: A review

S.K. Naik¹ and P.K. Chand^{2*}

¹Department of Botany, Ravenshaw University, Cuttack-753003

²Plant Cell and Tissue Culture Facility, Post Graduate Department of Botany, Utkal University, Vani Vihar, Bhubaneswar-751004

(Received : 01 September, 2010; Revision received : 01 November, 2010)

Pomegranate is widely cultivated for its delicious nutritive fruits and pharmaceutical properties. The fruit tree is gaining importance in India as well as in the world. Since last three decades micropropagation protocols of different cultivars of pomegranate have been developed to meet the demand of the horticultural industry. The purpose of this review is intended to describe the current information, limitations and future prospects of micropropagation of pomegranate via *in vitro* meristem culture.

Key words: Pomegranate (*Punica granatum* L), micropropagation, meristem culture, axillary bud proliferation

Introduction

The pomegranate, *Punica granatum* L., of Family Punicaceae is an ancient fruit tree of the tropics and sub-tropics, cultivated for its sweet edible fruits. Besides, the tree has been valued through ages for its medicinal properties. Studies indicate that pomegranate is a potent antioxidant and its potential therapeutic properties include treatment and prevention of cancer, cardiovascular diseases, diabetes, erectile dysfunction and protection from ultraviolet radiation (Julie Jurenka, 2008). The tree is also cultivated for ornamental value (Patil and Karale, 1985). Besides, the fruit rind, stem and root bark and leaves are a good source of tannins (Anonymous, 1982). Presently, the pomegranate is cultivated extensively in Iran, Afghanistan, Mediterranean countries including Tunisia, Turkey, Egypt, Spain, Morocco and to some extent in the USA, China, Japan and Russia (Kumar, 1990). In India, pomegranate grows wild in Western Himalayan regions that include states like Himachal Pradesh, Jammu & Kashmir and Uttarakhand (Pandey *et al.*, 2008); nevertheless it is widely cultivated throughout India. The total area under pomegranate cultivation in India is more than 100,000 ha yielding 0.45 million tons of fruits per year (Deepika and

Kanwar, 2010). There is a growing international demand of pomegranate fruits owing to their nutraceutical significance especially in prevention of diseases and ageing (Melgarejo *et al.*, 2009).

Conventionally pomegranates are propagated both by hardwood and softwood cuttings, the former being more commonly used. In addition, seed propagation, air-layering and grafting methods have also been employed. Conventional methods of propagation of pomegranate through stem cutting is time consuming and tedious as it requires about one year to raise the saplings. Seed propagated plants may not be true-to-type in cross pollinated ones (Anonymous, 1982). To circumvent these problems tissue culture has been extensively used for raising multiple clones of pomegranate cultivars; the method being widely referred as *in vitro* clonal propagation or micropropagation.

A range of tissue culture techniques have been developed for rapid true-to-type propagation of several elite pomegranates aimed at large-scale multiplication for commercialization. The *in vitro* culture of meristematic tissues existing either at the apex (shoot tips) or at the axils of branches/leaves (nodes) is the technique most commonly used in commercial micropropagation. This has been the most

*Corresponding author: Email: pkchanduubot@yahoo.co.in

convincingly documented/popular approach to obtain clonal plants because the cells in the apical/axillary meristems supposedly undergo rapid and sustained mitosis thereby resulting in cellular derivatives which are least susceptible to genetic drift under culture conditions. In contrast, although adventitious shoot organogenesis or regeneration of plants by callus mediated organogenesis or somatic embryogenesis are apparently endowed with high propagation rates, they fail to demonstrate trueness-to-type among the regenerated plants because of innate variation between cells of the source tissue and thus usually unsuitable for clonal propagation. Direct (adventitious) somatic embryogenesis, without precocious callusing intervention, shows the best balance of high propagation rates with relatively very few variants (off-types; Phillips and Hubstenberger, 1995). However, despite these advantages, direct somatic embryogenesis is not being used for commercial production of plants due to inadequate synchronization of embryogenesis and low conversion frequency of embryos (Cervelli and Senaratna, 1995).

The technique of micropropagation through axillary bud proliferation (enhanced axillary branching) exploits the normal ontogenetic route for shoot development from axillary vegetative meristems. Either the detectable vegetative bud conspicuous at the axil of a branch/leaf or the nodal region containing a hidden primordium can be a potential candidate to initiate *in vitro* culture. These are treated with plant growth regulator(s) to break dormancy and produce multiple shoots. The shoots are then individualized and induced to root for producing complete plantlets. Alternatively, the primary shoots are further utilized as nodal explants for successive shoot multiplication followed by plant regeneration (Phillips and Hubstenberger, 1995). Compared to conventional propagation this technique offers several advantages which include rapid multiplication of plants, availability of plants throughout the year (season-independence), production of clonally uniform (true-to-type) plants etc. This review presents a consolidated account of micropropagation protocols developed for large scale multiplication of different elite cultivars of pomegranate via meristem culture.

Micropropagation via meristem culture

A successful micropropagation protocol involves a predictable step-wise progress through a series of well-defined stages (i) initiation of aseptic cultures, (ii) *in vitro* bud-break and shoot proliferation, (iii) successive nodal multiplication of shoots, (iv) rooting of *in vitro* regenerated shoots and (v) acclimatization and field transfer of tissue culture raised plants. A chronology of events in micropropagation of pomegranate using axillary bud proliferation of nodal explants is presented in Fig. 1.

Initiation of aseptic cultures

Explant types and Surface disinfection

Explants which carry a pre-formed bud primordium such as mature nodal segments, shoot tips or cotyledonary nodes derived from axenic seedlings have been used for micropropagation of pomegranate. Microbial contamination is one of the serious limiting factors for culture initiation. The commonly adopted procedure used to avoid contamination involves initial washing of explants under running tap water for 30 min - 2h. Depending on the source, the explants were then surface-disinfected with either 0.01% mercuric chloride for 2-3 min (Naik *et al.*, 2000) or 0.1% mercuric chloride for 2-5 min (Naik *et al.*, 1999; Naik and Chand, 2001; El-Agamy *et al.*, 2009) or sodium hypochlorite (0.5 - 5.25%) mixed with a few drops of Tween-20 for 10 min (Al-Wasel, 1999; Sharon and Sinha, 2000) followed by rinsing (3-6 changes) with autoclaved double-distilled water. The surface disinfection procedures adopted in pomegranate micropropagation yielded 95-100% of the explants free of apparent microbial contamination.

Browning of media

Establishment of *in vitro* cultures of several plant species, especially woody plants is greatly hampered by intense browning of the medium because of exudates from cultured explants ultimately causing necrosis. Browning is generally considered to result from the oxidations of phenolic compounds, released from the cut ends of the explants by the enzymes like polyphenol oxidases or peroxidases. The problem is most prevalent in woody plant tissues as they

have high levels of phenols associated with secondary thickening and lignification. The oxidised products (quinones) are known to be highly reactive and to inhibit enzyme activity leading to the death of the explants (Hu and Wang, 1983). Browning of the medium has been a problem for micropropagation of pomegranate and the elimination of browning is necessary for the initiation and establishment of cultures.

In order to reduce phenolic exudation resulting in oxidative browning of shoot tips cultured *in vitro*, Mahishi *et al.* (1991) recommended the addition of

600 mg/l PVP-360 and shifting of explants onto fresh medium within 4-6 hours after initial inoculation. Naik *et al.* (1999) reported browning of the medium following inoculation of mature nodal explants of the pomegranate cultivar 'Ganesh', which inhibited shoot development and resulted in necrosis of the explants within a week. The browning problem was more intense with explants collected from the older shoots than those from younger shoots. To overcome the browning problem they employed a simple method i.e. repeatedly transferring cultured explants to a fresh medium of the same composition at 24 h

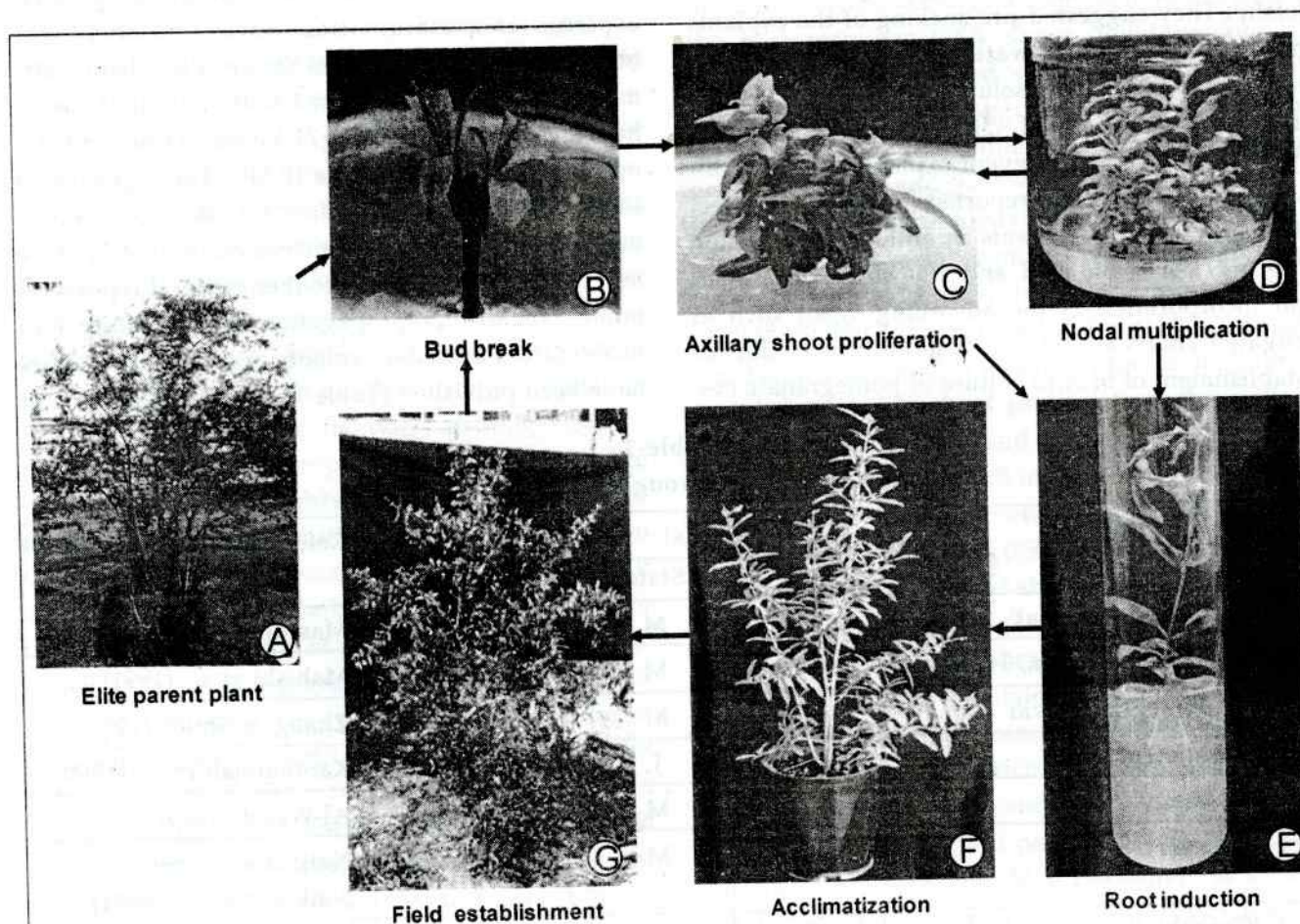


Fig. 1 A summary of events involved in micropropagation by proliferation of axillary buds of mature nodal segments (A) An elite mature tree of pomegranate; (B) Bud break and initial stage of shoot development from a nodal explant in MS + 2.0 mg/l ZR after 12 days of culture; (C) Development of multiple shoots from a nodal explant in MS + 2.0 mg/l ZR after 25 days of culture; (D) Prolific shoot cultures established using nodal segments derived from primary shoots of mature nodal explant in MS + BA (1.0 mg/l) + NAA (0.1 mg/l) on day 15 of culture; (E) A rooted shoot in $\frac{1}{2}$ MS + NAA (0.1 mg/l) on day 15 of culture; (F) A 3 month-old plant, derived from a mature nodal explant, established in garden soil; (G) A 18 month-old plant established in the field.

interval. Murkute *et al.* (2004) were also able to control the browning of cultures of cv. 'Ganesh' by regular subculturing of explants; the phenolic secretion was stopped after two consecutive transfers and the explants exhibited normal shoot development. Interestingly, browning was not observed during *in vitro* culture of cotyledonary nodes of cv. 'Ganesh' (Naik *et al.*, 2000). This was probably because of the juvenility of explants accounting for their inability to synthesize sufficient phenolic compounds. On the other hand, Al-Wasel (1999) reported the necessity for eliminating phenolics for successful establishment of *in vitro* culture of the pomegranate cultivar 'Al-Belehi'. They suggested pre-soaking of the explants in sterilized distilled water (1h) followed by 1h treatment in antioxidant solution containing 150 mg/l of each of ascorbic acid, citric acid and polyvinylpyrrolidone (PVP) to prevent explant browning. El-Agamy *et al.* (2009) also reported the beneficial effect of pre-soaking the explants in antioxidant solution (150 mg/l ascorbic acid and 100 mg/l citric acid) and incorporation of the adsorbing agent such as activated charcoal (3.0 mg/l) in culture media for establishment of *in vitro* culture of pomegranate cvs.

'Manfalouty' and 'Nab El-gamal'. To reduce heavy phenolic exudation leading to explant necrosis while working to establish *in vitro* culture using semi-hardwood nodal segments of cv. 'Jyoti', Singh and Khawale, 2006 also recommended the addition of activated charcoal (200 mg/l) and frequent subculturing of the explants to the fresh media.

In vitro shoot multiplication using mature nodal explants/shoot tips

Mascarenhas *et al.* (1981) were the first to have reported clonal multiplication of pomegranate (*Punica granatum* L.) cvs. 'Ganesh' and 'Muskat' using nodal explants of a mature tree. Shoot initiation was observed on Murashige and Skoog (1962) basal salts medium (MS) supplemented with 500 mg/l casein hydrolysate (CH), 0.5 mg/l kinetin (Kin) and 1.0 mg/l 6-benzylaminopurine (BAP). The regenerated shoots elongated only after transfer to a liquid medium containing a lower concentration of CH (100 mg/l) or without CH. Since then, several reports on pomegranate micropropagation using axillary bud proliferation of nodal explants of different cultivars have been published (Table 1). Micropropagation of

Table-1
Micropropagation through meristem culture

Cultivar / variety	Explant (s)		Response	Reference(s)
	Type	State		
cv. 'Ganesh' & 'Muskat'	NS	M	Msf, Se, R, Pl	Mascarenhas <i>et al.</i> (1981)
Pomegranate selection; HS-4,	ST	M	Msf, Se, R, Pl	Mahishi <i>et al.</i> (1991)
Dwarf pomegranate var. 'nana'	NS	M	Msf	Zhang & Stoltz (1991)
cv. 'Wonderful'	ANC	J	Msf, R, Pl	Kantharajah <i>et al.</i> (1998)
cv. 'Al-Belehi'	NS	M	Msf, R, Pl	Al-Wasel (1999)
cv. 'Ganesh'	NS	M	Msf, Se, R, Pl	Naik <i>et al.</i> (1999); Naik & Chand (2001)
cv. 'Ganesh'	CN	J	Msf, R, Pl	Naik <i>et al.</i> (2000)
cv. 'Ganesh' & 'Kabul'	CN	J	Msf, Se, R, Pl	Sharon & Sinha (2000)
cv. 'Ganesh'	ST & NS	M	Msf, R, Pl	Murkute <i>et al.</i> (2004)
cv. 'Jyoti'	NS	M	Msf, R, Pl	Singh & Khawale (2006)
cv. 'Manfalouty' & Nab-El-gamal	ST	M	Msf, R, Pl	El-Agamy <i>et al.</i> (2009)

ANC: Axenic nodal cuttings; CN: Cotyledonary node; NS: Nodal segment; ST: Shoot tip; M: Mature; J: Juvenile; Msf: Multiple shoot formation; Pl: Plantlet formation; R: Rooting; Se: Shoot elongation

a dwarf pomegranate var. 'nana' using nodal explants (from terminal shoots with 7-10 nodes) was reported; an average of 6.6 shoots per node were produced by culturing the nodal explants on modified MS medium supplemented with 1.0 μ M naphthaleneacetic acid (NAA) and 2.0 μ M BAP (Zhang and Stoltz, 1991). Mahishi *et al.* (1991) developed a micropropagation method using shoot tips of pomegranate selection (HS-4). They used MS supplemented with 8.0 mg/l BAP, 1.0 mg/l NAA and 600 mg/l PVP followed by modified woody plant medium (WPM; Lloyd and McCown, 1980) containing 6.0 mg/l indole-3-acetic acid (IAA), 0.6 mg/l Kin and 440 mg/l CaCl_2 for shoot bud activation and shoot proliferation (growth and elongation) respectively. They also observed that sub-culturing not only resulted in rapid elongation of the axillary buds but also accounted for better root initiation during subsequent cultures. Kantharajah *et al.* (1998) described a method for rapid multiplication of plants from nodal cutting of aseptically grown cultivar 'Wonderful' of pomegranate. They examined the effects of basal media formulations (MS; $\frac{1}{2}$ MS; White's Medium, 1963 and WPM) as well as plant growth regulators (BAP and NAA) on *in vitro* propagation of pomegranate. In their report, the rate and average number of shoots obtained via axillary bud proliferation of nodal cuttings was the maximum on $\frac{1}{2}$ MS medium supplemented with 0.5 mg/l BAP and 0.1 mg/l NAA. Naik *et al.* (1999) reported *in vitro* clonal propagation of an elite cultivar of 'Ganesh' using nodal stem segments of a mature tree. Bud break and axillary shoot proliferation was induced in nodal segments on MS medium. Of the different cytokinins tested (BAP; zeatin riboside [ZR] and thidiazuron [TDZ]) to observe their influence on bud break and shoot proliferation from nodal segments, ZR was found to be most effective. The highest number of shoots per explant (5.2) was recorded only on MS medium containing 2.0 mg/l ZR. These shoots elongated within 4-6 days following transfer to a medium containing a lower concentration of ZR (1.0 mg/l). However, Murkute *et al.* (2004) found that MS supplemented with BAP (1.0 mg/l) and NAA (0.5 mg/l) was necessary for *in vitro* plant regeneration using nodal segments or shoot tips. Al-Wasel (1999) reported *in vitro* clonal propagation of

pomegranate cultivar 'Al-Belehi' using nodes from one year-old plants grown in the greenhouse. The highest number of shoot formation was observed on MS media supplemented with BAP either at 2.0 mg/l (3.33 shoots/explant) or 4.0 mg/l (3.4 shoots/explant), without any significant difference between the two concentrations.

Naik and Chand (2001) reported that MS was more effective than WPM for *in vitro* propagation of pomegranate. Further with a view to upscaling propagation, the primary shoots obtained were multiplied further as stem nodal segment. Of the different concentrations and combinations evaluated, the best response (78% shoot development; 4.1 shoots/node) was elicited with MS augmented with 1.0 mg/l BAP and 0.1 mg/l NAA. In 60 days about 50-55 shoots were obtained from a single nodal explant. Singh and Khawale (2006) reported a procedure for micropropagation of cv. 'Jyoti' using semi-hardwood nodal segments from a mature tree. Half-strength MS medium augmented with a combination of 1.0 mg/l BAP, 1.0 mg/l Kin and 200 mg/l activated charcoal was the optimum for culture establishment. For shoot proliferation, the axillary shoots were excised and transferred to shoot proliferation medium (1.0 mg/l BAP, 1.0 mg/l Kin and 40 mg/l adenine sulfate), where highest frequency of proliferation (89.8%), maximum number of shoots (3.4) and longest shoot length (2.2 cm) was obtained on average. El-Agamy *et al.* (2009) developed a method for rapid multiplication of plants from shoot tips of two cultivars 'Manfalouty' and 'Nab El-gamal' of pomegranate. They examined the effects of basal media (MS; Nitsch & Nitsch, 1969; WPM) and plant growth regulators (BAP and Kin) on *in vitro* shoot regeneration. The highest percentage bud break (cv. 'Manfalouty': 100%; cv. 'Nab El-Gamal': 60%) and number of shoots formed (cv. 'Manfalouty': 6.8; cv. 'Nab El-Gamal': 5.8) was maximum on WPM supplemented with 1.0 mg/l BAP.

In vitro shoot multiplication using axenic cotyledonary node explants

The pomegranate is capable of both self and open pollination. The use of a marker gene, governing pigmentation of bud flower and petiole base

confirmed the fact that pomegranate (cvs. 'Ganesh' and 'Kabul Yellow') is usually self-pollinated, but in low proportion (13%), cross-pollinated (Jalikap and Sampath Kumar 1990). Keeping both self and cross pollination ability of pomegranate in view, the cotyledonary node-based regeneration system could be useful in two ways. First, the regeneration system based on using the cotyledonary node (excised from a seedling based on self pollination derived seeds) would generate a large number of true-to-type plants. On the other hand, the system based on cotyledonary node (excised from a seedling based on cross pollination derived seeds) could be an efficient strategy to obtain somaclonal variants of pomegranate possessing agronomically desirable traits. Nevertheless, in view of a relatively low heterozygosity in pomegranate populations associated with infrequent open pollination, plants raised from juvenile source via cotyledonary node culture will be expected to be clonally uniform.

Naik *et al.* (2000) reported high frequency axillary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate cv. 'Ganesh' for which MS medium was used for inducing shoot development. The highest percentile shoot regeneration (94%) and maximum number of shoots per explant (ca 10 shoots/cotyledonary node) was achieved with only a single cytokinin, BAP, at an optimal level of 9.0 μM . Contrarily, according to Sharon and Sinha (2000) Gamborg's B₅ medium (1968) supplemented with a combination of cytokinins and an auxin (1.0 mg/l BAP + 1.0 mg/l Kin + 0.05 mg/l NAA for cv. 'Ganesh' and 0.5 mg/l BAP + 0.05 mg/l NAA for cv. 'Kabul') was necessary for high frequency axillary shoot proliferation from cotyledonary node explants. In this medium they claimed to have induced multiple shoots (7-9 shoots/cotyledonary node). Nevertheless, the protocol described by Naik *et al.* (2000) was devoid of any shoot elongation problem. This fact was quite different from that reported by Sharon and Sinha (2000) in *P. granatum* L. cv. 'Kabul' and 'Ganesh', where the *in vitro* formed shoots were required to be isolated and transferred to an elongation medium (B₅ + 0.05 mg/l NAA + 0.5 mg/l BAP + 10% coconut milk) for 1 month prior to rooting. Naik *et al.* (2000) obtained about 30-35 shoots

within 60 days from a single cotyledonary node by the establishment of shoot culture by repeatedly sub-culturing the original cotyledonary node on fresh shoot multiplication medium (MS + 9.0 μM BAP) after each harvest of newly formed shoots and also culturing nodal segments excised from *in vitro* regenerated primary shoots. We further studied the influence of growth regulators on shoot proliferation in axenic nodal segments derived from cotyledonary-based shoot cultures and reported that about 89% of the nodes developed multiple shoots with an average of 7.6 shoots per node in 30 days on MS supplemented with 1.0 mg/l BAP + 0.1 mg/l NAA. Thus from a single cotyledonary node about 190-200 shoots were obtained within 60 days (Naik, 2002).

Rooting of in vitro regenerated shoots

Focal to a complete micropropagation protocol is the rooting of *in vitro* regenerated shoots, the latter being a mandatory requirement to facilitate plant establishment in soil. For pomegranate micro-propagation through meristem culture addition of auxin to the medium was essential to induce rooting in the regenerated shoots. Mascarenhas *et al.* (1981) reported rooting for cultivars 'Ganesh' and 'Muscat' in White's (1963) medium. They indicated that shoot culture in a combination of auxins (2.0 mg/l each of IAA, IBA [indole-3-butyric acid] and IPA [indole-3-propionic acid]) under 72 h dark incubation followed by transfer to an auxin-free medium was effective for rooting. Naik *et al.* (1999) achieved rooting (86%) of *in vitro* regenerated shoots of cultivar 'Ganesh' on half-strength MS medium supplemented with a single auxin IBA (1.0 mg/l). There was also a requirement for transfer of rooted shoots to an auxin-free half-strength MS medium for elongation of roots as well as development of additional roots. El-Agamy *et al.* (2009) used $\frac{1}{2}$ WPM supplemented with IBA (0.25 mg/l) for rooting while working with cv. 'Manfalouty'. Naik *et al.* (2000) successfully rooted the *in vitro* raised shoots in half-strength MS medium augmented with a different auxin, NAA (0.54 μM). But they still found the requirement of transfer of rooted shoots to an auxin-free $\frac{1}{2}$ MS medium for root elongation. Al-Wasel (1999) achieved rooting on half-strength MS medium supplemented with 0.5

mg/l NAA, while El-Agamy *et al.* (2009) found best rooting on ½ WPM supplemented with 0.25 mg/l NAA in cv. 'Nab El-gamal'. MS medium supplemented with NAA at a high concentration (3.0 mg/l) and 3 g/l activated charcoal was also reported to be the best rooting treatment for pomegranate by Mahishi *et al.* (1991). Some other laboratories reported the requirement of different basal media for *in vitro* rooting in pomegranate. Kantharajah *et al.* (1998) reported the use of WPM medium supplemented with NAA at an optimum concentration of 2.0 mg/l for rooting of shoots in cv. 'Wonderful'. B₅ medium augmented with 3.0 mg/l IAA was found to be effective for rooting of shoots in cultivar 'Ganesh' and 'Kabul' (Sharon and Sinha, 2000). Singh and Khawale (2006) observed the stimulatory effect of activated charcoal on rooting of *in vitro* regenerated shoots. They found MS medium supplemented with 2.0 mg/l IBA, 200 mg/l activated charcoal and 40 g/l sucrose was the best recipe for rooting of *in vitro* regenerated shoots of cv. 'Jyoti', in which 88.7% rooting with 4.2 roots per shoot was obtained.

Acclimatization and field transfer of tissue culture raised plants

The successful acclimatization of micropropagated plants and their efficient transfer to the field on large scale, at low cost and with negligibly low mortality rates is a crucial step for commercial exploitation of micropropagation. Naik *et al.* (1999, 2000) were able to acclimate *in vitro* regenerated plantlets of pomegranate cv. 'Ganesh' in vermicompost and subsequently in soil with a survival rate of 60-68% and 75-84% respectively. Al-Wasel (1999) reported more than 80% of survivability of plantlets (cv. 'Al-Belehi') in perlite inside a greenhouse under mist. Pots filled with either vermiculite or a 1:1 mixture of vermiculite and soil in the net house (80% RH, 14-18 klux light intensity and temperature below 30° C) was used for acclimatization of cv. 'Ganesh' and 'Kabul' plantlets with cent-per-cent survivability (Sharon and Sinha, 2000). Singh and Khawale (2006) reported the use of sterile mixture of peat: soilrite (1:1) moistened with ¼ MS macro and micro-salts without organics for maximum survival (78.7%) of plantlets after 45 days of transfer.

Unfortunately, little information is available regarding the acclimatization of tissue culture raised plants in many published reports on pomegranate. This is a bottleneck for the exploitation of micropropagation of pomegranate in commercial scale.

Conclusion

The genetic improvement of pomegranate for abiotic or biotic stress tolerance leading to increased productivity is essential to meet the future demand for its delicious fruits holding nutraceutical significance. Till date, the pomegranate improvement has been limited to the development of new elite cultivars/varieties through conventional genetics and breeding efforts. To complement conventional breeding, molecular approach offers an efficient alternative. The assessment of genetic diversity through molecular markers among the wild pomegranates or cultivars could be useful for identifying new germplasm sources (with biotic and abiotic traits) which, when crossed with existing varieties, would result in quality improvement coupled with enhanced yield. More importantly efforts are being made to develop efficient transformation protocols aiming at introduction of heterologous candidate genes encoding desired characteristics. Attempts may be worth towards production of asymmetric somatic hybrids following intergeneric protoplast fusion, thus enabling introgression of desirable genes into pomegranate from allied fruit tree species. Novel hybrids, through improved breeding program, somatic hybridization or molecular breeding via transgenic manipulations, endowed with desired traits are expected in near future with the prospect of enhanced quality and productivity of pomegranate. In this context, micropropagation holds a tremendous potential being a common traffic for clonal (true-to-type) multiplication thereby facilitating scaling-up of the limited number of improved varieties generated through various biotechnological interventions.

References

- Al-Wasel, A.S.A. (1999). *In vitro* clonal propagation of "Al-Belehi" pomegranate (*Punica granatum* L.). J. King Saud. Univ. 11(1): 3-14.

- Anonymous (1982). A Dictionary of Indian Raw Materials and Industrial Products, Vol. VIII (Ph-Re), CSIR Publication, New Delhi, India.
- Cervelli, R. and Senaratna, T. (1995). Economic aspects of somatic embryogenesis. In: Aitken-Christie, J. (ed.), Automation and Environmental Control in Plant Tissue Culture, Kluwer, Dordrecht, The Netherlands, pp. 29-64.
- Deepika, R. and Kanwar, K. (2010). *In vitro* regeneration of *Punica granatum* L. plants from different juvenile explants. J. Fruit Orn. Plant Res., 18(1): 5-22.
- El-Agamy, S.Z., Mostafa, R.A.A., Shaaban, M.M. and El-Mahdy, M.T. (2009). *In vitro* propagation of Manfalouty and Nab El-gamal pomegranate cultivars. Research J. Agric. Biol. Sci. 5 (6): 1169-1175.
- Gamborg, O.L., Miller, R.A. and Ojima, K.I. (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158.
- Hu, C.Y. and Wang, P.J. (1983). Meristem, shoot tip and bud cultures. In: Evans, D.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y. (eds.) Handbook of Plant Cell Cultures, vol. 1. MacMillan, New York, U.S.A. pp. 177-227.
- Jalikop, S.H. and Sampath Kumar, P. (1990). Use of a gene marker to study the mode of pollination in pomegranate (*Punica granatum* L.). J. Hort. Sci. 65(2): 221-223.
- Julie Jurenka, M.T. (2008). Therapeutic Applications of Pomegranate (*Punica granatum* L.): A Review. Alt. Med. Rev. 13 (2): 128-144.
- Kantharajah, A.S., Dewitz, I. and Jabbari, S. (1998). Auswirkungen unterschiedlicher Nahrmedien, Wachstums-regulatoren und Explantatquellen auf die in-vitro-Vermehrung von Granatapfel (*Punica granatum* L.) [The effect of media, plant growth regulators and source of explants on *in vitro* culture of pomegranate (*Punica granatum* L.). Erwerbsobstbau. 40: 54-58.
- Kumar, G.N.M. (1990). Pomegranate. In: Nagy, S., Shaw, P.E., Wordowski, W.F., (eds) Fruits of Tropical and Subtropical Origin, Florida Science Source, Inc, USA. pp. 328-347.
- Lloyd, G.B. and McCown, B.H. (1980). Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia* by use of shoot tip culture. Proc. Plant Prop. Soc. 30: 421-427.
- Mahishi, D.M., Muralikrishna, A., Shivashankar, G. and Kulkarni, R.S. (1991). Shoot tip culture method for rapid clonal propagation of pomegranate (*Punica granatum* L.). In: Prakash, J. and Pierik, R.L.M. (eds.) Horticulture-New Technologies and Applications: Proc. International Seminar New Frontiers in Horticulture, Kluwer Acad. Publ., Dordrecht, The Netherlands, pp. 215-217.
- Mascarenhas, A.F., Gupta, P.K., Kulkarni, V.M., Mehta, U., Iyer, R.S., Khuspe, S.S. and Jagannathan, V. (1981). Propagation of trees by tissue culture. In: Rao, A.N. (ed.) Proc. COSTED Symp. Tissue Culture of Economically Important Plants, Singapore Inst. of Biol., Bangalore, India, pp. 175-179.
- Melgarejo, P., Martinez, J.J., Hernandez, F., Martinez, R., Legua, P., Oncina, R. and Martinez-Murcia, A. (2009). Cultivar identification using 18S-28S rDNA intergenic spacer-RFLP in pomegranate (*Punica granatum* L.). Scient. Hort. 120: 500-503.
- Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
- Murkute, A.A., Patil, S. and Singh, S.K. (2004). *In vitro* regeneration in pomegranate cv. Ganesh from mature trees. Ind. J. Hort. 61 (3): 206-208.
- Naik, S.K. (2002) Studies on *in vitro* regeneration of pomegranate (*Punica granatum* L. cv. Ganesh). Ph.D. thesis, Utkal University, Bhubaneswar, Orissa, India. 120 p.
- Naik, S.K. and Chand, P.K. (2001). *In vitro* cloning of a medicinally important fruit tree *Punica granatum* L. by nodal culture. In: Sahoo, S., Ramesh, D.B., Rao, Y.R., Debata, B.K. and Mishra, V.N. (eds.) Conservation and Utilization of Medicinal and Aromatic Plants. Allied Publishers Limited, New Delhi, India pp. 313-322.
- Naik, S.K., Pattnaik, S. and Chand, P.K. (1999). *In vitro* propagation of pomegranate (*Punica granatum* L. cv. Ganesh) through axillary shoot proliferation from nodal segments of mature tree. Scient. Hort. 79: 175-183.
- Naik, S.K., Pattnaik, S. and Chand, P.K. (2000). High frequency axillary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate (*Punica granatum* L.). Scient. Hort. 85: 261-270.
- Nitsch, J.P. and Nitsch, C. (1969). Haploid plants from pollen grains. Science, 163: 85-87.
- Pandey, A., Tomer, A.K., Bhandari, D.C. and Pareek, S.K. (2008). Towards collection of wild relatives of crop plants in India. Genet. Resour. Crop Evol. 55: 187-202.

- Patil, A.V. and Karale, A.R. (1985). Pomegranate. In: Bose, T.K. (ed.) Fruits of India: Tropical and Subtropical, Naya Prokash, Kolkata, India, pp. 537-548.
- Phillips, G.C. and Hubstenberger, J.F. (1995). Micropropagation by proliferation of axillary buds. In: Gamborg, O.L. and Phillips, G.C. (eds.) Plant Cell, Tissue and Organ Culture, Fundamental Methods, Narosa Publ. House, New Delhi, India, pp. 45-54.
- Sharon, M. and Sinha, S. (2000). Plant regeneration from cotyledonary node of *Punica granatum* L. Ind. J. Plant Physiol. 5(4): 344-348.
- Singh, S.K. and Khawale, R.N. (2006). Plantlet regeneration from the nodal segments of pomegranate (*Punica granatum* L.) cv. Jyoti. In: Kumar, A. and Roy, S. (eds.) Plant Biotechnology and its Applications in Tissue Culture. IK International Pvt. Ltd. New Delhi, India, pp. 107-113.
- White, P.R. (1963). The Cultivation of Animal and Plant Cells. Ronalds Press, New York, U.S.A.
- Zhang, B. and Stoltz, L.P. (1991). *In vitro* shoot formation and elongation of dwarf pomegranate. Hort Sci. 26(8): 1084.

Low cost *in vitro* propagation: A review

A.K. Behera, S. Gochhait and U.B. Mohapatra^Ψ

P.G. Department of Botany, North Orissa University, Takatpur, Baripada-757003

(Received : 12 November, 2010; Revision received : 21 December, 2010)

Plant tissue culture is one of the most rapidly growing areas of biotechnology due to its high potential to develop improved qualities in plants. With advances in tissue culture technology, it is now possible to regenerate plants of any species in the laboratory from any plant part on appropriate nutrient media under aseptic condition. Because of the wide range of applications, plant tissue culture attracts the attention of molecular biologists, plant breeders and industrialists. In order to popularize the techniques among the developing countries and making it more useful for common farmers, low cost alternatives are needed, which is a challenging task. An effort has been made in this review to focus on such alternative strategies and their application in large scale *in vitro* propagation of plants.

Key words: Low cost propagation, micropropagation, plant tissue culture

Introduction

The commercial tissue culture technology is primarily based on micropropagation. This involves rapid proliferation achieved from stem nodal cuttings, axillary buds, and to a limited extent from somatic embryos, cell clumps in suspension cultures and bioreactors. The cultured cells and tissues can take several pathways. The pathways that lead to the *in vitro* production of plants in large numbers are either organogenesis or somatic embryogenesis. True-to-type plants by meristem culture or via synthetic seeds are preferred for commercial multiplication. The process of micropropagation is usually divided into several stages i.e. explant selection, shoot proliferation, rooting and hardening. Micropropagation technology is more expensive than the conventional methods of plant propagation, and requires skills. It is a capital-intensive industry, and in some cases, the unit cost per plant becomes unaffordable. Therefore, low cost alternatives are needed to reduce production cost of tissue-cultured plants. In less developed countries of Africa, Asia, and Latin America, where labour is relatively cheaper, consumables for micropropagation process such as media, culture containers, and electricity plus maintenance of culture room and hardening in greenhouse, make a comparatively greater contribution to production cost. Therefore, it is a

challenging task to adopt the low cost options of micropropagation without compromising the quality of the micropropagules and plants. An attempt has been made in this review to focus on the strategies of low cost production with respect to media making and handling, culture containers, low cost energy options, water sources, cost of labour, controlling somaclonal variations, culture contamination, and low cost hardening.

Strategies for cost reduction

Contamination control

The loss of culture increases the cost of production. Contamination in culture is caused from natural contaminants such as dust, air borne particles, bacteria and fungal spores, fibers and hair. Man made contamination occurs mainly from body, clothing and from faulty procedures in the laboratory. Wearing laboratory coats reduces contamination from clothing, skin and hair. There is continuous entry of contaminants with the worker and through air entering the growth room (Ahloowalia and Prakash, 2002). A set of routine procedures such as regular cleaning of working areas of laboratory, wearing laboratory coat, avoiding frequent opening of growth room etc. should be followed to reduce contamination in the laboratory; however, such procedures may vary with the location and requirement (Bruderere and Luwa, 1983; Schicht, 1985).

^ΨCorresponding author; E-mail: ubmbotnou@gmail.com

Washing and sterilizing operation

Costly dishwashing machines for cleaning the culture containers may be replaced by manual washing if labour is relatively cheap. The washed culture containers can be dried in sun instead of costly hot air ovens if weather permits. However, precautions must be taken to avoid dust accumulation. In small scale laboratories, the autoclaves can be replaced by large size pressure cookers, which are much cheaper. Autoclavable, less expensive, stainless steel containers can be used, for sterilization of glassware, pipettes etc. instead of wrapping these instruments with expensive aluminium foils (Ahloowalia and Prakash, 2002; Prakash, 1993).

Culture maintenance in growth rooms

Plant growth chambers are very expensive. Instead, plant culture can be maintained in rooms with air conditioners and tube lights. Downward illumination (conventional method) can be replaced by sidewise lighting systems, which reduce the number of lights but provides more uniform illumination to the culture (Ganapathi *et al.*, 1995). Electrical systems can be replaced by solar power, as done in the tropical and mediterranean regions (Ahloowalia and Prakash, 2002).

Production planning

Production planning means working procedures and monitoring the performance. Most plants are seasonal in demand. To meet the demand of market, commercial production has to be backed by production planning. Commercial laboratories should produce a range of plants for different seasons to maximize their annual production rate (Ganapathi *et al.*, 1995). This can lower the unit cost of plant production. To reduce organizational problems in these units, the management structure should be well planned. A large scale micropropagation unit needs some management operations, such as organizing the available personnel, supporting information system (inventory control, production scheduling, space utilization, daily targets etc.), maintenance of good co-ordination among the technicians, supporting staff, supervisors, the researchers, etc., strict

adherence to preparation of job chart or work description of each personnel in a unit, efficient reporting system, personnel selection and training, and periodical review of customers' need (Ahloowalia and Prakash, 2002), to enhance production.

Low cost alternatives

Low cost culture media

Chemical constituents of a tissue culture medium cost less than 15% of micro-plant production (Prakash, 1993). In some cases chemicals constituents cost less than 5%. Other media components e.g. the gelling agent such as agar contribute 70% of the cost. Other ingredients in the media, such as salt, sugar and growth regulators, have minimal influence on production cost and are reasonably cheap. However, low cost options are available to replace expensive gelling agents, sugar and reduce the cost of water (Prakash *et al.*, 2002).

Gelling agents

Growth of cultures and production of shoot or roots is strongly influenced by the physical consistency of the culture medium. Gelling agents are usually added to the culture medium to increase its viscosity, as a result of which plant tissue and organs remain above the surface of the nutrient medium. Many gelling agents are used for plant culture media. e.g. agar, agarose and gellan gum and are marketed under trade names such as phytigel, gelrite and gel-gro. Agar is the most commonly used gelling agent for preparation of solid and semisolid media. It contributes to the matrix potential, the humidity and affects the availability of water and dissolved substances in the culture containers (Debergh, 1983). Various brands and grades of agar are available commercially, which differs in the amounts of impurities and gelling capacity. Agar brands vary widely in price, performance and composition. It is the actual use and experience, which ultimately determines the choice of agar brand in a specific system and for a plant species (Prakash *et al.*, 2002). It is usually unnecessary to use high purity agar for large-scale micro-propagation. Cheap brand of agar have been successfully used for industrial scale micro-

propagation (Boxus, 1978). Lower concentration of agar can be used depends on its purity and brands. Agar is normally used at 0.6-0.8% (w/v). It is advisable to prepare sample media in small quantities using various concentrations of agar e.g. 0.7, 0.75, 0.8, 0.85 and 0.9%. The appropriate concentration should then be used for large scale production purposes. Use of low concentration of gelling agent is not only less expensive, but also it has other advantages. It results to form a semi-solid medium which ensures adequate contact between the plant tissue and the medium. It is beneficial for growth as it allows better diffusion of medium constituents and is easily removed from plantlets before their transfer to soil. For these reasons, a semisolid medium is often preferred over solid medium (Prakash *et al.*, 2002). Cheap alternative to agar include various types of starches and plant gums (Pierik, 1989; Nagamori and Kobayashi, 2001). Gelrite can be replaced with starch-gelrite mixture (Kodym *et al.*, 2001). For micro-propagation of ginger and turmeric, the combination of certain gelling agents gave growth as good as on agar-based media. The use of laundry starch, potato starch and semolina in a ratio 2:1:1 reduced the cost of gelling agent by 70-82% (Prakash, 1993). The addition of such gelling alternatives to the medium has some disadvantages since they contain inhibitory substances that hinder morphogenesis (Powel and Uhrig, 1987). Corn starch (CS) as a gelling agent has been used along with low concentration of gelrite

(0.5 g/l gelrite+50.0 g/l CS) for the propagation of fruit trees, such as apple, pear and raspberry, banana and sugarcane, ginger and turmeric (Zimmerman, 1995; Stanley, 1995). The shoot proliferation was better on CS medium than on agar. The cost of CS was about Rs 100/kg compared with about Rs 800/kg of agar. However, it becomes difficult to detect the contamination because the CS medium turned grayish-white (Stanley, 1995). Addition of 8.0% tapioca starch to the MS medium was found to be a good substitute for 'bacto-agar' for potato shoot culture (Getrudis and Wattimena, 1994). Barley starch (60 g/l) has also been used for culturing potato tuber discs, and for anther culture of barley (Sorvari, 1986; Sorvari and Schieder, 1987). Isubgol, a colloidal mucilaginous husk (chiefly composed of pentosans) derived from seeds of *Plantago ovata*, has good gelling activity and has reasonable clarity in gelled form. Isubgol at 3% in MS medium has been used for the propagation of *Chrysanthemum*. The cost of Isubgol is about Rs 150/kg (Babbar and Jain, 1988; Bhattacharya *et al.*, 1994). Sago, obtained from the stem pith of *Metroxylon* at 13% concentration was substituted for agar in MS medium for the multiplication of *Chrysanthemum*. The number of shoots, leaves and root length were significantly higher on sago than on agar. The cost of sago is Rs 20/kg (Bhattacharya *et al.*, 1994). Percentage of agar alternatives and their effect on medium and culture growth is presented in Table-1.

Table-1

Percentage of Agar alternatives	Effect on medium and culture growth
Wheat flour (10%)	Sloppy medium, Growth of culture poor
Wheat flour (8%)	Proper solidification, Growth of culture bellow average
Laundry starch (6%)	Proper solidification, Growth of culture bellow average
Semolina (5%)	Sloppy medium, Growth of culture average
Potato powder (7%)	Sloppy medium, Growth of culture average
Rice powder (11%)	Sloppy medium, Growth of culture poor
Sago (7%)	Proper solidification, Growth of culture normal
Laundry starch + Potato powder + Semolina (2:1:1)	Solidification, Growth of cultures as good as on agar media

Source: Prakash, 1993

Low cost matrices on liquid media

Suspension cultures without gelling agents are commonly used for culturing callus, cell clusters, buds and somatic embryos. Suspension system allows greater contact between the explants and the medium. Agitation of such media reduces the diffusion gradient in the nutrition supply. The toxic metabolites exuding from the tissue are also dispersed effectively (Prakash *et al.*, 2002). Sterilized, on-chlorine bleached rolled, pure cotton has been successfully used for culturing callus and proliferating shoots of *Taxus*, *Agrotis* and *Artemisia* (Moraes-cerdeira *et al.*, 1995). The use of cotton fiber support to cultures in liquid media has been reported in the commercial propagation of orchids, banana, *chrysanthemum* and potato in Pakistan and Bangladesh. Cotton fiber and MS liquid medium produced rapid growth of banana shoots, which could be sub-cultured after two instead of six weeks. Protocorm initiation and shoot development of orchids was much faster on cotton than on agar based medium. Other alternative culture supports includes foam plastic, filter paper bridges, glass wool, nylon cloth, polystyrene foam, glass beads etc. (Prakash *et al.*, 2002). Strips (2.5x15cm) of glass wool, nylon and filter paper have been used as supporting bridges for the propagation of *chrysanthemum* on MS medium. The shoot and root growth was almost identical on these matrices. Polystyrene foam blocks (2x2x1cm) have been used as supporting matrix for the propagation of *Chrysanthemum*. The growth response was similar to that on glass wool, except that explants on foam produced significantly fewer roots but longer shoots than on glass wool (Bhattacharya *et al.*, 1994). Ginger and turmeric plants multiplied on glass bead liquid-medium performed as good as on agar-based medium. A similar type of response was observed for *Vanila*, *Ficus cv. Mini Lucii*, *Saintpaulia*, *Syngonium*, *Philodendron* and *Spathiphyllum*. Glass bead based liquid media were successfully used for culturing several plant species and use of it can reduce the medium cost by 94%. With glass beads the amount of medium required is only 15-18 ml per culture container. By using 20 ml medium per culture container, one liter medium will give 50 culture containers, a substantial saving in medium cost

(Prakash, 1993). An additional advantage of liquid medium with glass beads is that the medium can be replaced without moving plants (Macleod and Nowak, 1990). It should be noted that the glass beads can be reused after washing with acid. Glass beads have been used for the propagation of raspberry and white clover resulting in 60% saving in media cost. The glass beads allowed easy removal of plantlets from the medium. Glass beads have also been used for the maintenance of callus and shoot organogenesis (Mc Culloch *et al.*, 1994). Liquid media have some disadvantages. Delicate tissues get damaged during agitation. In some species, shoot submerged in liquid media exhibit hyperhydricity (water soaked) and unsuitable for micropropagation (Prakash *et al.*, 2002).

Sugar alternative

Sucrose is the most commonly used carbon source in the micropropagation of plants. However, sucrose adds significantly to the cost of media. Household sugar and other sugar sources can be used to reduce the cost of the medium. Sugar sold in grocery stores is sufficiently pure for micropropagation. Use of common sugar reduces the cost of the medium up to 78 to 87% (Prakash *et al.*, 2002). Several alternate sources of sugar have been used to observe their effect on growth of cultured plants. Household sugar (3%), doubled refined sugar (3%), sugar crystals (3%) and sucrose LR grade (3%) in tissue culture media exhibited healthy cultures. Sugarcane juice (10%, v/v) caused drying of leaf tips during culture of ginger and turmeric plants (Prakash, 1993). As low cost sugar in the medium, refined white sugar (RWS) and unrefined brown sugar have been used for culture of zygotic embryos of coconut (*Cocos nucifera* L.) and *Brassica campestris* L. (Bonaobra *et al.*, 1994; Okuno *et al.*, 1996; Sorvari, 1986). Similarly table sugar is used for multiplication of banana, potato, orchids, and *chrysanthemum* and for shoot regeneration and rooting of lentil, peanut and chickpea (Kodym and Zapata, 2001). In Bangladesh, several laboratories have used locally available household sugar for culturing potato, banana, orchids, *chrysanthemum*, lentil, peanut, chickpea, medicinal plants and fruit trees. In Pakistan, local sugar was found to be as good as the high grade laboratory sugar for the multiplication of banana. Maple syrup (from *Acer*

saccharam) has been used for multiplication and rooting of cherry root stocks from nodal segment and shoot tips (Prakash *et al.*, 2002).

Alternatives to other ingredients

Several other ingredients can also be replaced by low cost alternatives. Commercial grade chemicals of lower purity than the analytical grades are quite suitable for commercial micropropagation unless deleterious effects are observed (Prakash *et al.*, 2002). Sugarcane molasses can provide many of the nutrients, namely, sugar, vitamins and inorganic metal ions required for sugarcane callus induction and shoot formation (Dhamankar, 1992). At Dhaka University, low cost media have been developed for the multiplication of orchids based on any of the Vacine and Went, Phytamax and Knudson's C media, supplemented with 10-20% banana extract (w/v) and 10-15% (w/v) coconut water (Hossain, 1995; Pervin, 1997; Komol, 1998; Hoque *et al.*, 1994, 1999; Zaheed, 2000). Use of the above media eliminated the need of the micro-salts and vitamins for the micropropagation. Similarly, media supplemented with only peptone, inositol, banana extract and coconut water could be successfully used for micropropagation of several orchids, namely, *Vanda*, *Dendrobium*, *Aerides*, *Acampe* and *Spathoglottis* (Pervin, 1997).

Sources of water

Water is the main component of all plant tissue culture media. Usually in tissue culture research, distilled or double distilled and de-ionised water is used. Distilled water produced through electrical distillation is expensive. In some cases, alternative water sources can be used to lower the cost of the medium. If tap water is free from heavy metals and contaminants, it can be substituted for distilled water (Prakash *et al.*, 2002). Tap water has been used for *in vitro* propagation of banana (Ganapathi *et al.*, 1995) and ginger (Sharma and Singh, 1995). Bottled water from the supermarket can also be used as low cost alternative. However, its mineral composition should be taken in to account as it may affect pH and nutrient uptake. In rural areas, rain water can be collected in clean glass jars and can be used for tissue culture. In Bangladesh, the changeover of water distillation from electrical to gas operated unit reduced the cost

from Rs 1200 to Rs 250 per month for producing 50 to 60 l water per day (Prakash *et al.*, 2002).

Low cost option for media making and handling

Media preparation is a time consuming process. Many companies sell ready-made media in liquid or powdered form. The pros and cons of laboratory making verses buying readymade media should be considered. Although pre-made media save time, their relative cost is high. Pre-pack media preparations are useful when requirement of medium is small. The errors in media preparation from pre-packed media are also less frequent. However, for large scale use, it is much more economical to prepare media by mixing the basic ingredients. By making large batches, the time for media preparation and sterilization can be reduced substantially. However, if mistakes occur, the bigger is the batch, the greater is the loss (Standaert and Metsenaere, 1991). The volume of medium prepared in large sized-laboratories can range from 200 to 500 l per day. The media should be made and used in small batches. Non-sterile media should not be stored at room temperature for more than 24 h especially when it contains high amount of sucrose. These should be refrigerated until further use. Media are usually dispensed in to containers either manually or with a peristaltic pump. Manually, one person can fill not more than 200 containers in an hour. In some large-scale laboratories, the distribution of sterile media is automated. To be economical, the automation must be able to handle many hundred containers per hour. The stock solution is usually prepared in 10X to 1000X concentrations. The stock solution consists of groups of chemicals, e.g. macronutrients, micronutrients, vitamins and plant growth regulators. The inorganic chemicals and vitamin solutions can be combined into a single, 10x concentration stock solution. The stock solutions can be frozen in plastic bags in volumes sufficient to make 1 to 5 l media at a time. The cost of media prepared in this way is much cheaper than the ready made powdered media. This is being practiced for long time in many developing countries. The cost of culture medium depends on the ingredients used, such as sugar, gelling agents and growth regulators used for the culture of the plant species. Based on

the current price of the various ingredients, the cost of one liter MS medium is high for both solid and liquid medium. If sucrose is not added, as in the case of autotrophic micropropagation, cost per litre of media will decrease for both solid and liquid media (Prakash *et al.*, 2002).

Autotrophic micropropagation

Plant with functional chloroplasts can grow *in vitro* on media without sugar, provided the micropropagation environment is modified to enable photosynthesis. The growth of plants on sugar-free medium, but with the carbondioxide enriched environment was similar to sugar-containing media (Kozai and Iwanami, 1988). Plants such as *Carnation*, *Chrysanthemum*, *Cymbidium*, *Primula*, potato and strawberry have been successfully grown using the above technique, termed as PTCS (Photoautotrophic Tissue Culture System). In the autotrophic system, plants are grown in large containers where the air contents (oxygen, carbon dioxide, relative humidity etc.) and the composition of the culture medium is easily controlled (Fujiwara *et al.*, 1987). This system has several advantages that include reduction in production cost because of large culture vessels, simple culture media formulations, and lower incidence of culture contamination. Plants grown in such strongly aerated vessels require little or no hardening (Prakash *et al.*, 2002).

Culture containers and lids

Different types of containers are deployed for culture initiation, maintenance of mother cultures and sub-culture for micropropagation. A wide variety of culture containers are available in the market. Irrespective of the type, the containers used for maintaining *in vitro* plants should be transparent to facilitate illumination and easy inspection. The culture performance and cost of the containers should be used as the prime criterion in choosing the appropriate type.

Types of culture containers

Glass test tubes have been universally used for culturing plant tissues as their narrow openings keep out contamination. Usually, only 1 to 2 explants are cultured in each test tube. As explants growth and

multiplication progress, the cultures are transferred to larger containers. Conical flasks of 150-250 ml capacity are mostly used for micropropagation of plants in liquid media, and are kept on shakers for agitation of the medium and suspension cultures. However, conical flasks are more expensive than glass bottles, and their narrow mouths make manipulations of cultures difficult. Glass and plastic petri dishes are used to culture explants, which are later transferred to larger vessels for elongation and multiplication. Glass petri dishes are sterilized before pouring medium which has to be sterilized separately. Glass petri dishes are more expensive than test tubes and conical flasks. Pre-sterilized disposable plastic petri dishes are much cheaper. Glass bottles and baby food jars with polypropylene caps are most widely used containers and are most economical. Such containers can be washed, sterilized and reused repeatedly. In Cuba, glass containers are washed with nylon brush mounted on an electric rotor, and then dried in the sun by inverting them on wooden trays. Transparent plastic containers, such as "Magenta" TM vessels, that withstand autoclaving and washing, are extensively used for micropropagation in many developed countries, but are very expensive. The repeated autoclaving made them cloudy, thus reduce the passage of light through them. Disposable, non-autoclavable food containers, such as sandwich boxes made from polystyrene have been used for micro propagation. Use of disposable containers eliminates the cost of washing. The high temperature during their manufacturing imparts relatively high degree of sterility, so that they can be used directly without sterilization and thus reduces cost of container sterilization. Non sterile disposable plastic type containers and bags can be bulk sterilized with gamma rays at industrial facilities. Being light weight material, these can be transported to distant places at a much lower cost. It is a new type of culture vessel manufactures by Agristar Inc., Sealy, Texas. It is a disposable bag, and is semi-permeable to gas but requires different methods of handling in contrast to test tubes and other containers (Prakash *et al.*, 2002). Watson Modules TM are disposable plastic type containers manufactured by Navrsh Ltd., Arkley, Barnett, Herts, Herts, U.K., have been used for production of micro- and mini-tubers of potato

(Ahloowalia, 1999a,b). These modules allow *in vitro* multiplication, plant hardening and soil growing in the same container, and permit batch handling of cultures during all stages of plant growth (Ahloowalia, 1995). Disposable containers have been made from fluorocarbon plastic films manufactured by Neoflon polymer film, Daikin Industries Ltd., Osaka, Japan, which can be autoclaved. These are transparent and impervious to water, but fairly permeable to gases (Tanaka *et al.*, 1988; 1991).

Low cost culture containers

It is important to ascertain the price, and the number of times the culture containers can be reused. The production cost of tissue-cultured plants can be reduced by 50-90% by using low cost and containers. In Bangladesh and India, juice, jam and jelly bottles have been used. The orchid producers in Thailand, Singapore, Indonesia, Malaysia and India have been using old whisky bottles for orchid culture for many years. Similarly, disposable plastic bags (approximately 10 x 15 cm) have been used for large scale micropropagation and are proved very cost effective. Using them eliminates the cost of washing and plugging. Plastic bags are sterile due to high temperature during their manufacture. After pouring pre-sterilized medium under the laminar flow hood, the bags are folded and several bags are held together with plastic cloth-hanging pegs. Then explants are transferred to the medium and closed with a heat-sealing machine. Plastic bags being light weight can also be hung by thread and do not need elaborate shelving (Prakash *et al.*, 2002).

Closures and lids

The culture containers are closed to keep out microorganisms. Closures and lids thus influence growth of the *in vitro* plants. Culture containers should not be sealed so tightly that gaseous exchange is prevented. Gaseous exchange is important for the availability of oxygen and carbondioxide, water vapour and elimination of ethylene gas build-up is detrimental to plant growth. The shape of the container influences the growth rate of cultures by modifying gaseous diffusion (Bateson *et al.*, 1987). The selection of containers should be done after

making sure that they would not affect growth *in vitro*.

Types of closures and lids

The different types of closures includes non-absorbent cotton plugs, polyurethane foam plugs, specially made plastic plugs, aluminum foil, stainless steel caps, polypropylene caps with bends, PVC films, microporous membranes, polythene films and silicon rubber.

Test tubes and flasks are closed with non-absorbent cotton wool, sometimes wrapped in muslin gauze. For large-scale production, preparing cotton plugs becomes cumbersome and time consuming. These can be replaced with autoclavable screw caps made of stainless steel or polypropylene (Prakash *et al.*, 2002). Test tubes with translucent caps, which facilitate good gaseous exchange, have been used for culturing carnation (Kozai and Iwanami, 1988). Polypropylene caps with bends, which are heat resistant, have been successfully used as closures. It is transparent, non-greasy, and can be used to seal large containers (Crisp and Walkey, 1974; Mahlberg *et al.*, 1980). Sandwich wrapping films, such as Cling film has been used as double wrap on top of the lids to prevent contamination after subculture (Prakash *et al.*, 2002). Culture containers can be vented by modifying lids, to facilitate optimal growth. Depending on the plant species, microporous membranes of different sizes are available (Osmotec, 2002a).

Low cost energy option

A large part of the electrical energy in tissue culture is used for autoclaving, lighting of growth room, air filtration in laminar-flow cabinet and air conditioning. In developing countries, the cost of electricity can account for up to 60% of the production costs. Reduction in the cost of energy is essential to lower the production cost of micropropagated plants. Artificial lighting of cultures through electricity in the growth rooms is the most expensive method in tissue culture technology. Erratic supply of electricity and fluctuation of voltage create major problem. Where the need for electrical energy is absolutely essential, adopting low cost options can be a major

saving. Changing illumination from artificial to natural light is a decisive low cost option in tissue culture (Ahloowalia and Savangikar, 2002).

Use of natural light

The maintenance and operation of artificial lighting of cultures in the growth rooms needs the light, chokes, fixtures, time controls, equipment to handle high electrical load etc. This system is one of the expensive and inefficient methods in tissue culture technology. The cool fluorescent lights used for illumination provide minimal energy required for photosynthesis. As a result, *in vitro* plants adapt to low-light intensity, and have reduced growth rate. Once they adapt to a set of conditions, re-adaptation to new conditions is rather slow or difficult. Therefore, if plants will be exposed to natural lighting, they can develop structural and anatomical features in their leaves and stems, e.g. cuticle and wax on leaves, thickness of leaf, fewer and closed stomata, and thickening of epidermal cell of the stems, all of which allow survival of the plants in the harsh environment under the field conditions (Ahloowalia and Savangikar, 2002).

Low cost lighting

Changing the method of illumination from artificial to natural light is a decisive low cost option in tissue culture. That not only reduces the electricity and capital costs, but also improves the plant quality. Expensive artificial lights can be replaced in several ways.

Diffused natural light under plastic or glass works very well in temperate climates, but not under tropical conditions. Heat build-up has to be reduced by installing thermostat-controlled exhaust fans. 'Solatubes' are bend tubes with multiple reflecting system to bring outer natural light in to culture room providing sufficient illumination for tissue culture. Replacing artificial lights with 'solartubes' (Anonymous, 2001) which can be installed in any roof, and redirects the day light through reflecting tubes into the culture room can be cost effective (Kodym and Zapata-Arias, 1999, 2001; Kodym *et al.*, 2001). Incorporation of southwest facing glass windows in the growth room can allow indirect diffused natural daylight. In addition to it, using

either muslin or Venetian curtains made of bamboo or plastics, the light can be diffused to the desired intensity. If growth room will be located on the upper storey of the house, it can allow maximum sunlight from all directions. *In vitro* culture have been successfully grown under natural light by using simple plastic bags (polypropylene bags) as culture containers. They are transparent, so incident illumination reaches the cultures with the least loss and the plant undergoes primary hardening while developing under culture. Hence, natural light has been successfully used in the above manners in Bio-factories in Cuba, based on conversion of village houses into tissue culture laboratories (Baezas-Lopez, 1995).

Low cost temperature regulation

Maintaining *in vitro* cultures at a regulated temperature with air conditioners adds to the cost of production. Rather it does not contribute to develop specific plant quality. It is a common belief that the day and night-temperature in the growth room must be strictly controlled at an even level. But growth of plant under a narrow temperature range is at a disadvantage, because it becomes difficult for *in vitro* plants to survive in the harsh field condition. Therefore, *in vitro* plants should be exposed to wide fluctuation in temperature. High daytime temperature up to 28-30 °C and as low as 10-12 °C at night do not damage plant growth. Such fluctuation in temperature promotes better growth. *In vitro* growing plants of banana and potato, kept at 16-41 °C at 750 $\mu\text{mole m}^{-2}/\text{s}$ under natural light, showed as good or better growth than in the controlled growth room (Kodym *et al.*, 2001).

Reducing energy cost for autoclaving

For large sized culture facilities, autoclaves operated on electricity are still the most economic. For small facilities, it is prudent to operate the units on a single phase electric connection. Autoclaving in pressure cookers heated with gas can also be cost effective (Ahloowalia and Savangikar, 2002).

Reducing energy cost in glass house and hardening area

Reduction of energy cost in glass house and hardening area can be done by following strategies.

Electrical usage can be reduced significantly from hardening plants in open shade if glasshouse is not available. *In vitro* grown plants can be hardened by covering them with net-shades after transfer to soil. *In vitro* grown plants can be hardened by placing containers in shade under thatched or plastic covered open huts. In case of culture in plastic containers, the lids are removed and containers are covered with a thin plastic sheet, and left for 3-4 days in shade. The medium is then removed by washing with tap water, and the plants are retained for another 3-5 days in the shade before transfer to soil (Ahloowalia and Savangikar, 2002).

Reducing labour costs

Production of plants based on tissue culture technology and their subsequent growing is a labour intensive system. In developed countries, labour is a major cost factor in micropropagation. On the other hand, in developing countries, labour is relatively less costly, which is a major advantage, but it is important to maintain high labour efficiency because the operators in the culture room are not highly trained. Further, to maintain high labour efficiency of the operators under laminar hoods, they should work not more than 4 hours per day and preferably on a single bench, afterwards they should undertake other operations, such as washing, drying of containers or work in glasshouse. The provision of music in culture transfer rooms also increases the efficiency. Usually, female operators are more efficient than male in the transfer rooms and for potting of plants (Ahloowalia and Savangikar, 2002).

Once the efficiency of labour has been achieved to the maximum possible level, there will be little need of automated or semi-automated system to be introduced. Accordingly, such a system can reduce cost of production by 50% in a production facility. The labour cost comes down from conventional to semi-automated model by 70% and in the fully automated model by 75%. Further, the use of plastic bags increased the output per person and reduces labour cost per propagule by 60% (Savangikar *et al.*, 2002). The manual transfer of explants in culture vessels has the upper ceiling of production per operator at about 5000 operations or transfer per

day. However, this peak rate is rarely achieved. In general, 2500 transfer per operation per day are considered to be good productivity, which is strongly influenced by the type of culture vessel used (Walker, 1986). For example, the transfers per person using test tubes are much lower than that in petri dishes and wide mouth culture vessels.

Reducing overhead costs

Overhead cost for commercial scale tissue culture based plant production includes salaries of managers-directors, scientists, marketing managers, market promotional expenses, payment of commissions, and perks to the management. Much of these cannot be avoided. The large sized companies can afford high overhead costs that match large volume of business. However, smaller units have to rely on a few permanent managers, but may hire the expertise of the specialists as and when required.

Production is prone to large-scale losses through contamination of cultures, sub-standard production, losses during hardening, bad plan and poor marketing. The advice and expertise of experienced persons is indispensable to reduce such losses. The expertise can be obtained for a reasonable fee either from established R & D laboratories or from colleges and universities. Further, for sustainable technology advancement, links should be established within the private sectors and between the private and public sector R & D organizations. Such shared costs of R & D in tissue culture technology by the public and private sector are thus a low cost option to reduce overhead cost (Ahloowalia and Savangikar, 2002).

Using low cost bioreactors

Bioreactors are vessels designed for large-scale cell, tissue or organ culture in liquid media. Functionally, plant culture bioreactors can be divided into two broad types: those in which the cultures are immersed partially or temporarily in the medium (Takayama and Akita, 1994) and those in which the cultures are continuously submerged (Osmotec, 2002b; Adelberg and Simpson, 2002). Bioreactors provide more precise control of the plant growth gaseous exchange, illumination, medium agitation, temperature and pH than the conventional culture

vessels. In spite of some disadvantages (Leifert and Waites, 1990; Leifert and Woodward, 1998; Leifert, 2000), bioreactor based propagation of plants can increase rate of multiplication and growth of cultures and reduce space, energy and labour requirements in commercial micro propagation. They can therefore be attractive to developing countries as regards new or expanding plant culture facilities, in combination with a conventional laboratory (Ziv, 2000; Ziv, 1999). However, to be cost effective, use of bioreactors requires indexed plant cultures, and attention to aseptic procedures during handling of plant materials (Gross and Levin, 1999). Hence, the integration of bioreactors into production systems should only be attempted by facilities with skilled and experienced propagators (Mascarenhas, 1999).

Selection of crops

Selection of crop is one of the strategies which should be considered before venturing in to commercial micropropagation. Micropropagation protocols have been developed for a large number of species; however, only a limited number of plants are being produced on a large scale through micropropagation. The life cycle of the species and the cropping system adopted by growers are important criteria to select plants for large scale micropropagation. Initially commercial micropropagation units focused on the production of ornamental plants, later they have ventured in to fruits, vegetables, landscape and medicinal plants, which provide options for production around the year. This leads to improve cash flow and optimal uses of equipments and facilities (Ahloowalia and Prakash, 2002).

Prevention of variation is a part of production cost

Establishment of mother culture from explants is a significant part of the production cost. Due to lack of sufficient mother cultures and maintenance of a large number of sub-cultures, the cost of production increases. There is a general consensus that the number of subculture for explants should be limited, otherwise there is greater chance of production of somaclonal variants. For example, in potato, the number of subcultures is recommended to be around twenty (Ahloowalia, 1999b). For many

plants, it is not known whether increase of subculture will increase the genetic variations. Many of the plants that are propagated from vegetative parts such as banana, potato, sweet potato, sugarcane, Chrysanthemum, and many fruits are polyploids and well buffered against the expression of mutation and somaclonal variation. If axillary bud proliferation is used as the strategy of multiplication, the frequency of variation is low. The initiation of cultures from meristem tips gives low variation in subculture. But, the high frequency of somaclonal variation has prevented the use of tissue culture for mass scale propagation of some plants e.g. oil palm. On the other hand, some of the novel somaclonal variants can be of value in expanding the market (Jain *et al.*, 1998).

Cost effective multiplication and hardening

Multiplication of tissue cultured plants should be cost effective. Use of liquid media can reduce the cost of multiplication (Levin *et al.*, 1997). Increasing in the multiplication rate through enhanced axillary bud proliferation helps in cutting the cost of production. Care should be taken to save plants at hardening stage, failing which; cost of production will be unbearable. Shoot elongation and rooting are two separate phases. Combining them in one step by using appropriate media with auxins and cytokinins is a valid cost reducing strategy. If natural light is used during multiplication and rooting, the plant needs shorter duration for hardening and will be cost effective. Thus, a cost effective system based on natural sunlight for *in vitro* acclimatization gives high survival (Savangikar *et al.*, 2002).

In a low cost hardening system, micropropagated plantlets should be acclimatized gradually by transforming them to a clean area under partial shade. The culture containers are kept in the shade with their lids loosened initially and then opened. Contamination can be controlled with regular spray of fungicides and pesticides. The plants are periodically sprayed with water to maintain humidity. Semi hardened elongated shoot clumps are then separated under non-sterile condition and rooted in peat or plugs under thin plastic sheets. This low cost procedure reduces the capital cost of

hardening by saving laboratory space, time and labour. This procedure has been successfully used for a number of plants such as *Gerbera*, *Spathiphyllum*, *Syngonium*, African violets, *Chrysanthemum*, *Ficus*, *Philodendron* and other ornamental plants (Prakash and Peirik, 1991; Prakash, 1996).

Low cost greenhouse facility

Sophisticated and costly greenhouse can be substituted by cost effective techniques for hardening *in vitro* derived plantlets. For this, it is necessary to understand the changes that tissue cultured plantlets go through during transfer from *in vitro* to *ex vitro* conditions. *In vitro* temperature changes from 25±2 °C to *ex vitro* 23-36 °C. *In vitro* light intensity 1200-2000 lux changes to *ex vitro* 4000-12000 lux, *in vitro* narrow spectrum light changes to *ex vitro* broad spectrum light, *in vitro* relative humidity 98-100% changes to *ex vitro* relative humidity 40-80%, *in vitro* non-functional roots changes to *ex vitro* functional roots, *in vitro* non-functional photosynthetic system changes to *ex vitro* functional photosynthetic system, *in vitro* exogenous source of hormones changes to *ex vitro* endogenous source of hormones, and the sterile condition of plant in culture room changes to harsh, non-sterile outer field environment. With reference to use of low cost greenhouse, the growers in Florida hardened tissue cultured foliage plants in cavity trays, thus saving costs of a greenhouse. In some parts of India, hanging the culture plastic bags in shade hardens tissue cultured sugarcane plants. Plants produced in this manner are semi-hardened, and can be delivered to the growers at a relatively cheaper price.

In cold climates, low cost plastic tunnels (polytunnel) with bio-fertilization can also be used for hardening. Another low cost greenhouse for cool climates is to use a rectangular pit dug on a site free of water logging. A bamboo frame or any other material is made above the pit with a gentle slope to one side. The frame is then covered with a polythene sheet, and tied using nylon ropes. The sheet is sealed with mud on three sides, leaving one side free. The temperature and humidity inside this structure is generally higher than the outside, and protect the plants from frost. The polythene sheet can partly be

opened to allow air circulation and sunlight as and when required.

The International Institute for Tropical Agriculture, Ibadan, Nigeria has developed another low cost hardening alternative. The Institute provides the farmers with plants in plastic bags and hardening instructions. For hardening the plants, the farmers hang the plastic bags under tree shade (Ahloowalia and Prakash, 2002).

Conclusion

Tissue culture technology has enough potential to contribute towards the improvement of agricultural productivity and food security around the world. However, it must be integrated into production systems in a cost effective manner. Regarding future prospects of the micropropagation industries, it is most desirable to incorporate the possible low cost options into the lay-out of building, laboratory working areas, design of equipment, lighting and production planning etc. Low cost *in vitro* propagation must be a choice of the cultivators without compromising the quality of the plants so produced.

References

- Adelberg, J.W. and Simpson, E.P. (2002). Intermittent immersion vessel apparatus and process for plant propagation. Internl. S/N; PCT/US01/06586.
- Ahloowalia, B.S. and Prakash, J. (2002). Physical components of tissue culture technology. In: IAEA-TECHDOC-1384. pp. 17-28.
- Ahloowalia, B.S. (1995). Watson module- A new system of plant micropropagation. Biolink 2: 17.
- Ahloowalia, B.S. (1999a). Production of mini-seed tubers using a modular system of plant micropropagation. Potato Res. 42: 569-575.
- Ahloowalia, B.S. (1999b). Stability of micropropagated plants and mini-tubers of potato. In: Cassells, A.C., Doyle, B.M. and Curry, R.F. (eds.). Methods and Markers for Quality Assurance in Micropropagation. Proc. Intl. Symp. Cork, Ireland. pp. 163-164.
- Ahloowalia, B.S. and Savangikar, V.A. (2002). Low cost options for energy and labour. In: IAEA-TECHDOC-1384. pp. 41-45.
- Anonymous, (2001). Solatube, the miracle skylight. <http://www.solaglobal.com>

- Babbar, S.B. and Jain, N. (1988). Isubgol as an alternative gelling agent in plant tissue culture media. *Plant cell Rep.* 17: 318-322.
- Baezas-Lopez, P. (1995). Cubans enlist the sun in virus-free propagation. *Ceres.* 156: 15-16.
- Bateson, J.M., Grout, B.W.W. and Lane, S. (1987). The influence of container dimensions on the multiplication rate of regenerating plant cell cultures. In: Ducate, G., Jacob, M. and Simeon, A. (eds.). *Plant Micropropagation in Horticultural Industries. Preparation, Hardening and Acclimatization Process.* Belgian Plant Tissue Culture Group, Symp. Florizel 87, Arlaon, Belgium pp. 275-277.
- Bhattacharya, P., Dey, S. and Bhattacharya, B.C. (1994). Use of low cost gelling agents and support matrices for industrial scale plant tissue culture. *Plant Cell Tiss. Org. Cult.* 37: 115-123.
- Bonaobra, Z.S., Rillo, E.P. and Ebert, A.W. (1994). Effect of table grade sugars on growth and development of coconut (*Cocos nucifera* L.) embryo *in vitro*. Poster paper. 24th International Horticultural Congress. Kyoto, Japan.
- Boxus, Ph. (1978). The production of fruit and vegetable plant by *in vitro* culture. Actual possibilities and perspectives. In: Hughes, K.W., Henker, R. and Constantin, M. (eds.) *Propagation of Higher Plants through Tissue culture.* Conf. 7804111. Technical Inf. Center, U.S. Dep. Energy Springfield, VA. pp. 44-58.
- Bruderere, J. and Luwa, A.G. (1983). An economic and efficient air distribution method for the establishment of clean working environment. *Swiss Pharma.* 5: 17-21.
- Crisp, F. and Walkey, D.G.A. (1974). The use of aseptic meristem culture in cauliflower breeding. *Euphytica* 23: 305-313.
- Debergh, P.C. (1983). Effects of agar brand and concentration on the tissue culture medium. *Physiol. Plant.* 59: 270-276.
- Dhamankar, V.S. (1992). Molasses, a source of nutrients for *in vitro* sugarcane culture. *Sugarcane* 4: 14-15.
- Fujiwara, K., Kozai, T. and Watanabe, I. (1987). Measurement of carbon dioxide gas concentration in closed vessels containing tissue cultured plantlets and estimates of net photosynthetic rates of the plantlets. *J. Agr. Meteorol.* 43: 21-30.
- Ganapathi, T.R., Mohan, T.S.S., Suprasanna, P., Bapat, V.A. and Rao, P.S. (1995). A low cost strategy for *in vitro* propagation of banana. *Curr. Sci.* 68: 646-665.
- Getrudis, D.J.M. and Wattimena, G.A. (1994). The effect of agar substitution on micro-shoot production of two potato cultivars (*Solanum tuberosum* L.). *Acta Hort.* 369: 447-450.
- Gross, A. and Levin, R. (1999). Design considerations for a mechanized micropropagation laboratory. In: Altman, A., Ziv, M. and Izhar, S. (eds.) *Plant Biotechnology and In vitro Biology in 21st Century.* Kluwer Acad. Pub. Dordrecht, The Netherlands, pp. 633-636.
- Hoque, M.I., Komol, N., Hossain, A., Parvin, S. and Sarkar, R.H. (1999). *In vitro* large scale propagation of two hybrid orchid species. *Dhaka Univ. J. Biol. Sci.* 8: 11-16.
- Hoque, M.I., Roy, A.R., Sarkar, R.H. and Haque, M.M. (1994). Micropropagation of *Cymbidium bicolor* through *in vitro* culture. *Plant Tissue Cult.* 4: 45-51.
- Hossain, A. (1995). Studies on *in vitro* seed germination, protocorm development and plant regeneration in twenty two indigenous and exotic orchids' species and hybrids. M.Sc. Thesis. University. Dhaka, Bangladesh.
- Jain, S.M., Brar, D.S. and Ahloowalia, B.S. (1998). Somaclonal variation and Induced mutations in crop improvement. Kluwer Acad. Pub. Dordrecht.
- Kodym, A. and Zapata-Arias, F.J. (1999). Natural light as an alternate light source for the *in vitro* culture of banana (*Musa acuminata*). CV. "Grande Naine". *Plant Cell Tiss. Org. Cult.* 55: 141-145.
- Kodym, A. Hollenthoner, S. and Zapata-Arias, F.J. (2001). Cost reduction in the micro propagation of banana by using tubular skylights as a source of natural lighting. *In vitro Cell. Biol. Plant* 37: 237-242.
- Kodym, A. and Zapata-Arias, F.J. (2001). Low cost alternative for the micropropagation of Banana. *Plant Cell Tiss. Org. Cult.* 66: 67-71.
- Komol, N. (1998). Study on the development of low cost medium for large scale propagation of some orchids. M.Sc. Thesis. Univ. Dhaka, Bangladesh.
- Kozai, T. and Iwanami, Y. (1988). Effects of CO₂ enrichment and sucrose concentration under high photosynthetic photon fluxes on growth of carnation in tissue culture during the preparation stage. *J. Jpn. Soc. Hot. Sci.* 57: 279-288.
- Leifert, C. (2000). Quality assurance systems for plant cell and tissue culture. In: Cassels, A.C. (eds.). *The problem of latent persistence of bacterial pathogens and Agrobacterium transformed vector systems.* Proc. Intl. Symp. methods and markers for quality assurance in micropropagation. *Acta. Hort.* pp.530.

- Leifert, C. and Woodward, S. (1998). Laboratory contamination management: the requirement for microbiological quality assurance. *Plant Cell Tiss. Org. Cult.* 52: 83-88.
- Leifert, C. and Waites, W.M. (1990). Contaminants of plant tissue culture. *Internl. Assoc. Plant Tiss. Cult. Newslett.* 60: 2-13.
- Levin, R. Alper, Y. Stav, R. and Watad, A.A. (1997). Methods and apparatus for liquid media and semi automated micropropagation. *Acta Hort.* 447: 659-663.
- Macleod, K. and Nowak (1990). Glass beads as an alternative solid matrix in plant tissue culture. *Plant Cell Tiss. Org. Cult.* 22: 113-117.
- Mahlberg, P.G. Masi, P. and Paul, D.R. (1980). Use of polypropylene film for capping tissue culture containers. *Phytomorphology*, 30: 397-399.
- Mascarenhas, A.F. (1999). Scope of the tissue culture industries in india. In: Altman, A, Ziv, M. and Izhar, S. (eds.). *Plant Biotechnology and in vitro Biology in the 21st Century*. Kluwer Acad. Publ. Dodrecht, The Netherlands, pp. 633-636.
- Mc Culloch, S.M., Briggs, B.A. and Spethmann, W. (1994). Rooting and establishment of *Rhododendron* micro cuttings. *Acta Hort.* 364: 27-34.
- Moraes-cerdeira, R.M., Krans, J.V., McChesney, J.D., Pereira, A.M.S. and France, S.C. (1995). Cotton fibre as a substitute for agar support in tissue culture. *Hort. Sci.* 30: 1082-1083.
- Nagamori, E. and Kobayashi, T. (2001). Viscous additive improves micropropagation in liquid medium. *J. Biosci. Bioeng.* 91: 283-287.
- Okuno, K., Matsumoto, K. and Hisajima, S. (1996). A trial of improvement of micropropagation of *Brassica campestris* cv. Kukitachina by cost simulation. *J. Soc. High Tech. Agric.* 8: 253-263.
- Osmotec, (2002a). The osmotic life line. Advanced products for plant tissue culture. <http://www.osmotek.com/product.htm>
- Osmotec, (2002b). L^o reactor multiplication. <http://www.osmotek.com/product.htm>
- Pervin, S. (1997). Studies on large scale plantlet development in different orchid species and hybrids through *in vitro* culture. M.Sc. Thesis. Univ. Dhaka, Bangladesh.
- Pierik, R.L.M. (1989). *In vitro* Culture of Higher Plants. Martinus Nijhoff, Dordrecht, The Netherlands.
- Powel, W. and Uhrig, H. (1987). Anther culture of *Solanum* genotypes. *Plant Cell Tis. Org. Cult.* 11:13-24.
- Prakash, J. (1996). Biovillage Biotechnologies, Rep. Asian Biotechnology and Biodiversity farmer centered Agriculture resource management (FARM) programme, UNDP/FAO/UNIDO. *In vitro* International, Bangalore, India pp. 56.
- Prakash, J. and Peirik, R.L.M. (1991). *Horticulture- New Technology and Applications*. Kluwer Acad. Publ. Dodrecht, The Netherlands, 414 p.
- Prakash, S. (1993). Production of ginger and turmeric through tissue culture methods and investigations in to making tissue culture propagation less expensive. Ph.D. Thesis. Bangalore University, Bangalore, India.
- Prakash, S., Hoque, M.I. and Brinks, T. (2002). Culture media and containers. In: IAEA, TECHDOC-1384.
- Savangikar, V.A., Savangikar, C., Daga, R.S. and Pathak, S. (2002). Reduction in cost in micropropagation: achievements and further prospects. 1st International Symp. Liquid system for *in vitro* mass propagation of plants, 29 May-2 June 2002. Agriculture Univ. Norway.
- Schicht, H.H. (1985). Clean room technology: The concept of tool environment control for advanced countries. *Rechno. Appl. Ion. Phy.* 485-491.
- Sharma, T.R. and Singh, B.M. (1995). Simple and cost effective medium for propagation of ginger (*Zingiber officinale*). *Ind. J. Agric. Sci.* 65: 506-508.
- Sorvari, S. (1986). Differentiation of potato tuber discs in barley starch gelatinized nutrient media. *Ann. Agric. Fenn.* 25: 135-138.
- Sorvari, S. and Schieder, O. (1987). Influence of sucrose and melibiose on barley anther cultures in starch media, *Plant Breed.* 99: 164-171.
- Standaert, De, Metsenaere, R.E.A. (1991). Economic considerations. In: Derbgh P.C. and Zimmerman R.H. (eds.). *Micropropagation*. Kluwer Acad. Publ. Dodrecht, The Netherlands, pp. 123-140.
- Stanley, D. (1995). Tissue culturing plants on corn starch. *Agric. Res.* 1-11.
- Takayama, S. and Akita, M. (1994). The types of bioreactors used for shoots and embryos. *Plant Cell Tiss. Org. Cult.* 39: 147-156.
- Tanaka, M., Jinno, K., Goi, M. and Higashiura, T. (1988). The use of disposable fluorocarbon film culture vessel in micropropagation. *Acta Hort.* 230: 73-80.
- Tanaka, M., Hirano, T., Goi, M., Higashiura, T., Sasahara, H. and Murasaki, K. (1991). Practical application of a novel disposable film culture vesselin micropropagation. *Acta Hort.* 300: 77-84.

- Walker, K.A. (1986). Automation of *in vitro* plant propagation: Has its time finally arrived? Genet. Eng. News, July-August, pp. 52.
- Zaheed, M.A.M. (2000). Studies on the morphogenesis of three elite species of orchids. M.Sc. Thesis. Univ. Dhaka, Bangladesh.
- Zimmerman, R.H. (1995). Use of starch-gelled medium for tissue culture of some fruit crops. Plant Cell Tiss. Org. Cult. 43: 207-213.
- Ziv, M. (1999). Organogenic plant regeneration in bioreactors. In: Altman, A., Ziv, M. and Izhar, S. (eds.). Plant Biotechnology and *In vitro* Biology in 21st Century. Kluwer Acad. Publ. Dodrecht, The Netherlands, pp. 673-676.
- Ziv, M. (2000). Bioreactor technology for plant micropropagation. Hort. Rev. 24: 1-30.

Effects of chelating agents and metal ions on photochemical activity, biochemical alterations and attenuation of nickel toxicity in wheat

N. Patnaik¹, B.Satpathy² and H.K. Patra^{1*}

¹P.G. Department of Botany, Utkal University, Vani Vihar, Bhubaneswar-751004

²Department of Botany, Sailabala Women's College, Cuttack-753001

(Received : 16 November, 2010; Revision received : 14 December, 2010)

The effects of nickel on plant growth and metabolism have been studied and reviewed from time to time. However, its interacting effects in presence of chelating agents and metal ions are less studied. The objectives of the study were to analyze the physiological and biochemical alterations and attenuation of nickel toxicity effects in wheat seedlings under combined applications of Ni ions, metal chelators (EDTA/citric acid) and metal ions (Zn^{2+}/Mg^{2+}). Wheat (*Triticum aestivum* L cv UP262) seedlings were grown hydroponically using different concentrations of Ni up to 7 days along with chelators and metal ions for study. The seedling growth was maximum with $NiCl_2-Zn^{2+}$ (100 μM) and minimum with $NiCl_2-EDTA$ (100 μM) treatments. Total chlorophyll content was maximum in the seedlings treated with $NiCl_2-Zn^{2+}$ (100 μM) and minimum in $NiCl_2-EDTA$ (100 μM). $NiCl_2-EDTA$ (100 μM) showed less F_o and F_m values and therefore, a trend in the decrease in OJIP transient indicates the maximum alteration of photochemical activity of PS-II in presence of $NiCl_2-EDTA$ (100 μM) treatment. Similar observation was found by $NiCl_2-EDTA$ (200 μM) treatment where F_o and F_m values were noted to decline. High nickel content in roots of the seedlings was noted as compared to shoots.

Key words: Nickel, chelating agents, chlorophyll-a fluorescence, biochemical alterations

Introduction

Soil contamination is a major environmental concern due to dispersal of industrial and urban wastes generated by human activities (Ghosh and Singh, 2005). Disposal of wastes, accidental and process spillage, mining and smelting of ores, sewage sludge application to agricultural soils are responsible factors for contamination of our ecosystem. However, elevated concentrations of both essential and nonessential heavy metals in the soil can lead to toxicity symptoms and growth inhibition in most plants and Ni is one of them (Hall, 2002). Its average worldwide concentration in soil is 8 ppm. Nickel concentration in plants is usually 1 μg while concentration above 50 $\mu g/g$ could be toxic. The increase in solubility and exchangeability of nickel in soils also results in an increase of this element in plant tissue (Alloway, 1990). Ni is considered an essential micronutrient for plants but is strongly phytotoxic at high concentrations and has a destructive effect on

growth, mineral nutrition, photosynthesis and membrane function (Woolhouse, 1983; Pandolfini *et al.*, 1992; Rao and Sresty, 2000; Peralta *et al.*, 2000; Reichman, 2002.) The toxicity of this metal has also been attributed to its negative effect on photosynthesis, mineral nutrition, sugar transport and water relations (Seregin and Kozhevnikova, 2006). Nickel toxicity is mainly dealt with organic acids in plants in terms of transportation and detoxification (Jocsak *et al.*, 2005) and results in the induction of reactive oxygen species (ROS) such as O_2^- and H_2O_2 (Gajewska and Sklodowska, 2007).

The objectives of the study were to analyze the interacting effects of Ni in presence of chelating agents and metal ions on physiological and biochemical alterations in wheat seedlings. The reduction of nickel toxicity effects in the seedlings under combined applications of Ni ions, metal chelators such as ethylene diamine tetraacetate (EDTA) and citric acid (CA) and metal ions (Zn^{2+}/Mg^{2+}) was also assessed by phytoremediation

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

protocols under *in vitro* hydroponic experiments using seedlings of wheat cultivar (*Triticum aestivum* L. cv UP262) as a model.

Materials and methods

Plant material

Graded dry seeds of wheat (*Triticum aestivum* L. cv. UP 262) were obtained from Orissa University of Agriculture and Technology, Bhubaneswar (India). Uniform sized seeds were selected and surface sterilized with 0.1% mercuric chloride (HgCl_2) for about 5 min and then was washed several times with tap water followed by distilled water. The surface sterilized seeds were placed in sterilized petriplates over saturated cotton pads for germination. Twenty five milliliters of either distilled water (control) or solutions of nickel chloride ($\text{NiCl}_2 \cdot 7\text{H}_2\text{O}$) containing specific concentrations of nickel were poured into each petriplates. The seeds were germinated under controlled condition at 25 °C in darkness for two days. Emergence of 2 mm primary root was used as the operational definition of germination.

Germination percentage

Uniform sized surface sterilized seeds were kept in the darkness in petriplates containing twenty five milliliters of either distilled water (control) or solutions of nickel chloride for two days. Different concentrations of Ni (10 - 1000 μM) were chosen for study. Chelating agents (EDTA, CA) and metal ions (Zn^{2+} , Mg^{2+}) were also used for treating the seeds for the study of germination percentage with reference to control.

Seedling growth

Two-day-old uniform surface sterilized germinated seeds were transferred to well aerated Hoagland's nutrient solution (half strength) and Hoagland's solution supplemented with varying concentrations of nickel chloride placed in hydroponic culture vessels for seedling growth. The seedlings were grown in the growth chamber and the white light was provided (12 h photo period) by white fluorescent tubes (36 W Philips TLD) with a photon flux density of 52 $\mu\text{E}/\text{m}^2/\text{s}$ (PAR).

Growth parameters

The growth parameters like root length, shoot length, fresh matter, dry matter, etc. of seven days old wheat seedlings were used for study. Different nickel concentrations were chosen for treatment. NiCl_2 -chelating agents (100 μM) and NiCl_2 -metal ions (100 μM) were also used for growth parameter analysis. For study of root and shoot length of 7 days old seedlings, the root and shoot were first detached from each other and individual length (cm) of root and shoot was measured. Fresh matter content of both control and nickel chloride treated samples were recorded for assessment. Both nickel treated and control seedlings were kept in an oven at 80 °C for a period of 3 days or more (till constant weight was attained) for dry weight determination.

Analysis of chlorophyll content

The extraction of chlorophyll using acetone was done following the method of Porra (2002). Half a gram of leaves cut into small pieces were taken in 10 ml of 80% acetone and kept in dark in refrigerator for 28 h or more (48 h) at 4 °C. The absorbancy of extracted liquid was observed at 663.6 nm and 646.6 nm for chlorophyll-a and chlorophyll-b respectively.

Analysis of chlorophyll-a fluorescence

The chlorophyll-a fluorescence was measured from the upper surface of the four fully open leaves from the growing apex using a handy PEA (Hansatech Instruments, Norfolk, UK). Prior to measurement, a leaf clip (4 mm diam measuring area) with closed lid was inserted into the leaf and dark adaption was made for 10 min, after which fluorescence was induced on application of red light saturation pulse (3000 $\mu\text{mole photon}/\text{m}^2/\text{s}$; Strasser *et al.*, 1995). The OJIP fluorescence levels were recorded after 50 μs (Fo), 2 ms (Fj), 30 ms (Fi) and 400 ms (Fm) respectively.

Analysis of nickel content

Hydroponically grown 7 day-old wheat seedlings were oven dried and grinded separately to fine powders. The nitric acid (HNO_3) and perchloric acid (HClO_4) in the ratio of 10:1 were added to the weighed and grinded plant powder samples (shoots and roots separately) and kept for 24 h overnight

(Bonet *et al.*, 1991). Then the acid mixed plant samples were digested in MDS-8 Microwave Chemistry Workstation until a clear soluble solution was obtained. The acid digested solutions were filtered and the final volume was made up to 50 ml. Ni^{2+} content of treatments were estimated by an atomic absorption spectrophotometer (Perkin Elmer, USA).

Results

Germination

The inhibition of seed germination was noted with application of $100 \mu\text{M}$ Ni^{2+} concentration leading to 50% inhibition at $800 \mu\text{M}$ (Fig.1). The seed germination was not hampered in presence of chelating agents and metals ions like CA, Zn^{2+} and Mg^{2+} except EDTA treatment.

Changes in growth parameters

Treatment of different concentrations of Ni^{2+} showed considerable changes in seven days grown wheat seedlings as observed from different growth parameters data (Table-1).

Shoot length decreased markedly with increase in Ni^{2+} concentrations (100 to $1000 \mu\text{M}$). The shoot length of the seedlings treated with $10 \mu\text{M}$ - Ni^{2+} was the highest as compared to all other Ni^{2+} treatments. The seedlings treated with $1000 \mu\text{M}$ - Ni^{2+} had minimum length as shown in Table-1. The seedlings treated with NiCl_2 and chelating agents/metal ions showed relative decrease in plant growth (measured in terms of shoot length) in the order: control > NiCl_2 -

Zn^{2+} ($100 \mu\text{M}$) > NiCl_2 - Mg^{2+} ($100 \mu\text{M}$) > NiCl_2 - CA ($100 \mu\text{M}$) > NiCl_2 - EDTA ($100 \mu\text{M}$). The root length also markedly decreased with increasing NiCl_2 concentrations in the growth medium. The seedlings treated with NiCl_2 and chelating agents/metal ions showed relative decrease in the root growth (measured in terms of root length) in the similar order as observed in shoot growth study.

The shoot fresh weight gradually decreased with increase in the concentrations of NiCl_2 . However, the seedlings treated with NiCl_2 - Mg^{2+} / Zn^{2+} ($100 \mu\text{M}$) showed a boost in the biomass production unlike addition of chelating agents like EDTA/CA. Analysis of the root fresh matter indicated that the seedlings treated with NiCl_2 - Zn^{2+} ($100 \mu\text{M}$) showed a boost in the biomass increase unlike addition of Mg^{2+} , EDTA and CA. Similar growth trend values were found for dry biomass production as compared to fresh weight values.

Analysis of chlorophyll content

The decrease in chlorophyll-a content was found with the increase in concentration of NiCl_2 except $10 \mu\text{M}$ (Table-2). The chlorophyll-a content was found maximum with $10 \mu\text{M}$ - NiCl_2 treatment and minimum in $1000 \mu\text{M}$ - NiCl_2 . Among the seedlings treated with metal ions (Zn^{2+} / Mg^{2+}), the chlorophyll-a content was maximum in the seedlings treated with $100 \mu\text{M}$ - Zn^{2+} - NiCl_2 and minimum in $100 \mu\text{M}$ - NiCl_2 as shown in Table-2.

There was a similar trend of increase/decrease in chlorophyll-b content of the seedlings treated with different concentrations of NiCl_2 as shown in Chlorophyll-a (Table-2). The chlorophyll-b content decreased with increasing concentration of NiCl_2 . The seedlings treated with metal ions (Zn^{2+} / Mg^{2+}) had maximum chlorophyll-b content with $100 \mu\text{M}$ - NiCl_2 - Mg^{2+} . The seedlings treated with chelating agents (EDTA/CA) had maximum chlorophyll-b content with $100 \mu\text{M}$ - NiCl_2 -CA application.

The total chlorophyll content was the highest in the seedlings treated with NiCl_2 ($10 \mu\text{M}$) and lowest in seedlings treated with NiCl_2 ($1000 \mu\text{M}$) as shown in Fig. 2. Among the seedlings treated with chelating agents (EDTA/CA) and metal ions (Zn^{2+} / Mg^{2+}), the

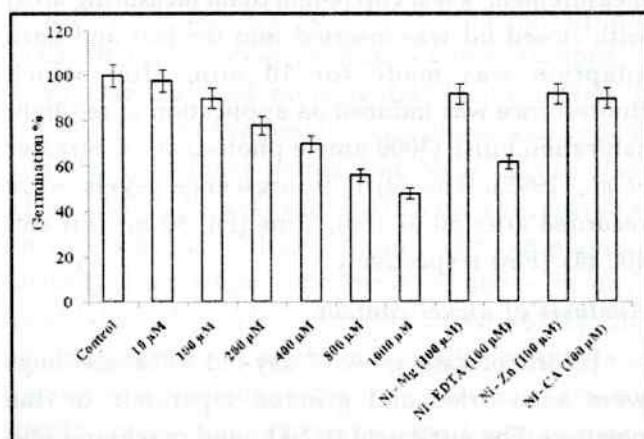


Fig. 1 : Effect of different concentrations of Nickel ions, chelating agents and metal ions on germination of wheat seeds (Mean $\pm$ S.E.).

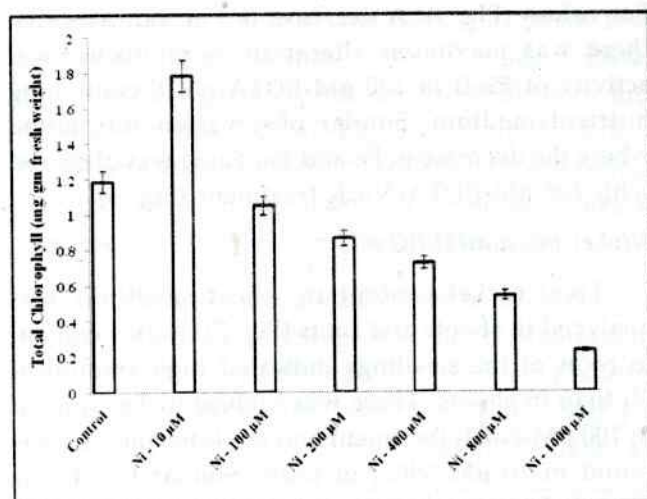


Fig. 2 : Effect of different concentrations of NiCl_2 on total chlorophyll content of wheat seedlings (Mean $\pm$ SE).

total chlorophyll content was found maximum in the seedlings treated with $100 \mu\text{M-Zn}^{2+}\text{-NiCl}_2$ and minimum in $\text{NiCl}_2\text{-EDTA}$ ($100 \mu\text{M}$) as shown in Fig. 3. The chlorophyll a/b ratio was highest in the seedlings treated with $1000 \mu\text{M-NiCl}_2$ and lowest in $800 \mu\text{M-NiCl}_2$ as shown in Table-2. The seedlings treated in $100 \mu\text{M-Zn}^{2+}\text{-NiCl}_2$ had the maximum chlorophyll a/b ratio and minimum in seedlings treated with $100 \mu\text{M-Mg}^{2+}\text{-NiCl}_2$.

Analysis of Chlorophyll-a Fluorescence

OJIP analysis with applications of different concentration of nickel ions indicated that there was no significant change in F_o value (Fig. 4) but F_m value along with the transient changed when treated

Table-1
Effect of nickel ions, chelating agents and metal ions on growth parameters of 7 day-old wheat seedlings

Treatments	Shoot length (cm)	Root length (cm)	Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)	Shoot dry wt./Root dry wt.
Control	18.491 ± 0.263	12.485 ± 0.157	1.914 ± 0.045	0.716 ± 0.011	0.178 ± 0.007	0.089 ± 0.003	2.0
10 μM	19.372 ± 0.238	10.652 ± 0.246	1.912 ± 0.034	0.619 ± 0.014	0.181 ± 0.004	0.083 ± 0.007	2.2
100 μM	16.424 ± 0.221	10.151 ± 0.365	1.559 ± 0.019	0.492 ± 0.006	0.151 ± 0.004	0.058 ± 0.002	2.6
200 μM	13.572 ± 0.249	7.472 ± 0.224	1.191 ± 0.029	0.362 ± 0.019	0.132 ± 0.003	0.044 ± 0.002	3.0
400 μM	10.684 ± 0.194	5.673 ± 0.049	0.935 ± 0.011	0.205 ± 0.009	0.117 ± 0.004	0.034 ± 0.002	3.4
800 μM	6.811 ± 0.055	2.602 ± 0.173	0.664 ± 0.021	0.161 ± 0.011	0.099 ± 0.006	0.022 ± 0.003	4.5
1000 μM	5.475 ± 0.263	1.864 ± 0.071	0.428 ± 0.014	0.052 ± 0.012	0.015 ± 0.007	0.014 ± 0.002	3.2
NiCl_2 - EDTA (100 μM)	10.562 ± 0.071	5.843 ± 0.537	0.820 ± 0.048	0.236 ± 0.014	0.136 ± 0.006	0.025 ± 0.002	5.4
NiCl_2 - CA (100 μM)	14.381 ± 0.118	7.623 ± 0.062	1.861 ± 0.011	0.458 ± 0.022	0.190 ± 0.005	0.065 ± 0.002	2.9
NiCl_2 - Mg^{2+} (100 μM)	15.384 ± 0.289	9.461 ± 0.222	2.359 ± 0.069	0.531 ± 0.011	0.258 ± 0.003	0.074 ± 0.002	3.5
NiCl_2 - Zn^{2+} (100 μM)	16.543 ± 0.118	10.511 ± 0.242	3.082 ± 0.451	1.482 ± 0.034	0.367 ± 0.015	0.082 ± 0.001	4.5

Data represents Mean $\pm$ S.E. of 3 separate experiments each with at least 4 replicates.

with NiCl_2 (10 μM). But in other treatments the OJIP transient did not show much variation. The 100 μM -EDTA- NiCl_2 treatment showed a decreased in F_0 and

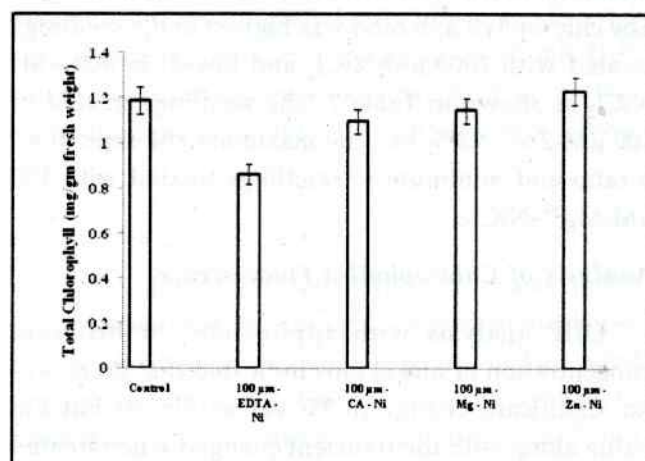


Fig. 3 : Effect of chelating agents and nickel ions total chlorophyll content of wheat seedlings (Mean $\pm$ S.E.).

F_m values (Fig. 5). A decrease in F_m indicates that there was maximum alteration of photochemical activity of PS-II in 100 μM -EDTA- NiCl_2 containing nutrient medium. Similar observation was noted where the decrease in F_0 and F_m value was observed with 200 μM -EDTA- NiCl_2 treatment (Fig. 6).

Nickel bioavailability

Total nickel content in wheat seedlings was analyzed in shoots and roots (Fig. 7). Nickel content in roots of the seedlings indicated high amount of Ni than in shoots. There was highest nickel content in 100 μM - NiCl_2 treatment and the least amount was found in 10 μM - NiCl_2 in roots. Similar trend was observed in shoots of the wheat seedlings.

Discussion

Heavy metals are detrimental because of their non-biodegradable nature, long biological half-life

Table-2
Effect of nickel ions, chelating agents and metal ions on chlorophyll contents of 7 day-old wheat seedlings grown in hydroponics conditions

Treatments	Chlorophyll-a mg/g f. wt.	Chlorophyll-b mg/g f.wt.	Chlorophyll a/b
Control	0.941 ± 0.004	0.180 ± 0.006	5.238
10 μM - NiCl_2	1.596 ± 0.004	0.192 ± 0.002	8.313
100 μM - NiCl_2	0.883 ± 0.004	0.170 ± 0.006	5.194
200 μM - NiCl_2	0.720 ± 0.004	0.143 ± 0.003	5.035
400 μM - NiCl_2	0.600 ± 0.006	0.114 ± 0.004	5.263
800 μM - NiCl_2	0.440 ± 0.005	0.101 ± 0.004	4.356
1000 μM - NiCl_2	0.234 ± 0.005	0.005 ± 0.002	46.80
100 μM - EDTA - NiCl_2	0.724 ± 0.005	0.132 ± 0.005	5.485
100 μM - CA - NiCl_2	0.934 ± 0.003	0.152 ± 0.005	6.145
100 μM - Mg^{2+} - NiCl_2	0.964 ± 0.004	0.176 ± 0.002	5.477
100 μM - Zn^{2+} - NiCl_2	1.074 ± 0.006	0.168 ± 0.003	6.393

Data represents Mean $\pm$ S.E. of 3 separate experiments each with at least 4 replicates.

and their potential to accumulate in different body parts (Behbahaninia *et al.*, 2009). The heavy metals that affect the plant growth and metabolism include Fe, Cu, Zn, Mn, Co, Ni, Pb, Cd, Cr etc. Nickel, on the other hand, has been considered as an essential micronutrient but at high concentrations it causes adverse effects in plants (Welch, 1995).

The study shows harmful effects of nickel chloride on growing wheat seedlings at higher concentrations. The study also depicts the roles of different chelating agents (CA, EDTA) and metal ions (Zn^{2+} , Mg^{2+}) in attenuating the toxic effects of nickel. Chelates are

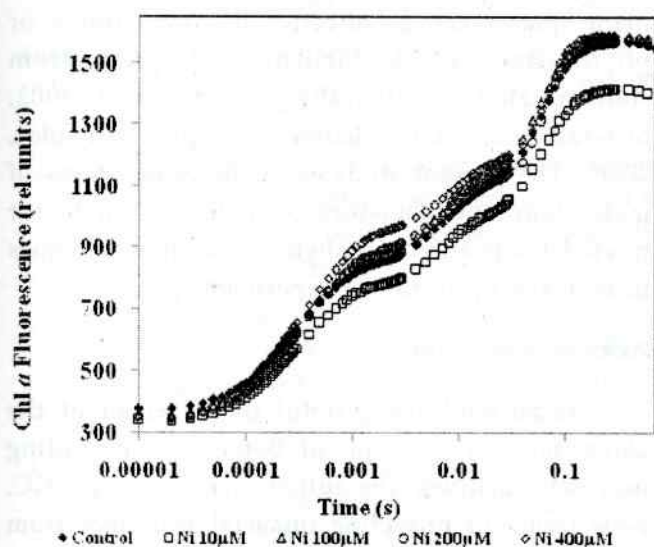


Fig. 4 : Effects of different concentrations of $NiCl_2$ on chlorophyll-a fluorescence in wheat seedlings.

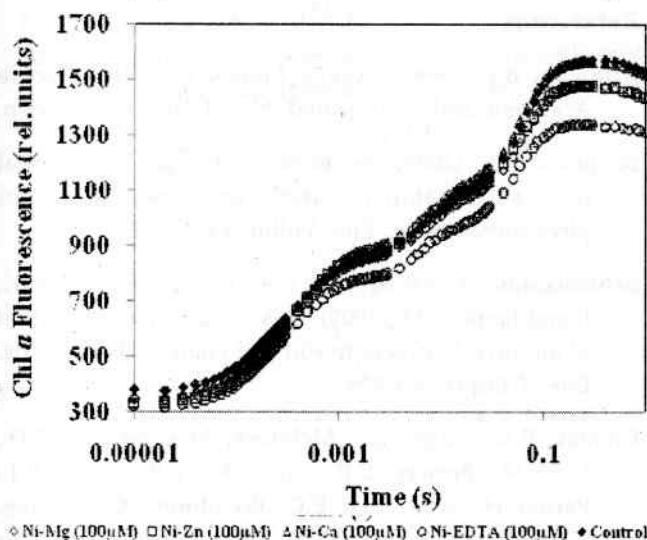


Fig. 5 : Effects of $NiCl_2$, metal ions and EDTA (100 μM) on chlorophyll-a fluorescence in wheat seedlings

substances that render insoluble cations soluble for their availability to the plants (Lindsay, 1974). Low toxicity multidentate chelating agents such as EDTA are used to enhance the bioavailability of heavy metals for plant uptake (Leyval *et al.*, 1995; Turnau, 1998). The enhancement performance of Ni by wheat seedlings in presence of different chelating agents has been undertaken.

The germination rate was significantly reduced with increasing concentrations of $NiCl_2$ in the nutrient solution which may be due to inhibition of biosynthetic activity (Peralta *et al.*, 2001). Growth parameters, viz. shoot and root length, fresh and dry weight of developing wheat seedlings were recorded in response to different concentrations of $NiCl_2$ and supplementation of chelating agents and

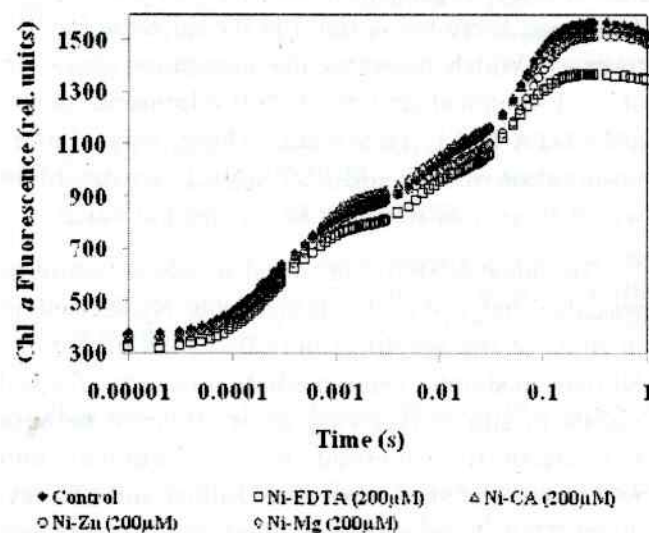


Fig. 6 : Effects of $NiCl_2$, metal ions and EDTA (200 μM) on chlorophyll-a fluorescence in wheat seedlings

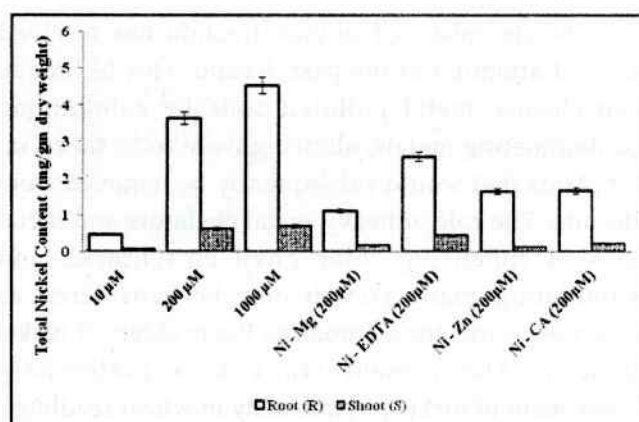


Fig. 7 : Effect of $NiCl_2$, chelating agents and metal ions on nickel bioavailability in wheat seedlings (Mean $\pm$ S.E.).

metal ions. The root length and shoot length were significantly reduced at 1mM NiCl₂ in wheat seedlings. However, at 10 µM NiCl₂ the growth was slightly stimulated. Supplementation of Zn²⁺ with NiCl₂ in nutrient solution stimulated the plant growth in comparison to other chelating agents and metal ions. The possible competitive uptake role of Zn with Ni caused growth stimulation in wheat seedlings suppressing the entry of Ni to plants, otherwise Ni would have exerted growth inhibitory effects as noticed with 100 µM-NiCl₂ application. The chlorophyll biosynthesis was also reflected in the similar manner using Zn²⁺-NiCl₂ and CA-NiCl₂ combinations for plant growth.

In OJIP analysis, the seedlings treated with 100 µM-EDTA-NiCl₂ showed less Fo and Fm values. Therefore, there was a trend in the decrease in OJIP transient which indicates the maximum alteration of photochemical activity of PS-II in presence of 100 µM-EDTA-NiCl₂ treatment. There was similar observation in 200 µM-EDTA-NiCl₂ treatment in which there was decrease in Fo and Fm value.

The bioavailability of nickel in wheat seedlings was also analyzed in shoots and roots. Nickel content in roots of the seedlings indicated high amount of Ni than in shoots. The remediation of excessive soil metals *in situ* is receiving great attention because the alternative method of soil removal and replacement is very expensive (Kukier and Chaney, 2000). Thus, bioremediation plays an important role for *in situ* bioremediation of soil contaminated with heavy metals.

Chelate-enhanced phytoextraction has received a lot of attention in the past decade. This technique can cleanse metal polluted soils by solubilizing contaminating metals, allowing them to be taken up by plants that would subsequently be removed from the site. The role of heavy metal chelators and metal ions in enhancing chlorophyll biosynthesis and stimulating plant growth may be considered as effective agents for attenuating the problem of nickel toxicity. The present study is a preliminary assessment of nickel phytotoxicity in wheat seedlings. The study further includes the effect of chelating agents in response to the growth and chlorophyll

biosynthesis. Nickel is a naturally occurring element which can be found in all environmental media: air, soil, sediment, and water. The concentration of nickel in plants generally reflects the concentration in soil although the relationship is more related to soluble and exchanged forms of nickel. Factors that increase solubility and exchangeability of nickel in soils also result in an increase of the element in plant tissue (Alloway, 1990) which is necessary for healthy growth and is essential for metabolic processes (Alloway, 1990; NRC, 2005). Chelation of heavy metals is a ubiquitous detoxification strategy described in wide variety of plants. Natural element hyperaccumulator plant species can be effective in phytomining or phytoextraction of particular elements from contaminated or mineralized soils (Lasat, 2002; McGrath *et al.*, 2002; Chaney *et al.*, 2005; Banuelos, 2006). The present study with the applications of metal ions and chelators provides an incite for phytodetoxification and phytoremediation strategies to be taken up in future experiments.

Acknowledgement

The authors are grateful to the Head of the Department, P.G. Dept. of Botany for providing necessary facilities. The authors acknowledge UGC, New Delhi for providing financial assistance from DRS-SAP-II and DRS-Research Fellowship in Science for Meritorious Students in Botany.

References

- Alloway, B.J. (1990). Heavy Metals in Soils. Blackie Academic and Professional. New Delhi, India. 339 p.
- Banuelos, G.S. (2006). Phyto-products may be essential for sustainability and implementation of phytoremediation. *Env. Pollut.* **144**: 19-23.
- Behbahani, A., Mirbagheri, S.A., Khorasani, N., Nouri, J. and Javid, A.H. (2009). Heavy metal contamination of municipal effluent in soil and plants. *J. Food Agric. Env.* **7** (3&4): 851-856.
- Chaney, R.L., Angle, J.S., McIntosh, M.S., Reeves, R.D., Li, Y.M., Brewer, E.P., Chen, K.Y., Roseberg, R.J., Perner, H., Synkowski, E.C., Broadhurst, C.L., Wang, S. and Baker, A.J.M. (2005). Using hyperaccumulator plants to phytoextract soil Ni and Cd. *Z. Naturforsch.* **60C**: 190-198.

- Gajewska, K. and Sklodowska, M. (2007). Effect of nickel on ROS content and antioxidative enzyme activities in wheat leaves. *Biometals* 20: 27-36.
- Ghosh, M. and Singh, S.P. (2005). A review on phytoremediation of heavy metals and utilization of its byproducts; *Appl. Ecol. Environ. Res.* 3(1): 1-18.
- Jocsak, I., Vegvari, Gy. and Droppa, M. (2005). Heavy metal detoxification by organic acids in barley seedlings. *Acta Biol. Szeged* 49: 99-101.
- Hall, J.L. (2002). Cellular mechanisms for heavy metal detoxification and tolerance. *J. Exp. Bot.* 53: 1-11.
- Kukier, U. and Chaney, R.L. (2000). Influence of the zinc hyperaccumulator. *Can. J. Soil Sci.* 80: 581-593.
- Lasat, M.M. (2002). Phytoextraction of toxic metals: A review of biological mechanisms. *J. Env. Qual.* 31: 109-120.
- Lindsay, W.L. (1974). Role of chelation in micronutrient availability. In: Carson, F.W. (ed.) *Plant Roots and its Environment*, Univ. Press of Virginia, Charlottesville, V.A. USA, pp. 507-524.
- Leyval, C., Singh, B.R. and Joner, E.J. (1995). Occurrence and infectivity of arbuscular mycorrhizal fungi in some Norwegian soils influenced by heavy metals and soil properties. *Water Air Soil Pollut.* 83: 203-216.
- McGrath, S.P., Zhao, F.J. and Lombi, E. (2002). Phytoremediation of metals, metalloids, and radionuclides. *Adv. Agron.* 75: 1-56.
- National Research Council (NRC) (2005). *Mineral Tolerance of Animals*. Second Edition. The National Academies Press. Washington, D.C., U.S.A. 496 p.
- Nieboer, E. and Richardson, D.H.S. (1980). The replacement of the nondescript term 'heavy metal' by a biologically and chemically significant classification of metal ions. *Env. Pollut. (Ser. B)* 1: 2-26.
- Pandolfini, T., Gabbrielli, R. and Comparini, C. (1992). Nickel toxicity and peroxidase activity in seedlings of *Triticum aestivum* L. *Plant Cell Env.* 15: 719-725.
- Peralta, J.R., Gardea-Torresdey, J.L., Tiemann, K.J., Gomez, E., Arteaga, S., Rascon, E. and Parsons, J.G. (2000). Study of the effects of heavy metals on seed germination and plant growth on alfalfa plant (*Medicago sativa*) grown in solid media. *Proc. 2000 Conf. Hazardous Waste Research*, Denver, Colorado, U.S.A. pp. 135-140.
- Rao, K.V.M. and Sresty, T.V.S. (2000). Antioxidative parameters in the seedlings of pigeon pea (*Cajanus cajan* (L.) Mullspauh) in response to Zn and Ni stresses. *Plant Sci.* 157: 113-128.
- Reichman, S.M. (2002). The responses of plants to metal toxicity: a review focusing on copper, manganese and zinc. *The Australian Minerals and Energy Environment Foundation*, p.54.
- Seregin, I.V. and Kozhevnikova, A.D. (2006). Physiological role of nickel and its toxic effects on higher plants. *Russ. J. Plant Physiol.* 53: 257-277.
- Turnau, K. (1998). Heavy metal content and localization in mycorrhizal *Euphorbia cyparissium* from zinc wastes in Southern Poland. *Acta Soci. Bot. Poloniae*, 67: 105-113.
- Welch, R.M. (1995). Micronutrient nutrition of plants. *Crit. Rev. Plant Sci.* 14: 49-82.
- Woolhouse, H.W. (1983). Toxicity and tolerance in the responses of plants to metals. *Encyclopedia of Plant Physiology*, Springer-Verlag, Berlin, Germany. 12C: 245-300.

Effect of salinity on biochemical changes and OJIP fluorescence parameters of *Rhizophora mucronata* L.

S. Acharya¹, M. Kar¹ and P.K. Mohapatra^{2*}

¹Environmental Biology Laboratory, P.G. Department of Botany, Utkal University, Vani Vihar, Bhubaneswar-751004.

²Department of Botany, Ravenshaw University, Cuttack-753003

(Received : 01 November, 2010; Revision received : 16 December, 2010)

The propagules of *Rhizophora mucronata* L. with a hypocotyl length of 25 ± 2 cm were collected from the interior region of Bhitarkanika National Park. After sprouting the propagules were transferred to the poly bags containing homogenized clay loam agricultural field soil. Three months old *R. mucronata* plants were supplemented with subsequent amount of sodium chloride to maintain 5-45 ppt salinity followed by control. NPK (20:20:20) 2.5 g/L solution was added as a source of fertilizer for adequate nutrition. There was a remarkable increase in average chlorophyll content from 5-20 ppt but significant decrease in all other higher salinities whereas no significant change was observed in control. The amount of soluble protein and carbohydrate in leaves showed a significantly negative correlation with salinity no such change was found in control. The growth and biochemical content was attributed to inhibition of photosynthetic activity and/or salt rejection of *Rhizophora mucronata*. The OJIP fluorescence rise showed an decrease in fluorescence at all levels (J, I and P levels) and increased rate of PS II closure leading to non-photosynthetic energy dissipation.

Key words: *Rhizophora mucronata*, salinity, OJIP fluorescence

Introduction

Mangrove forests occur in the inter-tidal zones along the tropical and subtropical coastal areas throughout the globe. These are vital inter-tidal wetland ecosystems which directly impact the welfare of coastal people. Because of long periods of human perturbation and incessant environmental stress, these unique coastal forests are among the most threatened habitats worldwide (Dagar *et al.*, 1991). The mangrove plants in this forest are adapted to harsh environments like marshy anoxic anaerobic soil and fluctuating salinity of the water bodies with several adaptive morphological and anatomical features (Takemura *et al.*, 2000; Das *et al.*, 2002; Nandy Datta *et al.*, 2007). Salinity gradient has long been recognized as a potential stressor and an important factor that regulates physiological processes such as growth, height, survival, and zonation patterns in mangroves (Lin and Sternberg, 1993; Jithesh *et al.*, 2006).

Mangroves may be found occurring naturally along a gradient of salinity, from riverine forests

with salinities close to zero parts per thousand (ppt) to fringe forests with typical salinities around 35 ppt and even in hyper saline areas where the salinity may reach 70 ppt (Hutchings and Saenger, 1987). Although mangroves are found growing over a wide range of salinities, some species have been found to grow ideally in low salinity of approximately two ppt (Lin and Sternberg, 1992) while other species tolerate much higher salinities before exhibiting signs of stress.

Photosynthetic rates are good indicators of physiological stress levels in all plants, including mangroves (Lichtenthaler, 1996). Mangroves growing in areas of high salinity show increasing stress as measured by decreased carbon dioxide assimilation, stomatal conductance and growth rates (Clarke and Hannon, 1967). Another useful and rapid measure of photosynthesis is the induction of chlorophyll fluorescence (Kautsky effect), a sensitive indicator of photosynthetic energy conversion that occurs during the light reaction (Schreiber *et al.*, 1995).

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

Chlorophyll fluorescence can be modified by any factor affecting the light reaction pathway of photosynthesis, including many environmental stressors (Lichtenthaler, 1996). This study has been undertaken to analyze the influence of salinity on seedlings of *Rhizophora mucronata* along with the physiological state of the photosynthetic apparatus which was determined by measuring the biochemical composition and chlorophyll fluorescence yield in seedlings of *R. mucronata* subject to different salinity levels.

Materials and Methods

Experimental design

Propagules of *Rhizophora mucronata* L. with a length of 25 ± 2 cm were collected during July 2009 from the interior region of Bhitarkanika National Park (covers an area of 214 sq. km extending between

$86^{\circ}48'-87^{\circ}03'E$ and $20^{\circ}33'N-20^{\circ}47'N$) in the east coast of India. The propagules were placed separately in 500 ml beakers containing 300 ml of tap water adjusted with NaCl to four levels of salinity (0, 5, 10 and 15 ppt). These were placed in garden with a maximum temperature of $30^{\circ}C$ and were replenished every 5 days. After sprouting the propagules were transferred to the poly bags containing clay loam agricultural field soil (7 kg/bag) having pH 6.3, electrical conductivity 0.33 mS/cm and moisture content 55.73% (Plate-1 and 2). During one month of acclimation period, commercially available soluble 20:20:20 NPK fertilizer for adequate nutrition was added to each poly bags at a rate of 30 ml once in 15 days. After the pre-treatment acclimation period, salinity in the poly bags was adjusted to 0-45 ppt by the successive addition of laboratory grade sodium chloride solution. Salinity was measured in every

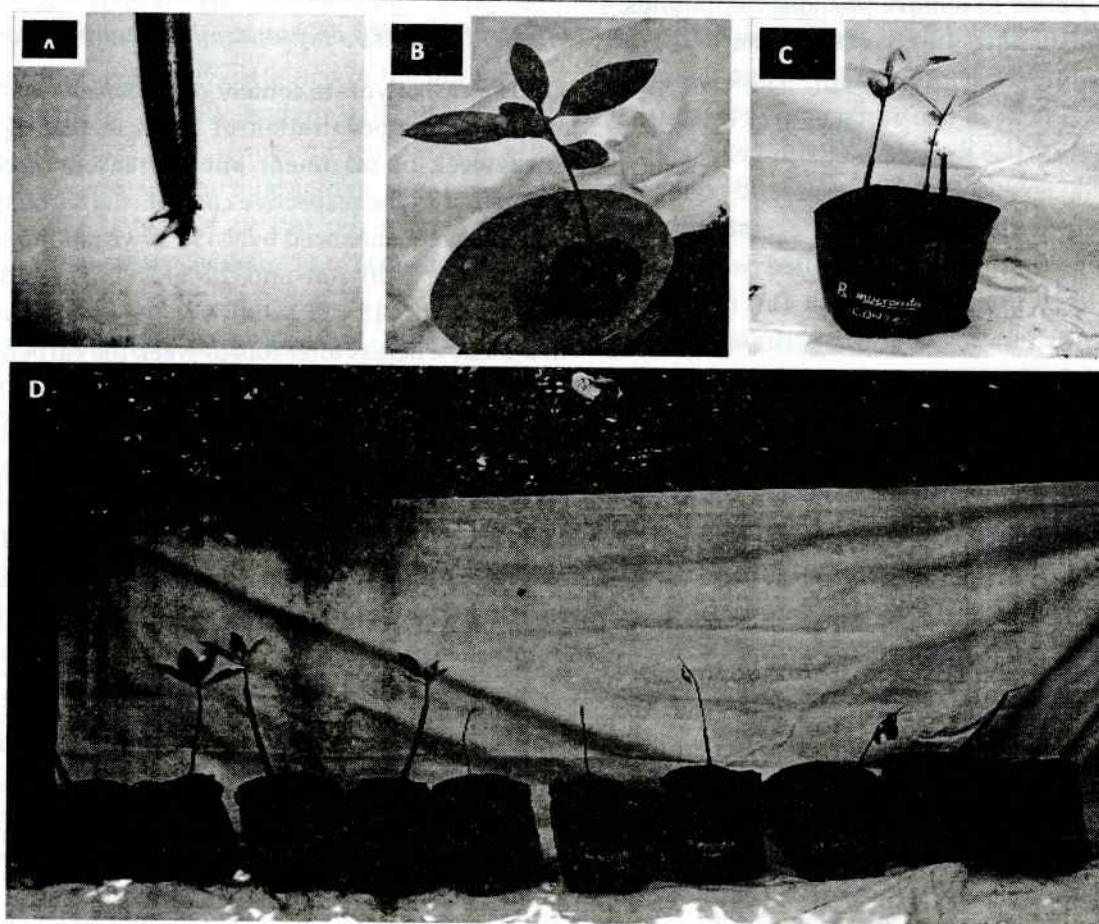


Plate 1 : Sprouting in *Rhizophora mucronata* (A), healthy saplings of the plant (B), Control (C) and (D) shows effect of salinity gradient on growth of *Rhizophora* saplings after 21 days of treatment.

week in terms of conductivity using a handy conductivity meter and adjusted as necessary. One month after, salinity levels were adjusted to experimental treatments and the plant responses were measured over a period of 30 days at 7 days interval.

Estimation of biochemical composition

The chlorophyll content of four fully open leaves from the growing apex were measured through a Chlorophyll meter (SPAD-502, Spectrum Technologies, Inc.). Total soluble protein was made according to Lowry *et al.* (1951). For extraction of protein, 0.5 g chilled sample tissue was homogenized in ice cold 10% TCA and was incubated overnight at 4 °C. The precipitates were centrifuged at 10000 rpm for 10 min, pellets were washed with 100% acetone and pigment free pellets were successively washed with 80% ethanol, 3:1 (v:v) ethanol : chloroform and ether to remove phenolic compounds. Pellets were suspended in known volume of 0.1 N NaOH and estimation of protein. Total soluble carbohydrate content of leaf samples was estimated following the anthrone reagent method. Leaf sample (100 mg) was taken and the photosynthetic pigments were extracted with methanol. The residue was homogenized with 1 ml of the distilled water, and then diluted to 30 times from which 1 ml of sample was taken for estimation of carbohydrate content. To this, 4 ml of the freshly prepared anthrone reagent was added and heated for 7 min. in water bath at 100 °C. It was cooled in ice cold water. Absorbance at 620 nm was taken for the samples using UV-visible spectrophotometer 118, (Systronics Instruments Pvt. Ltd.) against a blank prepared through the same procedure.

Measurement of OJIP fluorescence kinetics

The chl *a* fluorescence was measured from the upper surface of the four fully open leaves from the growing apex using a handy PEA (Hansatech Instruments, Norfolk, UK). Prior to measurement, dark adaptation of the leaf was made for 10 minutes, after which fluorescence rise was induced by a red light saturation pulse (3000 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$; Strasser and Strasser, 1995). The OJIP fluorescence levels were recorded continuously for 1 second. The

rise after 50 μs (F_0), 2 ms (F_i), 30 ms (F_1) and 400 ms (F_M) were noted to calculate other derived fluorescence parameters. The required fluorescence parameters, viz.- relative variables fluorescence at J level (V_j), net rate of PS II closure (M_0) and trapping probability (TR_0/Abs) were calculated from the OJIP fluorescence rise by using the equations of Force *et al.* (2003).

Each experiment for chl *a* fluorescence was conducted taking 10 replicates each time. The values presented in tables and figures are the means of the replicates. Since the OJIP fluorescence transient of control of subsequent treatments did not significantly differ from each other, a single control has been taken for comparison in figures. For protein and carbohydrate content the mean of three replicates of each plant were taken for estimation.

Results and Discussion

Effect of salinity on biochemical composition

The carbohydrate content of *Rhizophora mucronata* at different concentration of NaCl increased up to third week of treatment but decreased thereafter. Compared to the respective controls the carbohydrate contents were enhanced by 9-155 % within the salinity range from 5-20 ppt and 20-50 % increment were noticed for 25-45 ppt salinity range in first week of treatment whereas after fourth week the carbohydrate content were decreased by 14-50% for 5-20 ppt salinity. However, of 156% of increment of carbohydrate content was observed for 15 ppt salinity (Table 1) indicating that the metabolic performance of the plants was enhanced at this salinity level. After second and third week, there was an increase in carbohydrate content for 5 to 20 ppt along with a nominal decrease for 25-35 ppt.

The amount of total soluble protein in *Rhizophora mucronata* leaves at different concentrations of NaCl showed a decreasing trend for each week of observation, but without any noticeable changes in control (Table 1). The maximum decrease was noticed in 10 ppt NaCl treatment in which protein content decreased from 156.2 mg/g f.wt to 131.1 mg/g f.wt i.e. about 10% decrease with respect to control after fourth week of treatment. After first week there was

an enhancement of the soluble protein content of leaves at 15 ppt salinity but the reduction noticed at lower salinity were found insignificant indicating that at these salinity levels the seedling did not receive any stress. Appreciable decrease in protein content was observed for 20-45 ppt salinity range indicating that even though the seedlings tolerated higher salinity levels for shorter period, prolongation of exposure caused severe inhibition of the metabolic activities of the plants.

The reduction in the level of protein and carbohydrate is an indication of the fact that the cellular metabolism was significantly affected with the NaCl treatment. The increase in leaf protein content on fourth week for 15 ppt concentration of NaCl indicated enhancement of the metabolic performance of seedlings, which may be an indication of tolerance mechanism of *Rhizophora mucronata* against salt stress. The decreasing trend of carbohydrate content of autotrophic cells under NaCl stress condition has been attributed to the inhibition of photosynthetic activity and/or salt rejection of *Rhizophora mucronata* i.e. it was difficult for the salt to get to the leaves and the total soluble sugar content was always at low level or did not change a lot. There was an increase in carbohydrate content (Table-1)

for 15 ppt NaCl treatment after third week was obtained which may be due to improved water balance and/or enhancement in photosynthetic carbon dioxide fixation.

The salinity caused significant fluctuation in total chlorophyll content of *Rhizophora mucronata*. Increase in the total chlorophyll content was observed at 15 ppt salinity throughout the observation period. But at higher salinities there was a significant decrease in chlorophyll content for every passing week whereas there was a bit enhancement in chlorophyll content at 25-35 ppt salinity. On third week there was a sharp decrease in the chlorophyll content at 25 ppt salinity and complete chlorosis was recorded at 35 ppt salinity (Fig. 1).

The loss of pigment is the preferred and most easily measured parameter for describing the overall growth and metabolic efficiency of green plants. The cellular chlorophyll level in the present experiment showed a very good trend with different NaCl concentration. Reduced rate of photosynthesis and metabolic requirement for chlorophyll *a* biosynthesis to balance between chlorophyll *a* and photosynthetic carbon fixation may be considered as the causes of the reduction of chlorophyll *a* content under salinity stress.

Table-1

The Carbohydrate & protein content (mg/g f.wt) of *Rhizophora mucronata* treated with different concentrations of NaCl at different time durations

Salinity (ppt)	Carbohydrate content (Days after treatment)				Protein content (Days after treatment)			
	7	14	21	28	7	14	21	28
0	63.9	78.9	97.3	123.5	152.3	157.9	149.5	156.2
5	69.7	92.7	98.2	105.9	149.5	143.3	152.9	137.7
10	130	94.8	147	102.5	150.6	151.1	138.6	131.2
15	163.4	172.3	294.7	317.2	163.2	182.3	198.8	199.2
20	97.2	73.2	97.8	61.5	73.2	65.1	32.2	21.1
25	83.9	96.3	53.8	-	83.1	73.2	21.5	-
30	92.5	62.7	48.1	-	92.1	73.3	52.1	-
35	101.1	86.9	59.7	-	98.7	69.1	42.5	-
40	93.5	-	-	-	86.2	-	-	-
45	76.4	-	-	-	79.8	-	-	-

Note: '-' refers to no data due to death of plant during this period.

Effect of salinity on chlorophyll fluorescence induction

The OJIP fluorescence rise measured at different intervals after NaCl treatment showed characteristic difference at each phase (O-J, J-I, and I-P phase). The maximum difference was observed for 25-45 ppt salinity range compared to the rise in control during first week after treatment (Fig. 2A). During this period there was an increase in F_j (J rise) but decrease of F_i (I rise) and F_m (P rise) at ≥ 20 ppt salinities but there was a significant increase in F_m at lower salinities. No significant difference in F_o value, with change of salinity level, was observed after second week but decrease in F_m and F_j was more pronounced for each treatment (Fig. 2B). A decrease in F_i (I-rise) was noticed for 15-25 ppt treatment indicating the effect of salinity stress on electron transport beyond QA resulting in reduced multiple turnover events.

The F_o fluorescence showed no such variation for various concentrations (except 20 ppt) in the 21st days old plant (Fig. 3C). Maximum change in fluorescence transient was noticed at J-I and I-P phase for almost all the treatments. There was a decrease in fluorescence at J-level, I-level and P-level was noticed for all the treatments with minimal difference at 10 and 15 ppt. There was a significant decrease in fluorescence at all levels obtained with increase in NaCl concentration whereas at 10 and 15 ppt the difference was minimum. This indicated that no

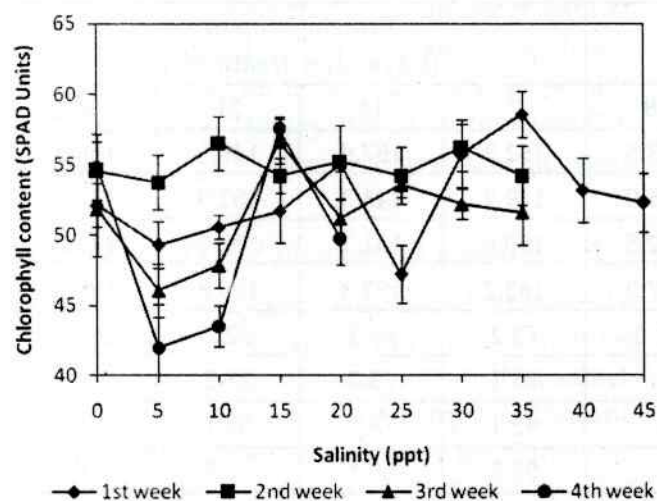


Figure 1 : Effect of salinity on total chlorophyll content of *Rhizophora mucronata* measured as a function of NaCl treatment.

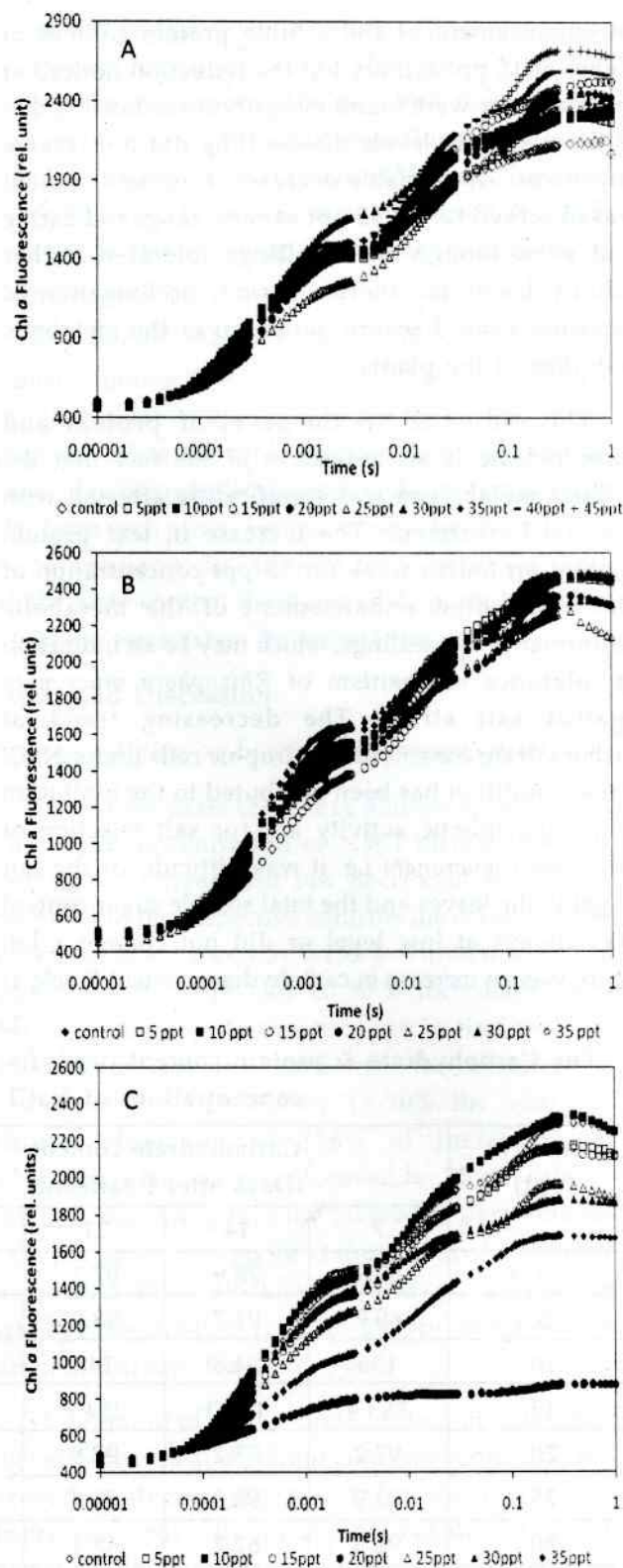


Figure 2 : The OJIP Fluorescence rise of dark adapted *Rhizophora mucronata* leaves in response to different concentrations of NaCl (ppt) after (A) First, (B) Second and (C) Third week of NaCl treatment.

significant increase of J fluorescence and thus insignificant variation at O-J phase of seedlings grown up to 15 ppt salinity showed similar physiological status of seedlings during the observation period.

The decrease in fluorescence rise may be due to two reasons, first by inhibition of electron transport at donor site of PS II which results in the accumulation of P_{680}^{+} (Govindjee *et al.*, 1995; Vernay *et al.*, 2008) and second due to a decrease in the pool size of Q_A^{-} . Also the increase of J-step reveals the fact that there was a lack of charge recombination between P_{680}^{+} and Q_A^{-} . As compared to control leaves the area over the fluorescence curve was decreased in 25 ppt NaCl treatment onwards on second and third week suggesting that high salt stress inhibits the electron transport rate at the donor site of PS II with poor photosynthetic performance (Strasser *et al.*, 2005).

F_v/F_m ratio was not affected significantly in high salt treatment up to second week whereas there was a reduction in yield parameter after third week of treatment (Table-2) which indicates reduction in photosynthetic trapping probability under NaCl stress with long exposure. The derived fluorescence

parameters Abs/RC i.e., effective antenna size of an active reaction center is calculated as a total number of photons absorbed by chlorophyll molecules of all RCs divided by total number of active RCs which is influenced by ratio of active RCs with increase in NaCl concentration the value of Abs/RC increased which results decrease of antenna size of active RCs. Here plants under 15 ppt salt stress shows better quantum efficiency than other treatments.

To localize the action of high salt stress in electron transport chain on acceptor site of PS II the kinetics of relative variables fluorescence (V_j) was calculated. V_j is equivalent to the fluorescence at J step, relative variable fluorescence (V_j at 2ms) for unconnected PS II units, equals to the fraction of closed RCs at J step expressed as a proportion of the total number of RCs that can be closed (Force *et al.*, 2003). Increasing value of V_j revealed a loss of QA-reoxidation capacity and an inhibition of electron transport at acceptor site of PS II (Congming and Vonshak, 1999) also beyond Q_A^{-} . V_j enhancement is an indication of the loss in PS II functionality, slower Q_A^{-} reduction events i.e. poor physiological response. But after third week of treatment, there was a slight

Table-2

The fluorescence parameters V_j , M_0 and quantum efficiency TR_0/Abs of *Rhizophora mucronata* treated with different concentrations of NaCl at different time durations.

Salinity (ppt)	V_j (Days after treatment)			M_0 (Days after treatment)			TR_0/Abs (Days after treatment)		
	7	14	21	7	14	21	7	14	21
0	0.549	0.563	0.513	0.003	0.003	0.003	0.752	0.780	0.780
5	0.502	0.518	0.579	0.968	1.025	0.819	0.768	0.788	0.746
10	0.516	0.506	0.509	0.949	0.967	1.008	0.760	0.791	0.761
15	0.467	0.410	0.503	1.036	0.805	0.958	0.794	0.807	0.767
20	0.485	0.510	0.731	0.770	0.571	0.807	0.794	0.787	0.308
25	0.382	0.521	0.507	0.726	0.818	1.400	0.785	0.754	0.729
30	0.564	0.593	0.614	0.639	0.918	0.826	0.776	0.751	0.695
35	0.489	0.470	0.435	0.911	1.047	1.108	0.796	0.793	0.704
40	0.471	-	-	0.798	-	-	0.801	-	-
45	0.409	-	-	0.858	-	-	0.829	-	-

Note: V_j - Relative variable fluorescence at 2 ms, M_0 - Net rate of PS II Closure, TR_0/Abs - Trapping Yield (F_v/F_m). '-' refers to no data due to death of plant.

decrease in V_j showing some improvement in PS II activity for plants under 20 ppt salinity. During this period the plants of salinity > 20 ppt failed to survive. Similarly V_j and M_0 showed an increment with respect to control up to second week whereas drastically decrease after third week for salinities less than 20 ppt show better adaptability. We may conclude that high salt stress inhibits the electron transport rates at donor as well as acceptor site of PS II as compared to acceptor to donor site of PS II is significantly affected. Inactive PS II centers increased with increase in salt concentration.

The study suggested we conclude that, there was no significant effect of salinity gradient on chlorophyll, carbohydrate and protein content of *R. mucronata* up to 15 ppt but adverse effect was reported thereafter. The study on photosynthetic performance (measured by chlorophyll fluorescence) decreased with increase in salinity stress and time of exposure. The plant showed better adaptability at 15 ppt salinity throughout the treatment period. The plants under salinity (>25 ppt) did not survive after third week. The use of chlorophyll fluorescence as a measure of photosynthesis allowed a rapid and reliable quantification of a known stressor like salinity, in the seedlings of *Rhizophora mucronata*. It is suggested that these techniques can be applied for rapid assessment of the health of mangrove plants in forestry, nursery and restoration activities.

Acknowledgement

The authors acknowledge the financial assistance of DRS SAP-II under UGC, New Delhi to carry out this research. We are also thankful to the Divisional Forest Officer, Batighar Reserve Forest, Orissa for his kind support during the collection of plant materials.

References

- Clark, L.D. and Hannon, N.J. (1967). The mangrove swamp and saltmarsh communities of the Sydney district, III. Plant growth in relation to salinity and waterlogging. *J. Ecol.* **58**: 351-369.
- Congming, L.U. and Vonshak, A. (1999). Characterization of PSII photochemistry in salt adapted cells of cyanobacterium *Spirulina platensis*. *New Phytol.* **141**: 231-239.
- Dagar, J.C., Mongia, A.D. and Bandyopadhyay, A.K. (1991). *Mangroves of Andaman and Nicobar Islands*. Oxford & IBH, New Delhi, India, 133 p.
- Das, A.B., Parida, A., Basak, U.C. and Das, P. (2002). Studies on pigments, proteins and photosynthetic rates in some mangroves and mangrove associates from Bhirarkanika, Orissa. *Mar. Biol.* **141**: 415-422.
- Force, L., Critchley C. and van Rensen J.J.S. (2003). New fluorescence of parameters for monitoring photosynthesis in plants: I. The effect of illumination on fluorescence parameters of the JIP test. *Photosynth. Res.* **90**: 1-19.
- Govindjee (1995). Sixty three years since Kautsky chlorophyll a fluorescence. *Aust. J. Plant Physiol.* **22**: 511-566.
- Hutchings, P. and Saenger, P. (1987). *Ecology of Mangroves*. University of Queensland Press, St Lucia, Australia. 388 p.
- Jithesh, M.N., Prashanth, S.R., Sivaprakash, K.R. and Parida, A.K. (2006). Antioxidant response mechanism in halophytes their role in stress defense. *J. Genet.* **85**: 237.
- Lichtenthaler, H.K. (1996). Vegetation stress: an introduction to the stress concept in plants. *J. Plant Physiol.* **148**: 4-14.
- Lin, G. and Sternberg, L. (1993). Effects of salinity fluctuation on photosynthetic gas exchange and plant growth of the red mangrove (*Rhizophora mangle*). *J. Exp. Bot.* **44**: 9-16.
- Lowry, O.H., Rosebrough, N.J., Farr, A.B.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagents, *J. Biol. Chem.* **193**: 265.
- Nandy Datta, P., Das, S., Ghose, M. and Spooner Hart, R. (2007). Effect of salinity on photosynthesis, leaf anatomy, ion accumulation and photosynthetic nitrogen use efficiency in five Indian mangroves, *Wetland Ecol. Man.* **15**(4): 347.
- Schreiber, U., Bilger, W. and Neubauer, C. (1995). Chlorophyll fluorescence as a non-intrusive indicator for rapid assessment of in vivo photosynthesis In : Schulze ED and Caldwell MM (ed) *Ecophysiology of photosynthesis*, Springer-Verlag, Berlin, pp 49-70.
- Strasser, B.J. and Strasser, R.J. (1995). Measuring fast fluorescence transients to address environmental questions: the JIP-test. In: Mathis, P. (ed.) *Photosynthesis: from Light to Biosphere*. Kluwer Acad. Publ., Dordrecht, The Netherlands, pp. 977-980.

- Strasser, R.J., Tsimilli-Michael, M. and Srivastava, A. (2005). Analysis of the chlorophyll a fluorescence transient. In. Papageorgiou, G.C., Govindjee (eds.) Chlorophyll Fluorescence: A Signature of Photosynthesis. Kluwer Acad. Publ., Dordrecht-Boston-London, pp. 1-50.
- Takemura, T., Hangata, N., Sugihara, K., Dubinsky, Z., Baba, S. and Karube, I. (2000). Physiological and biochemical response to salt stress in the mangrove, *Bruguiera gymnorhiza*. Aqat. Bot. 68: 15-28.
- Vernay, P., Gauthier-Moussard, C., Jean, L., Bordas, F., Faure, O., Ledoigt, G. and Hitmi, A. (2008). Effect of chromium species on phytochemical and physiological parameters in *Datura innoxia*. Chemosphere. 72: 763-771.

Nutrient-alginate encapsulated nodal explants of butterfly pea (*Clitoria ternatea* L.) as synseeds for germplasm conservation and exchange

S.S. Swain¹, A. Pal², L. Sahu¹, C. Pradhan¹, D.P. Barik³ and P.K. Chand^{1*}

¹Plant Cell & Tissue Culture Facility, P.G. Department of Botany, Utkal University, Bhubaneswar-751 004

²Department of Botany, Regional Institute of Education, Bhubaneswar-751 022

³Department of Botany, Ravenshaw University, Cuttack-753 003

(Received : 20 September, 2010; Revision received : 01 November, 2010)

In vitro shoot cultures of butterfly pea (*Clitoria ternatea* L.) were established using mature nodal segments from an outdoor garden-grown plant on MS medium augmented with 1.0 mg/l BA that elicited the maximum shoot proliferation (83.3%). Nodal segments from such axenic shoot cultures (source B) as well as from outdoor plants (source A) were encapsulated in calcium alginate hydrogel (4% sodium alginate complexed with 75 mM calcium chloride) containing MS supplemented with 1.0 mg/l BA to produce synseeds. Of the seven different planting media evaluated for morphogenic responses of encapsulated nodal segments, maximum sprouting percentage was observed in full-strength MS without any growth regulator, irrespective of explant source. A higher sprouting percentage was recorded for synseeds derived from source A (68.5%) producing a maximum number of multiple shoots (3-4 shoots/synseed) as compared to those from source B (57.7%; 1-2 shoots/synseed). Synseeds remained viable up to 65 days showing an effective sprouting efficiency (62%) following storage at 12 °C. *In vitro* sprouted shoots were rooted and the plantlets were hardened off and transferred to soil. The relevance of this investigation is discussed in the context of large-scale propagation of this pharmaceutically important plant species at low cost as well as its true-to-type germplasm conservation, distribution and academic exchange of *in vitro* seed-size propagules between laboratories for further genetic manipulations.

Key words: *Clitoria ternatea* L., *in vitro* shoot culture, nodal segments, synseeds

Introduction

Clitoria ternatea L. (English: Butterfly pea; Oriya: 'Aparajita'; Sanskrit: 'Aparajita'/'Girikarnika') of Family Fabaceae is an important plant species valued for its multi-medicinal and ornamental properties. The plant is widely used in the traditional Indian system of medicine as a brain tonic with rejuvenating property and is believed to promote memory and intelligence. It is also used in throat and eye infection, skin diseases, urinary troubles and ulcer. Seeds and roots are purgative, laxative and diuretic. Besides, seeds are cathartic and useful in visceralgia and eyesight deficiencies. In addition to possessing medicinal properties, it is very productive being a highly palatable forage legume, generally preferred by livestock. This plant is also used as a cover crop

or green manure. Conventionally, the plant is propagated through seeds and cuttings. However, the percentage of seed germination is low and cuttings are difficult-to-root. Biotechnological exploitation of this plant has been inadequate though plant regeneration via tissue culture has recently been reported in this pharmaceutically important plant species (Barik *et al.*, 2007).

In recent years, the nutrient-alginate encapsulation technique for producing synthetic seeds (synseeds) has become an important asset to micropropagation. Synthetic seed technology could be useful in conservation of clonal germplasm of elite and threatened plants as well as exchange of axenic plant material between laboratories due to small capsule size and relative ease in handling of

* Corresponding author; E-mail: pkchandubot@yahoo.co.in

these structures (Danso and Ford-Lloyd, 2003; Naik and Chand, 2006). Encapsulation of somatic embryos or non-embryogenic propagules such as meristems (apical/axillary meristem or nodal segments) and regeneration of complete plantlets from them has been reported for several plant species including vegetables, fruits, ornamentals conifers and woody crop plants (reviewed by Ara *et al.*, 2000). During initial days somatic embryos were essentially used for synseed production as in carrot (*Daucus carota*; Kitto and Janik, 1985); sandal wood (*Santalum album*; Bapat and Rao 1990), bamboo (*Dendrocalamus strictus*; Mukuntha Kumar and Mathur 1992), black-spruce (*Picea mariana*; Lusdorf *et al.*, 1993), interior spruce (*Picea glauca engelmannii* complex; Lusdorf *et al.*, 1993) and *Paulownia elongata* (Ipekci & Gozukirmizi, 2003). However, a large number of later investigations claimed the usefulness of meristems (from *in vivo/in vitro* sources) especially where either somatic embryogenesis had not been documented or there was an inordinately low embryo-to-plant conversion as in mulberries (Bapat *et al.*, 1987; Patnaik *et al.*, 1995; Patnaik and Chand, 2000), basil (Mandal *et al.*, 2000, 2001), pineapple (Soneji *et al.*, 2002), potato (Beura, 2002), common oak (Tsvekov & Hausman, 2005) and pomegranate (Naik and Chand, 2006).

In this work, we report a reproducible protocol for rapid and true-to-type propagation of *Clitoria ternatea* in a large scale using synthetic seeds (synseeds). Experiments were carried out to encapsulate the nodal segments collected directly from mature outdoor plant with a view to achieving plant retrieval within a shorter time. Nutrient-alginate encapsulation of nodal segments for synthetic seed production could be a useful strategy for rapid and efficient propagation of this important plant species of medico-horticultural significance for its *ex-situ* conservation and exchange of germplasm.

Materials and methods

Explant source

Nodal segments of butterfly pea (*Clitoria ternatea* L.) were collected from a mature plant grown in the botanical garden at the P.G. Department of Botany, Utkal University and cut into pieces. Nodal segments

were kept under running tap water for 30 min, followed by 10 min treatment with 5% (v/v) aqueous solution of Teepol (Reckitt's Colman, Kolkata) and rinsed with double-distilled water (5-6 changes). Then nodal segments were surface-disinfected with 0.1% (w/v) aqueous solution of mercuric chloride (HgCl_2) (Hi-media, Mumbai, India) for 8 min, followed by five rinses in autoclaved double distilled water inside the laminar air flow cabinet. Thereafter, explants were inoculated on Murashige and Skoog's (1962) medium (MS) augmented with benzylaminopurine (BA, 0.5-2.5 mg/l) or kinetin (Kin, 0.5-2.5 mg/l) for multiple shoot induction and proliferation. Nodal segments (2-4 mm) dissected out from multiple shoots developed from *in vitro* culture (source B) as well as *in vivo* nodal segment directly collected from the garden, following surface disinfection (source A) were used for the preparation of synseeds by nutrient-alginate encapsulation.

Nutrient-alginate encapsulation

Sodium alginate (Sigma, USA) in a range of concentrations (2-8% w/v) was added to MS augmented with 1.0 mg/l BA liquid medium containing 3% sucrose and 100 mg/l myo-inositol without agar. For complexation, different concentrations of calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 25-100 mM) were prepared in liquid MS containing the same growth adjuvants as in the sodium alginate preparation. Both the solutions were autoclaved at 104 kPa and 121 °C for 20 min after adjusting the pH to 5.8.

Encapsulation was accomplished by glueing the disinfected *in vivo* nodal segments (directly from the naturally occurring plant after surface sterilization) or *in vitro* nodal segments as source B (dissected from *in vitro* multiple shoots) into sodium alginate solution prepared in MS medium supplemented with 1.0 mg/l BA and dropping these into calcium chloride solution by the help of a dropping tube. Each drop contained one node. After 30 min in the calcium chloride solution when the drops had set as small white beads, these containing entrapped nodal segments (synseeds) were retrieved using a pre-sterilized tea-strainer.

Sprouting of synseeds in different planting media

In order to evaluate the germination percentage, synseeds from both the sources were planted separately in a range of planting media i.e. half-strength MS (MS1), full-strength MS (MS2), MS + 0.5 mg/l BA (MS3), MS + 1.0 mg/l BA (MS4), Soilrite mix + MS liquid (MS5), vermi-compost + MS Liquid (MS6) and natural soil + MS liquid (MS7).

Low temperature storage

When germination responses were to be recorded following prolonged storage, axillary buds encased in calcium alginate capsules were maintained in a laboratory refrigerator at 4 °C, 12 °C, and 27 °C and data collected up to 60 days following encapsulation.

Rooting of shoots derived from synseeds

Shoots proliferated from synseeds were individualized and transferred to root inducing medium containing ½ MS fortified with various concentrations and combinations of auxins (IBA/IAA; 0.1 - 3.0 mg/l). All the cultures were placed on culture racks inside a tissue culture room, the latter being maintained at 25 ± 1 °C, 55-60% relative humidity, 16 h photoperiod with illumination provided by cool white fluorescent tubes giving a photon flux density of 50 µmol/m² s at rack level.

Acclimatization and field transfer of plantlets

Rooted shoots retrieved from encapsulated nodal segments were transferred to plastic pots (12 cm diam) containing autoclaved vermi-compost (Ranjan Agrotech, Bhubaneswar) or garden soil moistened initially with ½ MS then with tap water. The pots were covered with polyethylene bags to maintain high humidity and kept in culture room at 25 ± 1 °C and a photon flux density of 50 µmol/m²/s provided by cool white fluorescent tubes (Phillips, Bangalore). After 10 days, plantlets were transferred to larger clay pots (18 cm diam) containing garden soil and kept outdoors under shade for another 8 days before transplanting in the field.

Data analysis

In low temperature storage and plant retrieval experiments, each treatment consisted of five replicates (culture jars) and the experimental units

was four synseeds/jar. In the rooting experiment each treatment consisted of 20 replicate tubes and one shoot/tube. Each experiment was conducted three times. Data were analysed using analysis of variance (ANOVA) for a completely randomized design (CRD). Duncan's new multiple range test (DMRT) (Gomez and Gomez, 1984) was used to separate the means for significant effect.

Results

Synseed production

Maximum shoot multiplication percentage (83.3%), highest number of shoots (averaging 4.3 per explant) with longest average shoot length (4.7 cm) was obtained on MS + 1.0 mg/l BA. Nodal segments from *in vitro* shoot cultures as well as those from the garden-grown plant were found ideal for production of synseeds by nutrient-alginate encapsulation. About 200-220 characteristic beads (hydrogels) were generated using 50 ml sodium alginate and 120 ml calcium chloride solution. The beads differed morphologically with respect to texture, shape and transparency with different concentration and combinations of sodium alginate and calcium chloride; bead diameters ranging 0.45 - 0.64 cm. An optimal ion exchange between Na⁺ and Ca⁺ produced firm clear, isodiametric beads resulting from complexation of a 4% solution of sodium alginate (Sigma) with 75 mM calcium chloride for 30 min thereby forming an insoluble gel matrix of calcium alginate (Fig. 1A). At higher concentrations of sodium alginate (5-8%) beads were iso-diametric but hard enough to cause considerable delay in sprouting.

Plant retrieval from freshly prepared synseeds

Morphogenic responses (shoot development) of encapsulated nodal segment (synseeds) of two different sources (*in vivo* & *in vitro*) were evaluated on seven different planting media. Of these full strength MS devoid of growth regulators (MS0) was found to be the best with respect to initiation and frequency of sprouting regardless of explants type (Table-1). On MS0 minimum period required for the initiation of sprouting was 12 days for synseeds obtained from source A (Fig. 1B) and 14 days for those from source B whereas, as long as 25 days

were necessary for sprouting of both the explant types on MS + 1.0 mg/l BA. On the other hand, sprouting percentage as recorded on day 30 assumed the highest value for source A synseed (68.5%) with 3-4 shoots from each synseed (Fig. 1C). Synseeds obtained from source B sprouted up to 57.7% with 1-2 shoot of stunted growth (Fig. 1D).

In order to induce rooting, the shoots developed from encapsulated nodal segments of source A were transferred to $\frac{1}{2}$ MS containing 0.25 mg/l IBA in which highest percentage (82.1%) of rooting with 4-5 roots/shoot with longest average root length (5.3 cm) was observed (Fig. 1E). Plantlets with 4-6 fully expanded leaflets and well-developed roots were successfully acclimated in the plant growth chamber and eventually established in the soil. The percentage of survival of the plantlets after transfer to vermicompost: soilrite mix (1 : 1) was 68% and about 73% of the plants transferred to garden soil survived. The regenerated plants did not show any detectable variation in morphology and growth characteristics when compared with that of donor plant and all of them grew to maturity producing flowers and pods with fertile seeds (Fig. 1F).

Plant retrieval after short-term storage of synseeds

Of the three different storage temperatures tested, 12 °C permitted the highest sprouting (78%) at day

15; synseeds remained viable up to 65 days showing an effective sprouting efficiency (62%) (Table-2). A lower percentage of sprouting was observed at 4 °C in which synseeds could be stored up to a maximum of 15 days. Storage at 27 °C was most unsuitable because synseeds stored at this temperature were able to maintain their viability (51%) only up to 15 days. Beyond 35 days of storage there was a gradual decrease in percentage sprouting and after 95 days synthetic seeds had a negligible sproutability (Table-2).

Discussion

During the past few years synseeds (synthetic seeds or artificial seeds) have received considerable attention as a valuable aid to plant propagation. Encapsulation of somatic embryos, axillary/apical shoot buds or nodal explants of *in vivo/in vitro* source in calcium alginate gel followed by retrieval of complete plantlets via sprouting/germination of the encased propagules on suitable planting substrates have been reported in a number of economically important plant species. Fundamental to a successful micropropagation programme routed through encapsulation is the selection of appropriate plant part as the starting experimental material, critical evaluation of factors influencing formation of gel matrix and above all optimization of the process of

Table-1

Sprouting percentage of encapsulated nodal segments on different planting media after 30 days (d) of inoculation

Planting media (Code)	Mean days to sprouting		% sprouting at 30 d*	
	<i>In vivo</i> node	<i>In vitro</i> node	<i>In vivo</i> node	<i>In vitro</i> node
Half-strength MS (MS1)	15	18	63.1 ^b	51.3 ^b
Full-strength MS (MS2)	12	14	68.5 ^a	57.7 ^a
MS + 0.5 mg/l BA (MS3)	16	18	61.3 ^{bc}	48.7 ^c
MS + 1.0 mg/l BA (MS4)	25	25	31.2 ^{fg}	30.1 ^g
Soilrite mix + MS liquid (MS5)	21	23	38.3 ^c	38.2 ^c
Vermi-compost + MS liquid (MS6)	20	25	33.3 ^f	35.1 ^f
Natural soil + MS liquid (MS7)	18	20	46.1 ^d	42.3 ^d

Data pooled from 3 separate experiments each with 5 jars containing 4 synseeds per jar. *Mean values within the column with the same alphabet (in superscript) are not significantly different ($p < 0.05$; Duncan's New Multiple Range Test).

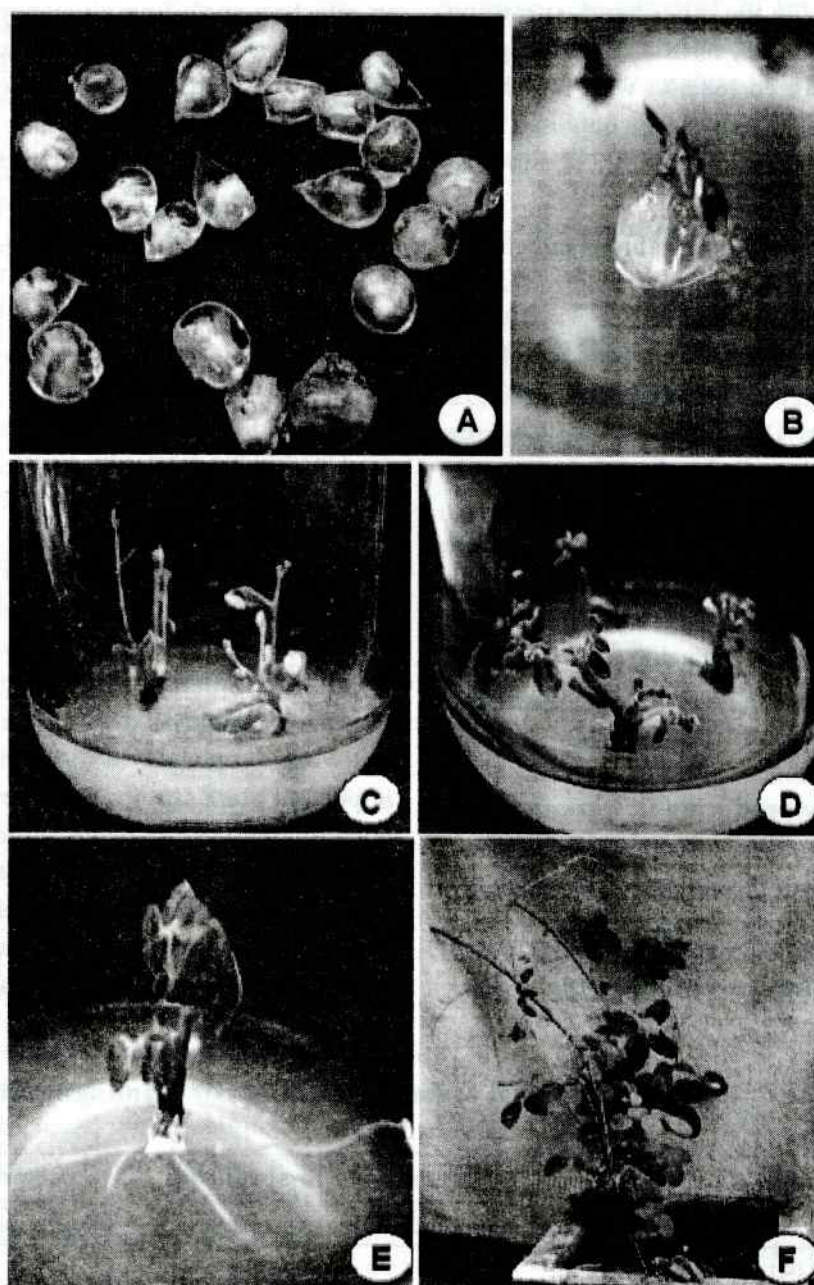


Fig. 1 : Synseed-to-plant formation in *Clitoria ternatea* L.

- (A) Freshly prepared synseeds by sodium alginate encapsulation of nodal segments of *in vivo* shoots (source A)
- (B) Initiation of sprouting of synseeds of source A on MS2 at day 12
- (C) Elongation of shoots derived from source A-synseeds on MS2 at day 30
- (D) Shoot development from source B-synseeds on MS2 at day 50
- (E) Rooting of an *in vitro* shoot developed from a source A-synseed on $\frac{1}{2}$ MS + 0.25 mg/l IBA
- (F) An acclimated plant in the pot containing vermi-compost : soilrite mix::1:1

germination for plant retrieval. Nodal segments were suitable for encapsulation studies as they were excellent plant material for preparation of synseeds besides possessing a great potential for plant development from pre-existing meristematic tissues. In the present study, *in vitro* shoot cultures of butterfly pea were established and nodal segments collected from such cultures were used for synseed production. It was also useful to collect nodal segments directly from naturally occurring field grown herbs and to use them for encapsulation in order that micropropagation via a synseed system could be simplified. Moreover, such a strategy could ensure maintenance of the propagules as near and natural to the plant level as possible so as to facilitate subsequent *in vivo* germination. It could also bypass an intervening step of *in vitro* maintenance in the form of axenic shoot cultures, which were neither necessary nor economical (Pattnaik *et al.*, 1995).

Synthetic seeds of *C. ternatea* were prepared by encapsulating the somatic embryos with 2.5% (w/v) sodium alginate and CaCl_2 (100 mM) (Malabadi & Nataraja, 2002). Similar types of results were obtained in tree species such as *Camellia* sp. (Janeiro *et al.*, 1997) or *Punica granatum* (Naik & Chand, 2006), except that in the latter species, synseeds were made from *in vitro* nodal segments. However, in the present study the beads prepared by 4% (w/v) sodium alginate and (75 mM) CaCl_2 were of ideal size, shape and consistency. At lower concentrations (1-3%), sodium alginate became unsuitable for encapsulation

perhaps because of a reduction in its gelling ability following exposure to a high temperature during autoclaving (Larkin *et al.*, 1988). At higher concentration of sodium alginate (5-8%) beads were iso-diametric but hard enough to cause considerable delay in sprouting (Naik & Chand, 2006). A desirable feature of the encapsulated somatic embryos or nodal segments or axillary/apical shoot buds is their ability to retain viability in terms of germination/sprouting potential even after a considerable period of storage. In case of *Clitoria ternatea* L. it was observed that 2 °C was found suitable for storage of synthetic seeds prepared from somatic embryos (Malabadi & Nataraja, 2002) whereas, in *Punica granatum* L. 4 °C was found suitable for short term storage (Naik & Chand, 2006). However, these not in agreement with results obtained in our experiment where synseeds stored at 12 °C have maximum sprouting percentage. Successful plant retrieval from encapsulated nodal segments following storage at 12 °C indicates that the method described in this paper could be potentially used to transfer of germplasm from lab-to-lab over a short period. It is assumed that any rate of decline in the germination/sprouting frequency observed among encapsulated propagules stored at low temperature may have resulted because of inhibited respiration of plant tissue perhaps due to the alginate capsule (Redenbaugh, *et al.*, 1987).

The potential of encapsulated nodal segments or apical/axillary shoot buds as synseeds is being increasingly realized considering the relative ease

Table-2
Effect of temperature and storage period in days (d) on the sprouting of synseeds

Explant type	Sprouting (%)														
	4 °C					12 °C					27 °C				
	15d	35d	45d	65d	95d	15d	35d	45d	65d	95d	15d	35d	45d	65d	95d
Encapsulated <i>in vivo</i> nodal segment (Source A)	67	63	52	43	14	78	72	64	62	33	51	46	36	27	12
Encapsulated <i>in vitro</i> nodal segment (Source B)	61	52	48	30	11	68	65	56	54	21	47	34	21	25	09

Mean data pooled from 3 separate experiments each with 5 jars containing 4 synseeds per jar.

and extensibility of the technology, as well as the possibility of moving small seed-sized micropropagated plant material between laboratories and countries, while reducing phytosanitary and quarantine problems (Mathur *et al.*, 1989; Bapat, 1993; Hasan & Takagi, 1995; Piccioni & Standardi, 1995; Maruyama *et al.*, 1997). Again, according to Hasan & Takagi (1995) and Piccioni & Standardi (1995), only sprouting ability of the encapsulated explants might be sufficient when an *in vitro* destination for further micropropagation purposes is intended. In this context, nodal segment encapsulation protocol for butterfly pea as developed in the present study could be useful for efficient transfer of sterile and elite material to extension centers and laboratories.

The production of synseeds has thus been elevated from a simple technique to a powerful biotechnology by virtue of its tremendous application potential for *ex situ* conservation of germplasm and mass propagation that guarantees genetic fidelity (trueness-to-type) of plants derived from synseeds (Chand *et al.*, 1994). Coupled with successful single-step *in vivo* germination the synseed system offers the possibility of direct field transfer of selected elite genotypes, hand-pollinated hybrids and genetically engineered plants. This technique would find an application in the cost-effective mass propagation of high value ornamental and medicinal species. On the other hand, synseeds could also provide an analytical tool for the study of zygotic embryology and to unravel the precise role of endosperm in embryo development and germination.

References

- Ara, H., Jaiswal, U. and Jaiswal, V.S. (2000). Synthetic seed: prospects and limitations. *Curr. Sci.* **78** (12): 1438-1444.
- Bapat, V.A. (1993). Studies on synthetic seeds of sandalwood (*Santalum album* L.) In : Redenbaugh, K. (ed.) *Synseeds: Application of Synthetic Seeds to Crop Improvement*. CRC, Boca. Raton, pp. 381-407.
- Bapat and Rao (1990). *In vitro* growth of encapsulated axillary buds of mulberry (*Morus indica* L.). *Plant Cell Tiss. Org. Cult.* **20**: 69-70.
- Bapat, V.A., Mhatre, M. and Rao, P.S. (1987). Propagation of *Morus indica* L. (mulberry) by encapsulation shoot buds. *Plant Cell Rep.* **6**: 393-395.
- Barik, D.P., Naik, S.K., Mudgal, A. and Chand, P.K. (2007). Rapid plant regeneration through *in vitro* axillary shoot proliferation of butterfly pea (*Clitoria ternatea* L.). *In Vitro Cell Dev. Biol. Plant*, **43**: 144-148.
- Beura, R. (2002). *In vivo* and *in vitro* methods for production of hybrid seeds, seed tubers & synseeds of potato (*Solanum tuberosum* L.). Ph.D thesis, Utkal University, Orissa, India. 138p.
- Chand, P.K., Sahoo, Y. and Pattnaik, S.K. (1994). Artificial seeds - A novel method for mulberry propagation. *Ind. Silk* **30** : 33-38.
- Danso, K.E. and Ford-Lloyd, B.V. (2003). Encapsulation of nodal cuttings and shoot tips for storage and exchange of germplasm. *Plant Cell Rep.* **21**: 718-725.
- Gomez, K.A. and Gomez, A.A. (1984). *Statistical Procedure for Agricultural Research*. New York: John Wiley & Sons: 680p.
- Hasan, S.M.Z. and Takagi, H. (1995). Alginate-coated nodal segments of yam (*Dioscorea* spp.) for germplasm exchange and distribution. *Plant Genet. Resour. Newslett.* **103**: 32-35.
- Ipekci, Z. and Gozukirmizi, N. (2003). Direct somatic embryogenesis and synthetic seed production from *Paulownia elongata*. *Plant Cell Rep.* **22**: 16-24.
- Janeiro, L.V., Ballester, A. and Vieitez, A.M. (1997). *In vitro* response of encapsulated somatic embryos of Camellia. *Plant Cell Tiss. Org. Cult.* **51**: 119-125.
- Kalamani, A. and Michael Gomez, S. (2002). *In vitro* propagation studies in *Clitoria* sp. *Int. J. Mendel.* **19**: 29-30.
- Kitto, S.L. and Janick, J. (1985). Production of synthetic seeds by encapsulating asexual embryos of Carrot. *J. Amer. Soc. Hort. Sci.* **110**(2): 277-282.
- Lakshmanan, K.K. and Dhanalakshmi, S. (1990). Callus, organogenesis and plantlet formation in tissue cultures of *Clitoria ternatea*. *Ann. Bot.* **66**: 451-455.
- Larkin, P.J., Davies, P.A. and Tanner, G.J. (1988). Nurse culture of low number of *Medicago* and *Nicotiana* protoplast using calcium alginate beads. *Plant Sci.* **58**: 203-210.
- Lulsdorf, M.M., Tautorius, T.E., Kikcio, S.I., Bethune, T.D., Dunstan, D.I. (1993). Germination of encapsulated embryos of interior spruce (*Picea glauca engelmannii* complex) and black spruce (*Picea mariana* Mill.). *Plant Cell Rep.* **12**: 385-389.
- Malabadi, R.B. and Nataraja, K. (2001). Shoot regeneration in leaf explants of *Clitoria ternatea* L. cultured *in vitro*. *Phytomorphology.* **51**: 169-171.

- Malabadi, R.B. and Nataraja, K. (2002). *In vitro* storage of synthetic seeds in *Clitoria ternatea* Linn. *Phytomorphology*, **52** (2-3): 231-237.
- Mandal, J., Pattnaik, S. and Chand, P.K. (2000). Alginate encapsulation of axillary buds of *Ocimum americanum* L. (hoary basil), *O. basilicum* L. (sweet basil), *O. gratissimum* L. (shrubby basil) and *O. sanctum* L. (sacred basil). *In Vitro Cell Dev. Biol. Plant*, **36** (4) : 287-292.
- Mandal, J., Pattnaik, S. and Chand, P.K. (2001). Growth response of the alginate-encapsulated nodal segments of *in vitro* - cultured basil. *J. Herbs Spic. Med. Plants*, **8**(4): 65-74.
- Mathur, J., Ahuja, P.S., Lal, N. and Mathur, A.K. (1989). Propagation of *Valeriana wallichii* using encapsulated apical and axial shoot buds. *Plant Sci.* **60**: 111-116.
- Mukunthakumar, S. and Mathur, J. (1992). Artificial seed production in male bamboo, *Dendrocalamus strictus* L. *Plant Sci.* **87**: 103-113.
- Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissues cultures. *Physiol. Plant.* **15**: 473-497.
- Naik, S.K. and Chand, P.K. (2006). Nutrient-alginate encapsulation of *in vitro* nodal segment of pomegranate (*Punica granatum* L.) for germplasm distribution and exchange. *Scient. Hort.* **108**: 247-252.
- Pattnaik, S. and Chand, P.K. (2000). Morphogenic response of the alginate - encapsulated axillary buds from *in vitro* shoot cultures of six mulberries. *Plant Cell Tiss. Org. Cult.* **60**: 177-185.
- Pattnaik, S.K., Sahoo, Y. and Chand, P.K. (1995). Efficient plant retrieval from alginate - encapsulated vegetative buds of mature mulberry trees. *Scient. Hort.* **61**: 227-239.
- Piccioni, E. and Standardi, A. (1995). Encapsulation of micropropagated buds of six woody species. *Plant Cell Tiss. Org. Cult.* **42**: 221-226.
- Redenbaugh, K., Slade, D.T., Viss, P.R. and Fujii, J.A. (1987). Encapsulation of somatic embryos in synthetic seed coats. *Hort. Sci.* **22** (5): 803-809.
- Rout, G.R. (2004). Effect of cytokinins and auxins on micropropagation of *Clitoria ternatea* L. *Biol. Lett.* **41** (1): 21-26.
- Rout, G.R. (2005). Micropropagation of *Clitoria ternatea* L. (Fabaceae) - an important medicinal plant. *In Vitro Cell Dev. Biol. Plant* **41**: 516-519.
- Soneji, J.R., Rao, P.S., and Mhatre, M. (2002). Germination of synthetic seeds of pineapple (*Ananas comosus* L. Merr.). *Plant Cell Rep.* **20**: 891-894.
- Standardi, A. and Piccioni, E. (1998). Recent perspective on synthetic seed technology using nonembryogenic *in vitro*-derived explants. *Intl. J. Plant Sci.* **159** (6): 968-978.
- Tsvetkov, I. and Hausman, J.F. (2005). *In vitro* regeneration from alginate-encapsulated microcuttings of *Quercus* sp. *Scient. Hort.* **103**: 503-507.

In vitro propagation of *Acalypha indica* L. : A potential medicinal plant

G. Rout¹, P.R. Biswal², L.P. Mishra³ and S.P. Rath^{4*}

Plant Tissue Culture Laboratory, P.G. Dept of Botany, Utkal University, Bhubaneswar-751004

¹IHSE, S 'O' A University, Bhubaneswar-751030, ²Trident Academy of Creative Technology, Bhubaneswar-751030

³Department of Botany, Rajsunakhala College, Rajsunakhala-752065, ⁴North-Orissa University.

(Received : 02 December, 2010; Revision received : 20 December, 2010)

A protocol has been established for large scale micropropagation of *Acalypha indica* L., an important medicinal plant of the Family Euphorbiaceae, by *in vitro* culture of cotyledon and cotyledonary node taken from one month-old *in vitro* grown seedlings. The explants were cultured in Murashige and Skoog (MS) medium (1962) supplemented with various concentration of cytokinins (BAP or Kn) either taken alone or in combination with gibberellic acid (GA₃) or auxins (IBA or NAA). Highest percentage (100%) of bud break and maximum number of shoots (8-9 shoots per explants) were observed in the MS medium supplemented with BAP (0.5 mg/l) and GA₃ (0.5 mg/l) in case of cotyledonary node explants. The regenerated shoots were best rooted in half-strength MS medium augmented with auxin IBA (0.5 mg/l). Fully-grown rooted plantlets were successfully transferred to land with 90% survival rate.

Key words: *Acalypha indica*, cotyledonary node, *in vitro* regeneration

Introduction

Acalypha indica L. is an important medicinal plant of Family Euphorbiaceae. It is commonly called 'Mokoi' in Hindi, Indian acalypha in English and 'Indramarish' in Oriya. It is an annual herb and found in field and waste places and has been used in Indian medicine being used for the prevention and reversal of the atherosclerotic disease (Shanmugasundaram *et al.*, 1983). The plant is also used as expectorant as a substitute to senega. It cures diseases of the teeth and gums, burns, stomach pain, bleeding piles, irritations, stabbing pain, wheezing, sinusitis and cough (Gangadevi *et al.*, 2008). It also has many uses as diuretic, purgative, anti-parasiticide, anti-bacterial, anti-helmintic and useful for bronchitis, asthma, pneumonia and rheumatism (Azmahani *et al.*, 2002). Its ethanolic extract possesses wound healing activity (Reddy *et al.*, 2002) and has neutralization potential of Viper venom (Shirwaikar *et al.*, 2004). Furthermore it possess antimicrobial activity against the fungus *Candida albicans* and bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*,

Salmonella typhi, *Bacillus subtilis*, *Klebsiella pneumoniae* (Gopalkrishnan *et al.*, 2000; Gangadevi *et al.*, 2008). It also possesses post-coital anti-fertility activity in rats (Hiremath *et al.*, 1999).

Due to its medicinal importance, the demand for *A. indica* plants has increased which has led to indiscriminate harvesting and threatened its biodiversity. This highly valued medicinal plant is conventionally propagated through seeds only and seeds show low germination potential. Again vegetative propagation through stem cutting is not possible. Hence plant tissue culture can act as an *ex situ* method for conservation of this medicinal plant as done for many other medicinal plant species (Chandel *et al.*, 1996; Sunnichen and Shivanna, 1998; Shivasubramanian *et al.*, 2002; Ghosh and Banerjee, 2003; Rout *et al.*, 2006, 2008). Keeping this in view, the present work has been undertaken to establish a protocol for large scale plant regeneration via micropropagation, which will be helpful to fulfill the demands of pharmaceutical firms by providing quality materials. The present work is first of its

* Corresponding author : E-mail: shibaprasad2001@yahoo.co.uk

kind as no previous record of micropropagation of *A. indica* has been reported.

Materials and methods

Seeds collected from the mature fruit of the *A. indica* grown in the medicinal plant garden of Post-Graduate Department of Botany, Utkal University were gently washed in running tap water and treated with 5% teepol (v/v) for 5 min and washed with distilled water. The seeds were surface sterilized with 0.1% mercuric chloride (HgCl_2) solution for 3-5 min followed by thorough wash with sterilized distilled water for 3-4 times in order to remove traces of HgCl_2 . Seeds were then inoculated in different strengths of Murashige & Skoog (1962) basal medium (MS) without growth regulators or in combination with GA_3 (0.5 mg/l) for germination and also to develop *in vitro* seedlings. Cotyledon and cotyledonary node explants of one month-old *in vitro* seedling were excised aseptically and used as primary explants for multiple shoot induction.

MS basal medium fortified with different concentrations of cytokinins viz. 6-benzylamino purine (BAP), kinetin (Kn) (0.2-3 mg/l) alone or in combination with gibberellic acid GA_3 (0.5 mg/l) or together with auxins viz. indole-3-butyric acid (IBA) or α -naphthaleneacetic acid (NAA) (0.2 mg/l) were taken as shooting medium. For *in vitro* rooting, half-strength MS basal medium ($\frac{1}{2}$ MS) devoid of phytohormone (control condition) and half-strength MS medium supplemented with various concentrations (0.1-0.8 mg/l) of auxins IBA or NAA were used.

All the media were fortified with 3% sucrose and 0.8% agar and pH was adjusted to 5.6-5.8 and then autoclaved at 121 °C for 15 min. The cultures were maintained at 25 ± 2 °C with 16 h photoperiod at 55% relative humidity. For proper growth and development, the cultures were frequently transferred onto fresh medium at fifteen days intervals.

For each treatment, ten replicates were taken and each experiment was carried independently and repeated thrice. The cultures were kept under regular observation and data were recorded at 7-day interval. The percentage of response, mean number of shoots,

mean number of roots, mean shoot and root length, standard error of mean (S.E.M.) per each treatment were calculated. Each and every development of culture was recorded by taking photographs from time to time in Kodak Easy share digital camera (model no. DX 6490).

Results & discussion

In vitro seed germination

Seeds inoculated on MS basal medium sprouted within 14-20 days. Germination potential of *A. indica* seeds was also tested in response to $\frac{1}{2}$ and $\frac{1}{4}$ strength MS medium. Full-strength MS medium was found to be more efficient for germination of seeds than $\frac{1}{2}$ and $\frac{1}{4}$ MS medium. Seeds inoculated on MS medium sprouted within 14-15 days of inoculation but took 18-20 days to sprout on $\frac{1}{2}$ and $\frac{1}{4}$ strength MS medium. Germination of seeds was 60% in full strength MS medium, 53% in $\frac{1}{2}$ MS and 46% in $\frac{1}{4}$ MS. Addition of GA_3 (0.5 mg/l) to aforesaid media also enhanced rate of germination (Fig. 1).

Multiple shoot induction

Aseptically germinated plants of *A. indica* acted as prime source for explants. Cotyledon and cotyledonary node of one month-old *in vitro* seedling were inoculated in the medium, fortified with different concentrations of BAP/Kn (0.2 - 3 mg/l), taken alone or along with GA_3 (0.5 mg/l) or with lower concentration (0.2 mg/l) of auxins (IBA or NAA). Out of the two types of explants tested, cotyledonary node showed a better response towards multiple shoot induction. Within 7-15 days of culture,

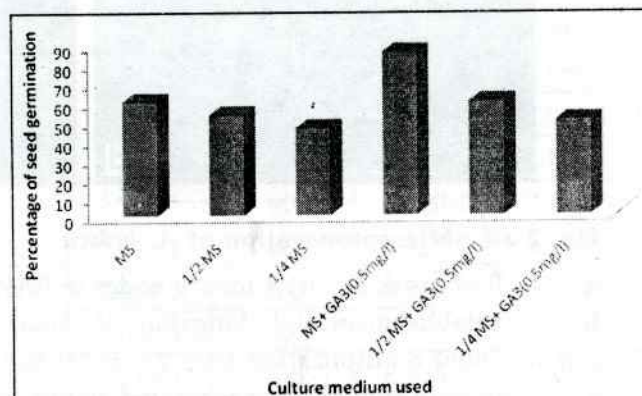


Fig. 1 : *In vitro* seed germination potential of *Acalypha indica*

cotyledonary node showed bud break (Fig. 2a). The basal portion of the cotyledonary nodes had also undergone slight callusing. Cotyledon explants exhibited multiple shoot induction after undergoing slight callusing. With increase in concentration of cytokinin (BAP/Kn) from 0.2 mg/l to 0.5 mg/l, increase in multiple bud induction was observed which declined thereafter. Transfer of cultures onto the same medium at 15-day interval also enhanced the number of multiple shoots per explants (Fig. 2b,

c, d). Better shoot multiplication occurred in medium containing a combination of cytokinin and GA₃ (0.5 mg/l) than the medium containing cytokinin alone. Synergistic effects of GA₃ on *in vitro* culture of medicinal plants have been studied earlier by many workers (Arora and Bhojwani, 1989; Pattnaik *et al.*, 1995; Pattnaik and Chand, 1996; Rout *et al.*, 2008). In the present investigation it was also found that medium containing high cytokinin (0.5 mg/l) and auxin (0.2 mg/l) to be better in shoot induction than

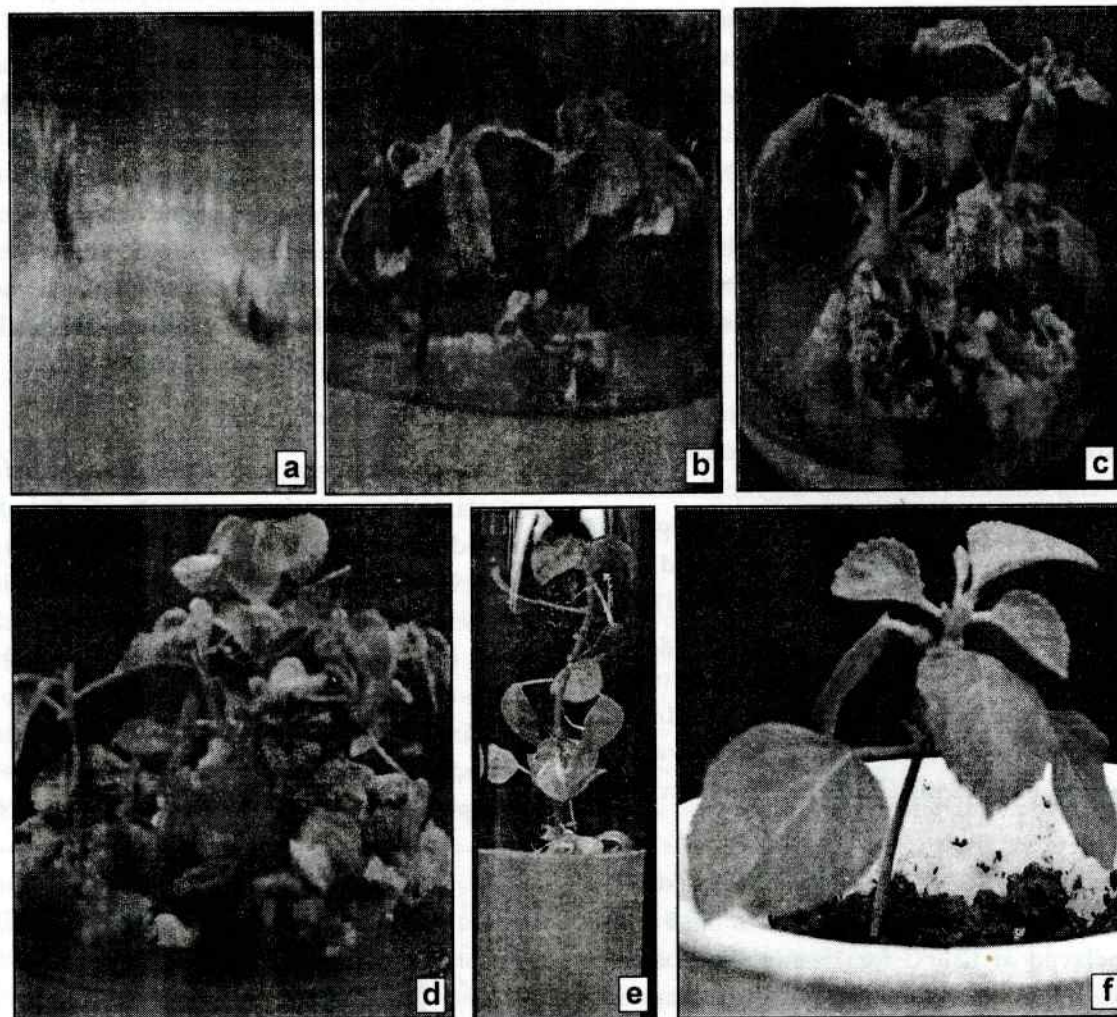


Fig. 2 a-f : Micropropagation of *A. indica*

- a : Bud break in cotyledonary nodes in BAP (0.5 mg/l) & GA₃ (0.5 mg/l) after 7 days of culture
- b : Establishment and elongation of shoots
- c & d : Shoot multiplication after 2nd & 3rd sub-culture respectively
- e : *In vitro* rooting of regenerated shoots in MS medium augmented with IBA (0.5 mg/l)
- f : A fully acclimatized one-month-old *A. indica* plant

the medium containing cytokinin alone. Enhancing effect of high cytokinin and low auxin concentration on shoot multiplication rate has been studied earlier in micropropagation of several medicinal plants (Phukan and Mitra, 1984; Islam, *et al.*, 1994; Anand and Bansal, 2002; Azad *et al.*, 2003).

In all the combinations tested, BAP was found to be more effective than Kn for multiple bud induction whether taken alone or in combination with GA₃ or auxin. Superior role of BAP on Kn in plant tissue culture has also been observed by earlier workers (Benjamin *et al.*, 1987 in *Atropa belladonna*; Arulpragasam and Latiff, 1986 in *Camellia sinensis*; Begum *et al.*, 2002 in *Ocimum basilicum*; Ghanti *et al.*, 2004 in *Phyllanthus amarus*; Rout *et al.*, 2006 in *Adhatoda vasica*; Rout *et al.*, 2008 in *Solanum surattense*).

Highest number of shoots per explants (8-10 shoots/explant) was produced on the MS medium fortified with BAP (0.5 mg/l) and GA₃ (0.5 mg/l) from cotyledonary node and 5-6 shoots/explants from cotyledon (Table-1). Out of the two types of explants tested, cotyledonary nodes were found to be more effective for multiple shoot induction.

In vitro rooting

For *in vitro* rooting, one-month-old regenerated shoots (2.0-2.5 cm) were cut aseptically and transferred onto the culture tube containing ½ MS (control condition) or ½ MS fortified with IBA or NAA (0.02-0.8 mg/l). The shoots were rooted in all the auxin supplemented medium within 7-10 days but maximum number of rooting (10-12 roots/shoot) (Fig. 2e) were observed on ½ MS medium fortified

Table-1
Effect of growth regulators on multiple shoot induction* in cotyledonary node and cotyledon explants of *A. indica*

Growth regulators (mg/l)					Cotyledon			Cotyledonary node		
BAP	Kn	NAA	IBA	GA ₃	% Response	Shoots/explant*	Shoot length	% Response	Shoots/explants	Shoot length
0.2					46	1.3±0.3	0.5±0.1	73	1.8±0.1	1.0±0.2
0.5					60	2.5±0.3	0.8±0.1	76	3.2±0.1	1.0±0.1
1.0					56	1.1±0.1	0.6±0.2	63	3.0±0.2	1.4±0.2
	0.2				40	1.0±0.4	0.6±0.3	60	2.5±0.2	1.2±0.3
	0.5				53	1.8±0.2	0.9±0.1	76	2.9±0.2	0.8±0.1
	1.0				50	1.8±0.2	1.7±0.1	70	2.0±0.1	0.9±0.2
0.5		0.2			83	3.0±0.1	1.2±0.2	96	4.4±0.1	1.6±0.1
1.0		0.2			80	2.1±0.2	1.0±0.1	90	4.0±0.2	2.4±0.1
	0.5	0.2			80	2.0±0.3	1.2±0.1	80	3.8±0.1	1.3±0.2
	1.0	0.2			76	1.8±0.2	1.1±0.2	86	3.0±0.2	2.0±0.1
0.5			0.2		100	3.6±0.4	1.4±0.3	100	5.3±0.2	2.0±0.1
1.0			0.2		93	3.0±0.1	1.3±0.1	90	4.2±0.3	1.9±0.2
	0.5		0.2		100	3.1±0.1	1.5±0.1	90	4.5±0.4	2.4±0.3
	1.0		0.2		90	2.5±0.2	1.0±0.2	86	3.6±0.1	2.1±0.1
0.5				0.5	95	5.5±0.1	2.2±0.3	100	8.5±0.3	3.2±0.1
1.0				0.5	90	3.8±0.1	2.0±0.1	93	7.2±0.2	3.0±0.1
	0.5			0.5	85	4.0±0.3	2.0±0.1	100	6.6±0.1	3.1±0.2
	1.0			0.5	80	3.1±0.1	1.1±0.1	96	6.0±0.2	3.0±0.2

* Data (Mean ± S.E.M.) based on 3 experiments and each experiment had ten replicates.

with IBA (0.5 mg/l), in comparison to medium fortified with the same concentration of NAA (Table-2). A superior role of IBA over NAA in tissue culture for *in vitro* rooting has already been studied by many workers (Ghanti *et al.*, 2004 in *Phyllanthus amarus*; Abhyankar and Reddy, 2007 in *Adhatoda vasica*; Pattanaik and Chand, 1996 in *Ocimum americanum*). No rooting had occurred on ½ MS medium devoid of phytohormones (control condition). With increase in auxin concentration up to 0.5 mg/l, the percentage of rooting increased which decreased thereafter. With increase of auxin concentration beyond 0.5 mg/l, callusing was observed from the cut end, which retarded the number of roots.

Acclimatization

The well rooted plantlets were transferred to polypots containing a sterile mixture of pure soil, sand and compost in the ratio of 1:1:1. They were irrigated with MS liquid medium at two days interval for fifteen days and kept in the culture room at 25±2 °C with 16 h photoperiod at 55% relative humidity. Gradually they were irrigated with ½ MS liquid medium and then by plain water and transferred to less humid conditions in order to acclimatize the plants to normal atmospheric conditions. Ninety percent of the regenerated plants

survived after the hardening procedure (Fig. 2f). The plants also set flowers and fruits within 8-10 weeks and there were no significant variations between *in vivo* and *in vitro* regenerated plants.

The present work has established a protocol for rapid propagation of *A. indica* by using explants from one month old *in vitro* grown seedlings. This method of plant regeneration can meet the demand of pharmaceutical firms and will help in conservation of the plant species.

Acknowledgements

Authors are thankful to National Medicinal Plant Board, New Delhi for their financial assistance to carry this research work. Authors are also thankful to Head, P.G. Department of Botany, Utkal University for providing necessary laboratory facilities.

References

- Abhyankar, G. and Reddy, V.D. (2007). Rapid Micropropagation via axillary bud proliferation of *Adhatoda vasica* Nees. from nodal segments. *Ind. J. Exp. Biol.* 45: 268-271.
- Azmahani, A., Somchit, M.N. and Rosyilah, A.R. (2002). *In vitro* Anti-bacterial and Anti-fungal Properties of *Acalypha indica* (Kucing Galak). *Proc. Reg. Symp. Env. Natural Resour.* 1: 503-508.

Table-2
Effect of auxins on *in vitro* rooting of regenerated shoots of *A. indica*

Auxin concentration	% rooting	*Roots/explant	*Root length (cm)
½ MS (Control)	0	0	0
IBA			
0.1	80	3.2± 0.1	1.0±0.1
0.2	93	5.5±0.2	1.1±0.2
0.5	100	10.2±0.2	2.0±0.1
0.8	96	6.5±0.3	1.5±0.1
NAA			
0.1	76	2.4±0.2	0.8±0.1
0.2	96	4.3±0.3	1.0±0.1
0.5	96	9.3±0.4	1.9±0.3
0.8	80	2.8±0.3	1.0±0.2

*Data (Mean ± S.E.M.) based on 3 experiments and each experiment had ten replicates.

- Azad, M.A.K., Amin, M.N. and Begum, F. (2003). Rapid clonal propagation of a medicinal plant-*Adhatoda vasica* Nees. using tissue culture technique. *Online J. Biol. Sci.* 3(2): 172-182.
- Anand, Y. and Bansal, Y.K. (2002). Micropropagation of medicinal plant *Adhatoda vasica* Nees. through nodal culture. *Proc. Nat. Acad. Sci. India.* 72 B (III) & (IV): 313-318.
- Aroora, R. and Bhojwani, S.S. (1989). Micropropagation of *Saussurea lappa*, *Plant Cell Rep.* 8: 44-47.
- Arulpragasam, P.V. and Latiff, R. (1986). Studies on the tissue culture of tea (*Camellia sinensis* L.O. Kuntze). 1. Development of a culture method for the multiplication of shoots. *Sri Lanka J. Tea Sci.*, 55: 44-47.
- Benjamin, B.D., Roja, P.C., Heble, M.R. and Chadha, M.S. (1987). Multiple shoot cultures of *Atropa belladonna*: effect of physico-chemical factors on growth and alkaloid formation. *J. Plant Physiol.* 129: 129-135.
- Begum, F., Amin, M.N., and Azad, M.A.K. (2002). *In vitro* Rapid clonal propagation of *Ocimum basilicum* L.. *Plant Tiss. Cult.* 12(1): 27-35.
- Chandel, K.P.S., Shukla, G. and Sharma, N. (1996). Biodiversity in medicinal and aromatic plants in India: Conservation and utilisation, Indian Council of Agricultural Research: New Delhi, India.
- Gangadevi, V., Yogeswari, S., Kamalraj, S., Rani, G. and Muthumary, J. (2008). The antibacterial activity of *Acalypha indica* L. *Ind. J. Sci. Tech.* 1(6): 1-5.
- Ghanti, K.S., Govindraju, B., Venugopal, R.B., Ramgopal Rao, S., Kaviraj, C.P., Jabeen F.T.Z., Barad, A. and Rao, S. (2004). High frequency shoot regeneration from *Phyllanthus amarus* Schum. & Thonn. *Ind. J. Biotechnol.* 3: 103-107.
- Ghosh, K.C. and Banerjee, N. (2003). Influence of plant growth regulators on *in vitro* micropropagation of *Rauwolfia tetraphylla* L., *Phytomorphology.* 53: 11-19.
- Gopalakrishnan, V., Rao, K.N.V., Loganathan, V., Shanmuganathan, S., Bollu, V.K. and Sharma, T.B. (2000). Antimicrobial activity of extracts of *Acalypha indica* Linn. *Ind. J. Pharmac. Sci.* 62(5): 347-350.
- Hiremath, S.P., Rudresh, K., Badami, S., Patil, S.B. and Patil, S.R. (1999). Post-coital antifertility activity of *Acalypha indica* L. *J. Ethnopharmacol.* 30(3): 253-258.
- Islam, R., Hossain, M., Joarder, O.I. and Naderuzzaman, A.T.M. (1994). *In vitro* clonal propagation of *Eucalyptus globosus*. *Plant Tiss. Cult.* 4: 85-88.
- Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Planta.* 15: 473-497.
- Pattanaik, S.K., Sahoo, Y. and Chand, P.K. (1995). *In vitro* culture of *Morus alba*: A economically important plant. *Scient. Hort.* 61: 227-239.
- Pattanaik, S. and Chand, P.K. (1996). *In vitro* propagation of the medicinal herbs *Ocimum americanum* L. syn. *O. canum* Sims. (Hoary basil) and *Ocimum sanctum* L. (Holy basil), *Plant Cell Rep.* 15: 846-850.
- Phukan, M.K. and Mitra, G.C. (1984). Regeneration of Tea (*Camellia sinensis* L.) shoot from nodal explants in tissue culture. *Curr. Sci.* 53: 874-876.
- Reddy, J.S., Rao, P.R. and Reddy, M.S. (2002). Wound healing effects of *Heliotropium indicum*, *Plumbago zeylanicum* and *Acalypha indica* in rats. *J. Ethnopharmacol.* 79(2): 249-251.
- Rout G., Swain, P.K. and Rath, S.P. (2006). *In vitro* propagation of *Adhatoda vasica* Nees.: A potential medicinal Plant, *Plant Sci. Res.* 28 (1&2): 23-26.
- Rout, G., Biswal, P.R., Mishra, L.P. and Rath, S.P. (2008). Large scale propagation of *Solanum surattense* Burm.f.: An important medicinal plant. *Plant Sci. Res.* 30 (1&2): 52-56.
- Shanmugasundaram, K.R., Seethapapathy, P.G. and Shanmugasundaram, E.R. (1983). Anna Pavala Sindhoooram, an anti-atherosclerotic Indian drug. *J. Ethnopharmacol.* 7(3): 247-265.
- Shirwaikar, A., Rajendran, A.K., Bodla, R., and Kumar, D.C. (2004). Neutralization potential of *Viper russelli* (Russell's viper) venom by ethanol leaf extract of *Acalypha indica*. *J. Ethnopharmacol.* 94(2-3): 267-273.
- Sunnichen, V.K. and Shivanna, K.R. (1998). Micropropagation of *Eremostachys superba* - an endangered endemic species from India. *Curr. Sci.* 74: 699-702.
- Shivasubramanian, S. Vallinayagam, S., Patric, R.D. and Manickam, V.S. (2002). Micropropagation of *Plecranthus vetiveroides* (Jacob) Singh and Sharma- A Medicinal Plant, *Phytomorphology.* 52: 55-59.

***In vitro* response of cotyledon and hypocotyl explants of flax *Linum usitatissimum* L.**

S. Gochhait, S. Dwibedy, A.K. Behera, D. Behera and U.B. Mohapatra^ψ

P.G. Department of Botany, North Orissa University, Takatpur, Baripada- 757003

(Received : 22 November, 2010; Revision received : 17 December, 2010)

Flax (*Linum usitatissimum* L.) is an important crop possessing industrial as well as medicinal value. *In vitro* response of two seedling explants (cotyledons & hypocotyls) of four different cultivars of flax was investigated. Results showed that all the four flax cultivars were amenable to *in vitro* plant production. Calli were observed from both the explants but shoot regeneration was observed only in case of hypocotyl derived callus, though calli derived from cotyledons had a better growth response. MS medium supplemented with 1.0 mg/l IAA and 1.5 mg/l BAP resulted in the formation of higher callus weight and enhanced shoot regeneration in callus derived from hypocotyls.

Key words: Flax, seedling explants, callus, shoot regeneration

Introduction

Flax or linseed (*Linum usitatissimum* L.) is a triple purpose crop, serving as a source of oil, fibre and fodder. It is a cool, temperate, annual, dicotyledonous herb belonging to the family Linaceae. It has a long history of cultivation. The plant has industrial as well as medicinal importance. The linseed oil is also used for edible purposes. Because of its wide application genetic improvement of this crop is imperative. Unlike other crops, mainly cereals, flax improvement has not been developed at a significant rate (Burbulis *et al.*, 2005). Nevertheless, the application of tissue culture techniques could facilitate flax improvement by incorporating valuable, desirable traits in terms of resistance against diseases, oil quality improvement and herbicide tolerance through somatic hybridization and somaclonal variation (Barisan *et al.*, 1987; Marshall & Courduries, 1992; Rutkowsska-Krasue *et al.*, 2003). Since it has a smaller nuclear genome, it has been used as a model system for genetic engineering (Yildiz & Celal, 2002). Flax is easy to regenerate under *in vitro* condition. Moreover, *in vitro* regeneration is greatly influenced by genotype, explants, developmental stage and media composition (Chen & Dribenki, 2004; Mundhare & Rashid, 2002; Tang *et al.*, 2003). Genetic transformation has been achieved by *Agrobacterium*

infection (Barison *et al.*, 1987). Thus, *in vitro* regeneration of plant should be enhanced to get high frequency of transgenic plants; efficient regeneration protocols being a pre-requisite for genetic manipulations.

In the present investigation, we evaluated the response of four flax cultivars as regards *in vitro* responsiveness of hypocotyl and cotyledon explants towards callusing, shoot induction and role of IAA (indole-3-acetic acid), and BAP (benzylaminopurine), in shoot regeneration.

Materials & methods

Plant material

Seeds of four flax cultivars namely 'OL-98-7-3', 'OL-98-15-2', 'Padmini' and 'Sharada' were collected from the Regional Research & Technology Transfer Substation, Jashipur, Mayurbhanj, Orissa. Hypocotyls and cotyledons were collected from axenically grown 4-5 days old seedlings, which were germinated from seeds on MS (Murashige & Skoog, 1962) medium without any growth regulator (MS0 medium). MS medium, IAA and BAP were procured from Hi-media Pvt. Ltd., Mumbai.

***In vitro* culture**

Seeds of four cultivars of flax were surface sterilized by 2% sodium hypochlorite solution for 3

^ψCorresponding author; E-mail: ubmbotnou@gmail.com

min. Subsequently, the seeds were washed three times with sterile distilled water and plated on to MS0 medium. Plates were sealed with parafilm and incubated in dark. Hypocotyls and cotyledons were excised from 4-5 day-old *in vitro* grown seedlings (Fig. 1a). Aseptically explants were transferred to MS medium containing different concentrations of auxin (IAA) and cytokinin (BAP) (Table-1 & 2). Prior to auto-claving, pH of the medium was adjusted to 5.6 and cultures were incubated at $22 \pm 2^\circ\text{C}$ under cool white fluorescent light with a 16 h light/8 h dark photoperiod. Subculture to fresh medium was carried out at every 2 weeks interval. Callus growth was measured after 28 days. Shoot number per explant was counted after 2 weeks of subculturing to fresh MS media. Regenerated shoots were transferred to rooting medium (MS medium containing 0.5 mg/l auxin (NAA alone) for development of plantlets.

Results and discussion

In the present investigation, both types of explants (cotyledons & hypocotyls) formed callus (Fig. 1 b,c) on MS medium containing different concentrations of auxin and cytokinin (Tables-1 & 2). Among the two types of explants, the fresh weight gain of callus derived from cotyledons was relatively more as compared to that of callus derived from hypocotyls. Great variation was found in the callus weight derived from the cotyledons (Table-1). The maximum callus weight was noted in the cultivar 'Padmini' (2.9 g) on MS medium supplemented with 1.5 mg/l IAA and 1.0 mg/l BAP and also in cultivar OL-98-7-3 (2.9 g) on MS medium supplemented with 2.0 mg/l each of IAA and BAP. This was followed by cultivar OL-98-15-2 (2.8 g) on MS medium supplemented with 2.0 mg/l of both IAA and BAP and also on MS medium supplemented with 2.0 mg/l

Table-1
Response of cotyledon explants towards callus growth & shoot regeneration in MS with different concentrations of IAA & BAP

IAA/BAP (mg/l)	OL-98-7-3		OL-98-15-2		'Padmini'		'Sarada'	
	Callus g.f.wt	Shoots/ explant	Callus g.f.wt	Shoots/ explant	Callus g.f.wt	Shoots/ explant	Callus g.f.wt	Shoots/ explant
0.5/0.5	1.6 $\pm$ 0.8	-	1.1 $\pm$ 0.5	-	1.8 $\pm$ 1.1	-	1.0 $\pm$ 0.1	-
1.0/0.5	1.6 $\pm$ 0.9	-	1.5 $\pm$ 0.0	-	2.4 $\pm$ 1.0	-	1.7 $\pm$ 1.6	-
1.5/0.5	1.6 $\pm$ 0.9	-	1.5 $\pm$ 0.8	-	2.2 $\pm$ 0.1	-	2.2 $\pm$ 1.3	-
2.0/0.5	2.4 $\pm$ 1.3	-	2.8 $\pm$ 0.3	-	1.9 $\pm$ 1.3	-	1.9 $\pm$ 1.0	-
0.5/1.0	2.3 $\pm$ 1.3	-	2.2 $\pm$ 1.2	-	0.8 $\pm$ 0.4	-	1.9 $\pm$ 1.1	-
1.0/1.0	2.2 $\pm$ 1.2	-	1.9 $\pm$ 1.3	-	1.5 $\pm$ 0.8	-	0.8 $\pm$ 0.7	-
1.5/1.0	1.3 $\pm$ 0.4	-	2.2 $\pm$ 0.5	-	2.9 $\pm$ 0.2	-	1.7 $\pm$ 0.8	-
2.0/1.0	1.6 $\pm$ 0.4	-	2.4 $\pm$ 1.3	-	2.1 $\pm$ 1.2	-	0.8 $\pm$ 0.8	-
0.5/1.5	2.3 $\pm$ 1.3	-	1.9 $\pm$ 1.1	-	1.5 $\pm$ 0.7	-	1.6 $\pm$ 0.9	-
1.0/1.5	2.0 $\pm$ 1.2	-	2.6 $\pm$ 0.4	-	2.2 $\pm$ 0.4	-	1.3 $\pm$ 1.1	-
1.5/1.5	2.3 $\pm$ 1.3	-	2.3 $\pm$ 1.3	-	1.2 $\pm$ 0.8	-	2.4 $\pm$ 0.7	-
2.0/1.5	2.0 $\pm$ 1.1	-	2.6 $\pm$ 0.5	-	1.6 $\pm$ 0.6	-	1.8 $\pm$ 1.0	-
0.5/2.0	2.1 $\pm$ 1.2	-	1.9 $\pm$ 0.8	-	2.5 $\pm$ 1.4	-	1.3 $\pm$ 0.8	-
1.0/2.0	2.1 $\pm$ 0.4	-	1.8 $\pm$ 1.1	-	1.5 $\pm$ 0.9	-	1.2 $\pm$ 0.9	-
1.5/2.0	2.4 $\pm$ 0.3	-	2.4 $\pm$ 0.5	-	1.8 $\pm$ 0.1	-	1.6 $\pm$ 0.7	-
2.0/2.0	2.9 $\pm$ 0.1	-	2.8 $\pm$ 0.4	-	2.6 $\pm$ 0.6	-	1.3 $\pm$ 0.6	-

Data represent Mean $\pm$ S.D. of 3 separate experiments each with at least 4 replicates.

g.f.wt. : gram fresh weight

IAA and 0.5 mg/l BAP. In case of cultivar 'Sharada' the highest callus weight (2.4 g) was observed on MS medium supplemented with both IAA & BAP each of 1.5 mg/l. The lowest callus weight was noted in cultivar 'Sharada' (0.8 g) on medium supplemented with 1.0 mg/l each of IAA & BAP or with 2.0 mg/l IAA & 1.0 mg/l BAP. The same callus weight was observed in cultivar 'Padmini' (0.8 g) on MS medium supplemented with 0.5 mg/l IAA & 1.0 mg/l BAP. In case of cultivar 'OL-98-7-3' and 'OL-98-15-2' the gram fresh weight of callus was 1.3 g on MS medium supplemented with 1.5 mg/l IAA & 1.0 mg/l BAP and 1.1 g on MS medium supplemented with 0.5 mg/l both IAA & BAP respectively. In cultivar 'Sharada' the highest callus weight, 2.4 g was noted on MS medium supplemented with 1.5 mg/l of both IAA & BAP. However, there was uniformity observed in callus weight that were derived from the hypocotyls. In the latter case, callus weight varied from 2.5 g to

2.1 g among the four different flax cultivars (Table-2; Fig. 2). Maximum callus weight was observed in case of cultivar 'Sharada' (2.5 g) whereas the minimum was in cultivar 'Padmini' (2.1 g). In all the cultivars, increase in callus weight was observed in medium supplemented with 1.0 mg/l IAA & 1.5 mg/l BAP. While considering the shoot induction, hypocotyls were more responsive towards shoot regeneration in comparison with cotyledons. Shoot regeneration was found only in case of hypocotyl derived callus but, not in the case of callus derived from cotyledons. This result corresponded to the findings of Friedt (1990), Mathews & Narayanswamy, (1976) and Yildiz *et al.* (2002). Similar types of results were also described by Bretagne *et al.* (1994). In case of callus derived from hypocotyls, the shoot number per explant varied from 5-7 (Table-2 & Fig. 3). Highest number of shoots was found in the cultivar 'OL-98-15-2' (7) followed by 'OL-98-7-3' (6.6), 'Padmini' (6.4)

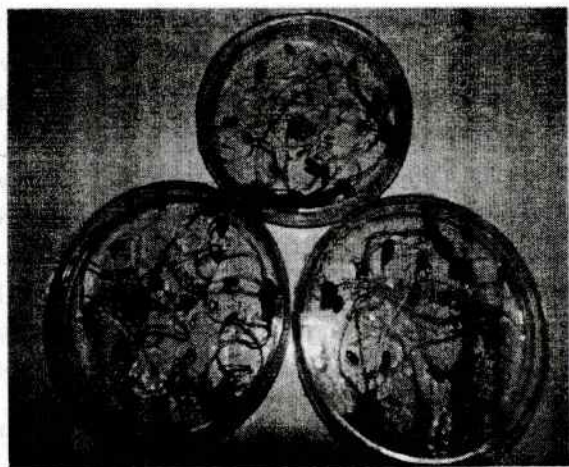
Table-2

Response of hypocotyl explants towards callus growth & shoot regeneration in MS with different concentrations of IAA & BAP

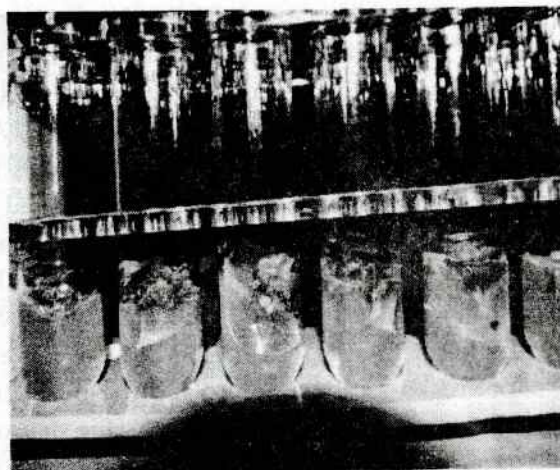
IAA/BAP (mg/l)	OL-98-7-3		OL-98-15-2		'Padmini'		'Sarada'	
	Callus g.f.wt.	Shoots/ explant	Callus g.f.wt.	Shoots/ explant	Callus g.f.wt.	Shoots/ explant	Callus g.f.wt.	Shoots/ explant
0.5/0.5	1.8 ± 0.2	4.0 ± 1.8	1.0 ± 0.5	3.8 ± 1.3	1.6 ± 0.7	2.8 ± 1.4	1.9 ± 0.2	3.6 ± 1.5
1.0/0.5	1.6 ± 0.7	2.2 ± 1.3	1.2 ± 0.4	4.0 ± 1.5	1.6 ± 0.5	2.8 ± 1.3	1.7 ± 0.5	2.0 ± 0.0
1.5/0.5	1.3 ± 0.4	2.0 ± 1.0	1.5 ± 0.2	3.4 ± 0.8	1.2 ± 0.6	5.4 ± 2.3	2.0 ± 0.3	4.0 ± 1.5
2.0/0.5	1.9 ± 0.2	1.8 ± 0.4	1.5 ± 0.2	1.6 ± 0.5	1.2 ± 0.3	3.0 ± 2.0	1.5 ± 0.4	1.8 ± 0.8
0.5/1.0	1.9 ± 0.1	3.4 ± 1.1	1.3 ± 0.0	2.0 ± 0.0	1.0 ± 0.2	4.2 ± 1.6	1.9 ± 0.1	2.6 ± 1.1
1.0/1.0	2.0 ± 0.1	3.6 ± 1.1	2.2 ± 0.3	3.4 ± 0.8	2.1 ± 0.2	3.0 ± 0.7	2.2 ± 0.2	2.0 ± 0.7
1.5/1.0	1.9 ± 0.1	3.2 ± 0.4	2.0 ± 0.3	4.8 ± 1.3	2.0 ± 0.4	3.6 ± 1.6	2.3 ± 0.3	1.6 ± 0.5
2.0/1.0	2.0 ± 0.2	4.6 ± 1.6	2.0 ± 0.2	6.2 ± 2.4	2.1 ± 0.3	4.8 ± 1.9	2.0 ± 0.5	3.6 ± 1.1
0.5/1.5	1.9 ± 0.6	4.2 ± 1.0	1.7 ± 0.1	5.6 ± 2.3	1.8 ± 0.1	2.6 ± 1.1	1.9 ± 0.5	5.0 ± 1.5
1.0/1.5	2.1 ± 0.4	6.6 ± 2.0	2.2 ± 0.2	7.0 ± 1.8	2.1 ± 0.2	6.4 ± 1.1	2.5 ± 0.2	5.6 ± 1.1
1.5/1.5	1.2 ± 0.4	5.2 ± 2.3	1.4 ± 0.2	4.6 ± 1.5	1.3 ± 0.4	6.2 ± 2.4	1.8 ± 0.3	3.0 ± 1.5
2.0/1.5	1.9 ± 0.2	4.4 ± 1.1	1.3 ± 0.3	3.8 ± 0.4	1.5 ± 0.5	3.0 ± 1.2	1.7 ± 0.4	3.0 ± 1.8
0.5/2.0	1.4 ± 0.4	3.4 ± 1.1	1.8 ± 0.3	3.3 ± 0.5	1.9 ± 0.4	3.4 ± 1.1	2.1 ± 0.2	3.4 ± 1.3
1.0/2.0	1.8 ± 0.5	2.8 ± 1.1	1.8 ± 0.2	4.6 ± 1.1	1.6 ± 0.2	2.0 ± 1.0	1.7 ± 0.2	3.6 ± 1.1
1.5/2.0	1.3 ± 0.6	3.6 ± 0.5	1.7 ± 0.4	4.0 ± 1.5	1.8 ± 0.5	1.6 ± 0.5	1.7 ± 0.6	2.2 ± 0.8
2.0/2.0	1.6 ± 0.5	2.2 ± 0.4	1.6 ± 0.3	2.8 ± 0.8	1.4 ± 0.1	1.6 ± 0.5	1.6 ± 0.4	2.0 ± 1.0

Data represent Mean ± S.D. of 3 separate experiments each with at least 4 replicates.

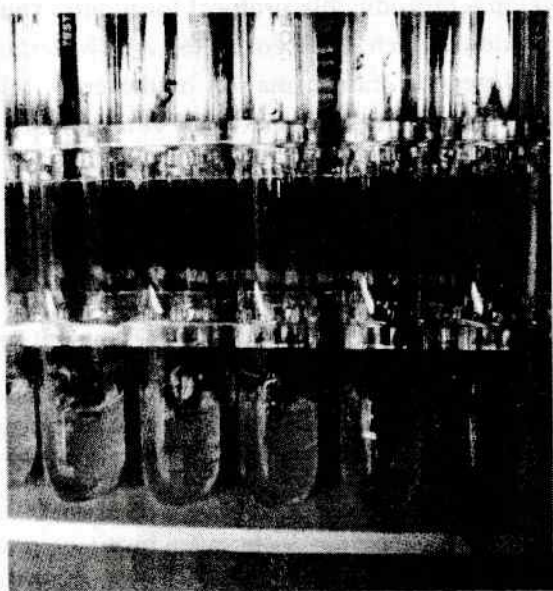
g.f.wt. : gram fresh weight



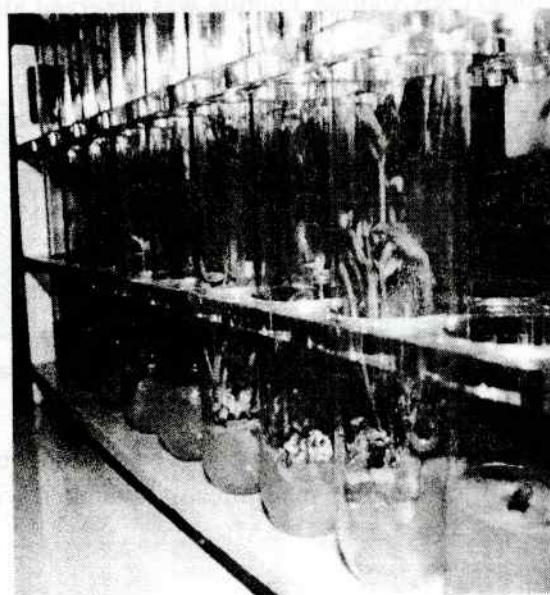
(a)



(b)



(c)



(d)

Fig. 1 : Callus-mediated shoot regeneration in flax (*Linum usitatissimum* L.

- (a) *In vitro* seed germination
- (b) Cotyledon-derived callus
- (c) Hypocotyl-derived callus
- (d) Shoot regeneration from hypocotyl-derived callus

and 'Sharada' (5.6) in MS medium supplemented with 1.0 mg/l IAA & 1.5 mg/l BAP. Thus, it was confirmed that in addition to cytokinin, lower concentration of auxin promotes callusing and shoot regeneration which corroborates the findings of Koronfel (1998), Marshall & Courduries (1992) and Cunha & Ferreira (1996). In contrast to our findings, Burbulis *et al.* (2009) reported that 2.0 mg/l BAP & 0.1 mg/l NAA (α -naphthaleneacetic acid) was most effective in the flax cultivar 'Szaphir', associated with a high shoot regeneration ability (22 shoots/explant) and shoot regeneration frequency (100%). Yildiz & Ozen (2006) reported that MS medium supplemented with 1.0 mg/l BAP & 0.02 mg/l NAA, was the best for shoot regeneration among the different cultivars tested in his experiment while comparing the role of growth regulators in adventitious shoot regeneration from hypocotyls of flax. The regenerated shoots were then transferred to MS medium supplemented with 0.5 mg/l NAA alone. Rooting was observed after 2-3

weeks. The complete regenerants were then ready for acclimatization in subsequent steps. Thus, the variation in the callus weight derived from cotyledons at the same concentration of IAA & BAP and also the variation in the shoot number obtained from the hypocotyl-derived callus in different linseed cultivars on MS medium supplemented with 1.0 mg/l IAA and 1.5 mg/l BAP may be due to the effect of genotype. Burbulis *et al.* (2009) also reported that genotype strongly affects adventitious bud regeneration from linseed hypocotyls among the different cultivars investigated by him. It is widely accepted that morphogenesis is strongly affected by the genotype and exogenous factors (Bhaskaran & Smith, 1990).

In conclusion, data investigated in this study indicate that genotype, auxin-to-cytokinin ratio and explant type strongly affect *in vitro* callusing and shoot regeneration in flax. Therefore, it is essential to develop a reproducible protocol for *in vitro* shoot regeneration, which is essential for *Agrobacterium*-mediated genetic transformation of the crop, with minimum possibility of somaclonal variation.

Reference

- Barisan, N., Armitage, P., Scoot, R.J. and Drapper, J. (1987). Genetic transformation of flax (*Linum usitatissimum* L.) by *Agrobacterium tumefaciens*: Regeneration of transformed shoots via a callus phase. *Plant Cell Rep.* 6: 396-399.
- Bhaskaran, S. and Smith, R.H. (1990). Regeneration in cereal tissue culture: A Review. *Crop Sci.* 30: 1329-1336.
- Bretagne, B., Chupeau, MCh., Chupeau, Y., and Fouilloux, G. (1994). Improved flax regeneration from hypocotyls using thidiazuron as a cytokinin source. *Plant Cell Rep.* 14: 120-124.
- Burbulis, N., Blinstrubiene, A., Venskutoniene, E. and Katauskyte, L. (2005). Organogenesis in callus cultures of *Linum usitatissimum* L. *Biol.* 691: 129-135.
- Burbulis, N., Blinstrubiene, A. and Kupriene, R. (2009). Regeneration of adventitious shoot of linseed (*Linum usitatissimum* L.) from hypocotyl explants. *Zemdirbyste-Agric.* 96(3): 169-175.
- Chen, Y. and Dribnenki, P. (2004). Effect of medium osmotic potential on callus induction and shoot regeneration in flax anther culture. *Plant Cell Rep.* 23(5): 272-276.

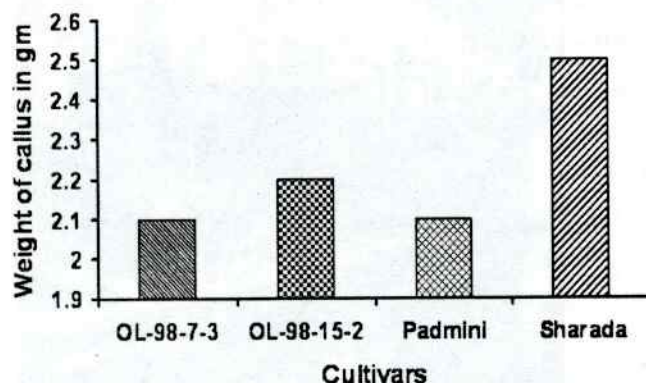


Fig. 2: Weight of hypocotyl derived callus from four flax cultivars on MS medium supplemented with 1.0 mg/l IAA & 1.5 mg/l BAP.

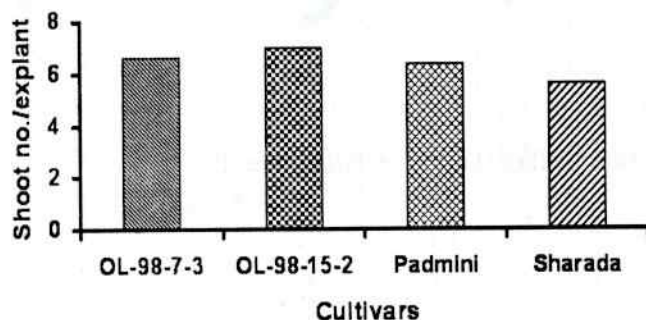


Fig. 3 : Shoots/explant from hypocotyl-derived callus from four flax cultivars on MS medium supplemented with 1.0 mg/l IAA & 1.5 mg/l BAP.

- Cunha, A.C.G. and Ferreira, M.F. (1996). Somatic embryogenesis, organogenesis and callus growth kinetics of flax. *Plant Cell Tiss. Org. Cult.* **47**: 1-8.
- Friedt, W. (1990). Biotechnology in breeding of industrial oil crops: the present status and future prospects. *Fat Sci. Technol.* **90**: 51-55.
- Koronfel, M. (1998). Development of genotype and efficient regeneration procedure for flax (*Linum usitatissimum* L.). *Arab J. Biotechnol.* **1**(1): 87-92.
- Marshall, G. and Courduries, P. (1992). An assessment of somaclonal variation in linseed (*Linum usitatissimum* L.). *Ann. Appl. Biol.* **120**: 501-509.
- Mathews, V.H. and Narayanswamy, S. (1976). Phytohormone control of regeneration in cultured tissue of flax. *Z. Pflanzenphysiol.* **80**: 436-442.
- Mundhare, R. and Rashid, A. (2002). Stimulation of shoot bud regeneration on hypocotyls of *Linum* seedlings, on a transient withdrawal of calcium: effect of calcium, cytokinin & thidiazuron. *Plant Sci.* **162**: 211-214.
- Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant.* **15**: 473-479.
- Rutkowska-Krause, I., Mankowska, G. and Lukaszewicz, M. (2003). Regeneration of flax (*Linum usitatissimum* L.) plants from anther culture & somatic tissue with increased resistance to *Fusarium oxysporum*. *Plant Cell Rep.* **22**(2): 110-116.
- Tang, G.X., Zhou, W.J. and Li, H.Z. (2003). Medium, explants, genotype factors influencing shoot regeneration in oilseed *Brassica* sp. *J. Agron. Crop Sci.* **189**: 351-358.
- Yildiz, M., Ozcan, S. and Celal, Er. (2002). The effect of different explants sources on adventitious shoot regeneration in flax (*Linum usitatissimum* L.). *Turk J. Biol.* **26**: 37-40.
- Yildiz, M. and Ozgen, M. (2006). A comparison of growth regulators for adventitious shoot regeneration from hypocotyls of flax. *J. Food, Agric. Env.* **4**(3&4): 171-174.

Metabolic performance of microbes in rice field under conventional and organic farming systems

D. Chhotaray¹, P.K. Mohapatra^{2Ψ} and C.S.K. Mishra³

¹P.G. Department of Botany, Utkal University, Bhubaneswar - 751 004

²Department of Botany, Ravenshaw University, Cuttack - 753 003

³P.G. Department of Zoology and Biotechnology, College of Basic Sciences and Humanities, OUAT, Bhubaneswar - 751003

(Received : 06 November, 2010; Revision received : 14 December, 2010)

Agricultural practices that reduce soil degradation and improve agricultural sustainability are need of the day particularly for tropical soils where the economy is mostly agro-based. The soil under organic agriculture showed higher biomass of bacteria and fungi and higher respiration rate than of the soil under conventional agriculture. The activity of enzymes such as amylase, cellulase, invertase, proteases and dehydrogenase also followed the same pattern. In both cases there were significant seasonal variations of the microbial biomass and metabolic activity in soil with minimum activity in summer while the soil depth and physico-chemistry significantly influenced the microbial performance. Soil water and moisture contents were found to be the most important factors regulating soil microbial activity and the regulation was more intense in conventional system than in the other. When these two were not limiting, the activity was controlled by the availability of oxygen while temperature played a secondary role. Always the field under organic farming was metabolically more active compared to that in case of conventional farming indicating that long term organic fertilization has a positive effect on soil fertility.

Key words: Organic farming, conventional farming, enzyme activity, soil microbial biomass, soil respiration.

Introduction

Conventional agriculture with intensive use of chemicals and fertilizers has been responsible for improving the productivity especially after the green revolution. With more dependence on chemicals and pesticides the sustainable productivity of soil under intensive chemical agriculture is now at stake. It is reported that conventional agricultural practices greatly affect the soil microbial density and subsequently the remineralization cycle (Melero *et al.*, 2005; Araujo *et al.*, 2009). However, this agricultural practice is still followed in many parts of the country without much evaluation of its adverse impact on soil microbial efficiency. Alternatively other environment friendly agricultural practices have emerged to replace the conventional agriculture of which organic practice is significant (Singh *et al.*, 2004; Liu *et al.*, 2008; Araujo *et al.*, 2009; Zhao *et al.*,

2009). The latter is gaining worldwide acceptance in recent years and is expanding at an annual rate of 20% in the developed countries, whereas in India, it is still in infancy (Sunita and Singhcharya, 2003; Krishnakumar *et al.*, 2005). Under Indian conditions there is hardly any report on evaluation of microbial activity in soil with prolonged conventional or organic cultivation practices though it is being regularly done in other rice growing countries like China, Japan and South Korea (Wang and Zhao, 1995; Zhang *et al.*, 2008; Liu *et al.*, 2008; Zhao *et al.*, 2009). On the other hand, there is a need to measure the metabolic performance of soil microbes of different agroecosystems before recommending the appropriate farm practices to the farmers. The present paper deals with a comparative account of metabolic performance of microbes in case of conventional and organically managed rice agroecosystems.

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

Materials and methods

Study sites and sampling

The study sites are located in the Khurda district of Orissa where only rice-rice cultivation is the practice. Samples were collected from the sites every fortnight for a period of two years from 2007-2009, using steel corer (10 cm diam) and brought to the laboratory in sterilized polybags. After collection from the top 20 cm soil, each sample was segregated to five depth classes with 4 cm interval. The soil of each 4 cm class was properly homogenized before use. Each experimental plot was sampled from five locations after which the soils were mixed to form a single sample. Fifteen such sites were sampled each of which served as a replicate.

Microbial activity measurement

Soil microbial biomass and enzyme activities were measured on the day of collection. The bacterial and fungal biomass was measured separately by dilution plate counting (Parkinson *et al.*, 1971) and agar film technique (Jones and Mollison, 1948) respectively. Activity of soil amylase, cellulase and invertase was measured spectrophotometrically using starch, carboxymethylcellulose and sucrose as substrate, respectively and the absorbance values were quantified taking glucose as standard (Mishra *et al.*, 1979). Protease activity was estimated spectrophotometrically (UV-VIS spectrophotometer, Perkin-Elmer EZ 201, Perkin-Elmer, USA) as per the method described by Speir and Ross (1975) taking sodium caesinate as substrate and quantification was made using tyrosine as the standard. The activity of dehydrogenase was done using 2,3,5- triphenyl tetrazolium chloride (TTC) as substrate and quantified taking TPF as standard (Casida *et al.*, 1964). All enzyme activities were measured at room temperature after a standard incubation of 24 hours. Respiration was measured directly from the soil surface with the help of an IRGA equipped with a soil respiration chamber (CIRAS II, PP Systems, UK).

Statistical analysis

All samples were taken in replicates and values presented in figure and tables as means and SD of fifteen replicates. The statistical comparison of

microbial activity among seasons, depths and farming systems was made through analysis of variance. Two factor and three factor Anova were performed for analyzing the interaction of factors and its effect on microbial activity. The regulation of microbial efficiency by local environmental conditions was analysed through correlation and regression analysis. Statistical calculations were made using M Stat C software (Russelfreed, Michigan State University, 1991) following standard procedures (Gomez and Gomez, 1984).

Results

Microbial biomass

There was significant variation of bacterial and fungal biomass between the two agricultural systems considered in this study. In both organic and conventional systems minimum microbial biomass was recorded in summer and maximum in winter (Fig. 1). In general, organic systems had a greater microbial biomass than of conventional systems. The annual mean of the bacterial biomass was 1.87 and 1.25 $\mu\text{g/g}$ soil in organic and conventional systems, respectively. Thus the bacterial biomass in conventional system was only 66.8% of the organic system. Similarly, the annual means of fungal biomass were 22.35 and 16.41 $\mu\text{g/g}$ soil in organic and conventional system, respectively indicating that the fungal biomass in conventional systems was 73.34% of organic one. In all the cases the differences of microbial biomass between two farming systems were found significant.

Soil respiration

Like in case of microbial biomass soil respiration was minimum in summer and maximum in rainy season (Fig.2). The soil respiration observed in summer was only 43.1% and 67.5% of that observed in rainy season in organic and conventional system, respectively. This indicated that reduced soil water content in summer significantly reduced the respiratory CO_2 evolution in both the agroecosystems. Compared to the difference in the microbial biomass in two farming systems the difference in respiratory evolution of CO_2 was found to be significantly less. Annual mean values of respiratory evolution of CO_2

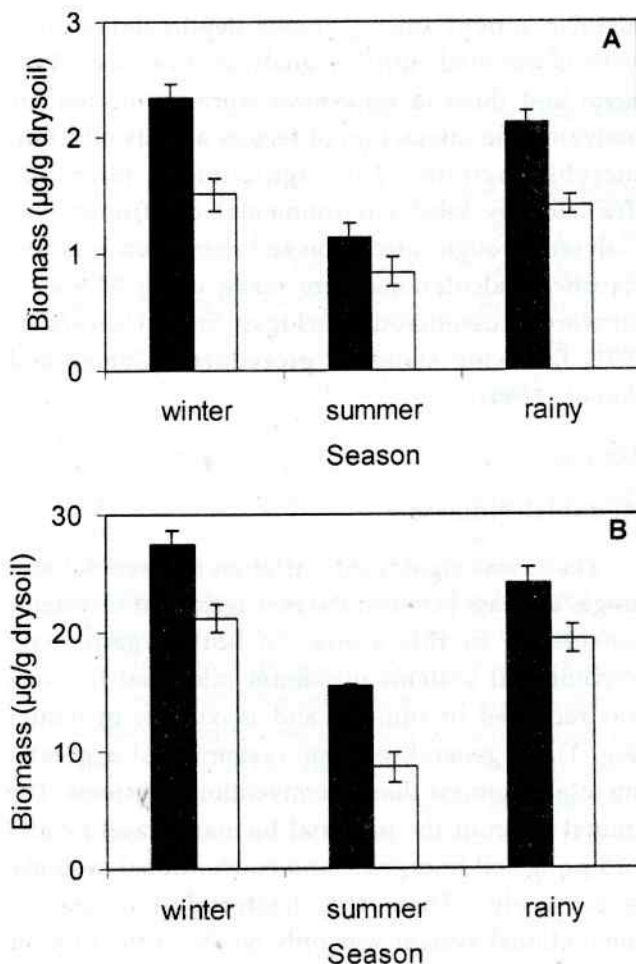


Fig. 1 : The biomass ($\mu\text{g/g}$ soil) of (A) bacteria and (B) fungi recorded in different seasons in (■) organic and (□) conventional farming systems.

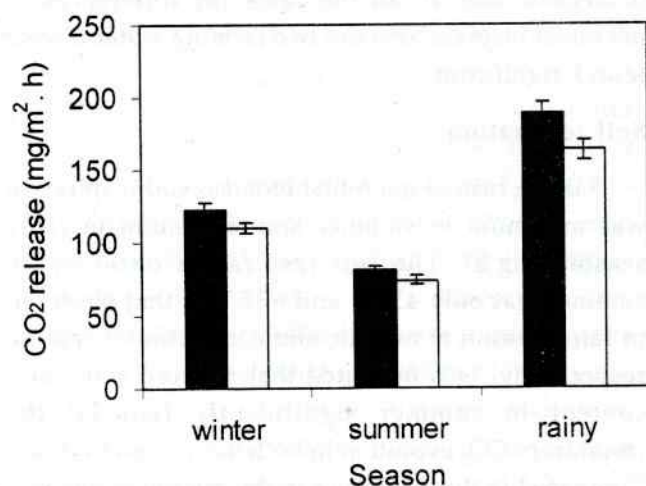


Fig. 2 : Seasonal variation of respiratory CO₂ evolution ($\text{mg/m}^2 \text{ h}$) from soil in (■) organic and (□) conventional farming systems.

were 130.64 and 115.78 $\text{mg/m}^2 \text{ h}$, in organic and conventional system, respectively. The respiration recorded in the conventional system was 88.62% of that of organic system. Seasonal analysis showed that the maximum difference of soil respiration between two farming systems was observed in rainy season while the minimum difference was observed in summer season.

Enzyme activity

As expected the activity of carbohydrate enzymes in soil was found minimum in summer and maximum in winter (Table-1). However, the activity of protease and dehydrogenase was minimum in summer and maximum in rainy season. In both the farming systems there was significant depth wise variation of enzyme activity, which differed with season. The activity decreased with increase in depth in winter and rainy season whereas the reverse was noted in summer. Comparison among the three carbohydrate enzymes showed that with few exceptions the invertase activity was more in the soil than the other carbohydrate enzymes. Minimum activity was reported in case of cellulase. Depth wise comparison was also made in order to determine the rate of change of carbohydrate enzyme activity with variation in soil depth. There is almost same rate of reduction in the rate of activity of the three enzymes with increase in soil depth in winter and rainy season but variation was observed in summer. The activity of cellulase increased more significantly with increase of depth in summer. The rate of increase in activity of all enzymes was more in the organic system than in the conventional one indicating that the organic carbon content of soil played an important role in facilitating the soil enzyme activity.

Discussion

Soil microbial biomass represents a small fraction of the living biomass of the soil but is responsible for most of the soil metabolic activities. There is a direct relation between the density and diversity of soil microbes and their metabolic performance in soil (Wardle, 1998; Biohne *et al.*, 2005). Soil organic carbon is the principal determinant of the soil microbial biomass, observed in the present study and also earlier (Chhotaray *et al.*, 2010). The high microbial

Table-1

Depth wise variation of amylase (μg glucose/g soil), cellulase (μg glucose/g soil), invertase (μg glucose/g soil), protease (μg tyrosine/g soil), and dehydrogenase (μg TPE/g soil) in different seasons and soil depths in organic (O) and conventional (C) farming systems

Enzyme	Season	Soil depth (cm)											
		0-4		4-8		8-12		12-16		16-20			
		O	C	O	C	O	C	O	C	O	C		
Amylase	Winter	102.75 ±2.31	71.26 ±1.56	97.98 ±3.03	66.51 ±1.46	90.75 ±3.06	65.37 ±3.03	86.01 ±2.08	60.72 ±2.07	78.18 ±2.06	58.29 ±1.11		
	Summer	52.95 ±2.32	44.51 ±1.31	59.42 ±2.04	52.27 ±2.03	63.87 ±2.05	57.16 ±2.04	69.59 ±1.47	60.85 ±1.75	72.83 ±1.07	65.62 ±1.02		
	Rainy	95.35 ±2.32	66.95 ±1.28	90.62 ±1.21	64.85 ±1.21	86.46 ±1.89	63.64 ±2.08	81.04 ±2.05	59.95 ±3.01	75.09 ±2.03	52.13 ±1.27		
Cellulase	Winter	92.19 ±1.47	70.95 ±0.68	85.33 ±0.35	68.31 ±1.35	80.36 ±1.45	63.87 ±1.47	75.39 ±2.12	59.81 ±1.04	73.51 ±1.34	56.03 ±1.57		
	Summer	50.31 ±1.31	31.93 ±1.35	52.95 ±1.45	35.25 ±1.23	57.47 ±1.24	41.57 ±1.81	61.91 ±1.25	48.05 ±2.04	69.59 ±1.09	52.27 ±1.27		
	Rainy	90.04 ±1.05	60.78 ±1.06	90.05 ±1.06	56.03 ±1.04	77.95 ±1.12	57.84 ±1.08	75.16 ±1.15	55.36 ±1.08	76.89 ±1.42	50.46 ±1.08		
Invertase	Winter	111.47 ±3.05	80.36 ±3.01	102.20 ±3.13	75.39 ±2.46	93.54 ±2.89	71.02 ±2.06	99.57 ±1.99	66.21 ±1.34	80.44 ±2.14	60.85 ±3.01		
	Summer	53.17 ±2.01	51.74 ±1.13	59.15 ±1.03	54.68 ±1.07	64.39 ±1.06	55.21 ±2.11	70.87 ±3.03	65.07 ±1.28	75.24 ±1.74	68.31 ±1.71		
	Rainy	92.86 ±2.11	72.31 ±1.25	88.57 ±2.15	67.11 ±1.19	84.73 ±2.05	64.85 ±1.02	81.87 ±1.06	60.55 ±1.13	76.97 ±1.19	57.31 ±1.08		
Proteases	Winter	105.11 ±1.92	80.67 ±1.02	104.11 ±1.71	74.13 ±1.04	105.44 ±2.06	71.78 ±2.78	103.44 ±1.14	68.44 ±1.21	85.89 ±0.46	60.67 ±0.91		
	Summer	71.78 ±0.67	61.22 ±0.59	77.33 ±1.03	63.22 ±0.68	82.89 ±0.84	64.11 ±0.61	84.05 ±0.82	66.25 ±0.62	87.36 ±1.13	70.68 ±1.55		
	Rainy	144.07 ±0.77	94.12 ±1.82	139.56 ±1.92	82.89 ±1.03	138.46 ±1.09	75.14 ±2.11	135.16 ±1.78	72.89 ±1.95	121.78 ±1.85	66.22 ±1.81		
Dehydrogenase	Winter	16.26 ±0.14	12.15 ±0.34	16.01 ±0.22	10.12 ±0.68	16.32 ±0.31	9.92 ±0.53	15.87 ±0.28	7.81 ±0.41	11.82 ±0.12	6.71 ±0.62		
	Summer	9.15 ±0.21	7.34 ±0.31	9.45 ±0.24	7.36 ±0.45	10.16 ±0.44	8.11 ±0.31	11.08 ±0.18	8.21 ±0.44	11.67 ±0.44	9.15 ±0.47		
	Rainy	27.17 ±0.34	13.12 ±0.45	25.13 ±0.61	10.91 ±0.38	23.15 ±0.47	9.51 ±0.53	21.34 ±0.51	8.23 ±0.39	21.87 ±0.32	7.47 ±0.46		

biomass in case of organic farming may be attributed to the favourable organic carbon load.

Soil respiration is a direct parameter to indicate the metabolic performance of microbes in soil. Metabolically active soil consumes more oxygen than of metabolically poor soil, which again is dependent on many biological and physicochemical parameters. In the present study higher respiration was found in organically managed soil than the conventional but the difference in the rate of respiration in both the farming systems was not proportional to the difference in the microbial biomass. Though there was a big difference in microbial biomass of the two systems the difference in soil respiration was less, with conventional systems having 88.6% of respiration observed in organic system. This indicated that the soil respiration was not dependent only on the soil microbial biomass but also other biological components of which root biomass is a significant one. It may be noted that the fields had a greater standing crop with higher root biomass in rainy season though the microbial biomass was maximum in winter. The respiratory CO₂ evolution was mostly contributed by rhizosphere in both the agroecosystems. The conventional systems were added with nutrients to facilitate plant growth due to which root growth of organic and conventional systems were insignificantly different even though the microbial biomass in the bulk soil significantly varied.

The rate of respiration is also dependent on soil, water, moisture, temperature and organic matter content. Correlation between the soil respiration and all these soil factors indicated that in all cases under both the farming systems the correlation was significant (Table-2). Maximum slope value for respiratory CO₂ evolution was found with variation of rainfall while minimum with change of temperature. This indicates that soil respiration is more dependent on soil water and moisture content than on soil temperature. This result is in agreement with the observation of previous workers (Alef, 1995; Araujo *et al.*, 2009). The temperature played a regulatory role as long as the soil moisture was not limiting but with limitation of soil moisture temperature had secondary importance in regulating the soil respiration. Thus in the present study even though there was not much difference in temperature of different soil layers in summer, greater respiration and enzymatic activity were reported in inner layers, most probably due to high moisture content.

Single factor Avova showed that microbial metabolic performance vis-à-vis enzymatic activity varied significantly with season, depth and farming systems (Table-3). Similarly in case of the two factor analysis season and depth as well as season and farming systems significantly influenced the metabolic performance but when depth with farming systems were taken together, the variation was insignificant in some cases. Same was also noted with

Table-2
The slope (mg CO₂/m² h) and coefficient of correlation between soil respiration and the environmental conditions

Environmental parameter	Output	Season					
		Winter		Summer		Rainy	
		O	C	O	C	O	C
Temperature	Slope	0.579	0.321	0.583	0.966	1.613	2.217
	Coefficient	0.97	0.99	0.97	0.91	0.94	0.96
Moisture	Slope	0.795	0.561	0.952	1.407	2.196	5.393
	Coefficient	0.99	0.99	0.81	0.77	0.86	0.90
Rainfall	Slope	1.630	1.628	1.669	1.601	0.754	0.872
	Coefficient	0.99	0.97	0.99	0.94	0.88	0.99

All correlation coefficients were found significant at $p = 0.05$; $n = 90$

Abbreviations: O - organic; C - conventional

Table-3

Analysis of variance among seasons, soil depth and farming systems for determination of dependence of soil enzyme activity

Parameter	Season (S)	Depth (D)	Farming systems (O/C)	S x D	S x O/C	D x O/C	S x D x O/C
Amylase	4630.32	97.58	14654.73	755.26	1391.69	76.21	17.8
Cellulase	5203.63	57.47	10792.21	507.52	180.36	46.85	20.35
Invertase	192.37	6.54	357.84	25.17	45.01	13.05	24.79
Protease	256.59	8.23	762.26	10.05	98.63	2.41 ^{ns}	0.16 ^{ns}
Dehydrogenase	991.65	42.14	2604.07	44.83	595.91	2.67 ^{ns}	3.70

ns indicates "not significant". All other F values are significant at $p = 0.05$.

three factor analysis. This showed that depth wise variation of metabolic performance was found to remain same and was not influenced by the farming systems. However, when total metabolic activity was considered, microbial performance was better in organic farming systems than the conventional (Wang and Chao, 1995; Singh *et al.*, 2004; Gajda and Martyniuk, 2005; Zhao *et al.*, 2009).

Irrespective of the farming systems the soil enzyme activities were more on the surface layer than in the inner layers. Since in rainy and winter seasons soil has higher level of moisture and greater activity of microbes, lower enzyme activity in inner soil layers than in surface can be attributed to limitation by O_2 . The reverse trend in summer is because of more favourable temperature and moisture level in the inner layer of soil than on the surface.

Acknowledgement

The authors thank the UGC, New Delhi for the financial assistance under SAP (DRS - II) Programme in the P.G. Department of Botany, Utkal University to carry out this research.

References

- Alef, K. (1995). Estimation of soil respiration. In *Methods in Soil Microbiology and Biochemistry*. Academic Press, New York, NY, USA, pp: 464-470.
- Araujo, A.S.F., Leite, L.F.C., Santos, V.B. and Carneiro, R.F.V. (2009). Soil microbial activity in conventional and organic agricultural systems. *Sustainability* 1: 268-276.
- Bohme, L., Langer, U. and Bohme, F. (2005). Microbial biomass, enzyme activities and microbial community structure in two European long term field experiment. *Agric. Ecosyst. Env.* 109: 141-152.
- Casida, L.E. Jr., Klein, D.A. and Santoro, T. (1964). Soil dehydrogenase activity. *Soil Sci.* 98: 371-376.
- Chhotaray, D., Mohapatra, P.K. and Mishra, C.S.K. (2010). Impact of farm management on soil microbial density and metabolic activities in rice agroecosystems. In: Patra, H.K. (ed) *Environmental Stress Impacts on Plants*. Utkal University, Bhubaneswar, pp: 50-60.
- Gajda, A. and Martyniuk, S. (2005). Microbial biomass C and N and activity of enzymes in soil under winter wheat grown in different crop management systems. *Pol. J. Env. Stud.* 14: 159-163.
- Gomez, A.A. and Gomez, K.A. (1984). *Statistical Procedures for Agriculture Research*. John Wiley and Sons, New York, U.S.A. pp. 680.
- Jones, P.T.C. and Mollison, J.E., (1948). A technique for the quantitative estimation of microorganisms. *J. Gen. Microbiol.* 2: 54-69.
- Krishnakumar, S., Saravanan, A., Natarajan, S.K., Veerabadran, V. and Mani, S. (2005). Microbial population and enzymatic activity as influenced by organic farming. *Res. J. Agric. Biol. Sci.* 1(1): 85-88.
- Liu, X., Li, Q., Liang, W. and Yong, J. (2008). Distribution of soil enzyme activities and microbial biomass along a latitudinal gradient in farmlands of Songliao Plain, Northeast China. *Pedosphere* 18(4): 431-440.
- Parkinson, D., Gray, T.R.G. and Williams, S.T. (1971). *Methods to Study Ecology of Soil Microorganism*. IBP Handbook No. 19. Blackwell Scientific Publ. Oxford, U.K.. pp: 116.

- Melero, S., Porras, J.C.R., Herencia, J.F. and Madejon, E. (2005). Chemical and biochemical properties in a silty loam soil under conventional and organic management. *Soil Till. Res.* 90: 162-170.
- Mishra, P.C., Mohanty, R.K. and Dash, M.C. (1979). Enzyme activity in subtropical surface soils under forest pasture. *Ind. J. Agric. Chem.* 12: 19-24.
- Singh, Y., Singh, B., Ladha, J.K., Gupta, R.K., Meelu, O.P. and Pasuquin, E. (2004). Long term effects of organic inputs on yield and soil fertility in the rice wheat rotation. *Soil Sci. Soc. Amer. J.* 68: 845-853.
- Speir, T.W. and Ross, D.J. (1975). Effect of storage on the activities of protease, urease, phosphatases and sulphatase in three soils under pasture. *New Zealand J. Sci.* 18: 231-237.
- Sunitha, V. and Singara-Charya, M.A. (2003). Accumulated soil enzymes in a soil profile under different agro-ecosystems. *Ind. J. Env.. Ecoplan.* 7(1): 71-74.
- Wang, Y. and Chao, C. (1995). The effect of organic farming practices on the chemical, physical and biological properties of soil in Taiwan. *Sustainable food production in the Asian and pacific region*, FFTC book Ser. 46: 33-39.
- Wardle, D.A. (1998). Controls of temporal variability of soil microbial biomass: A global scale synthesis. *Soil Biol. Biochem.* 30: 1627-1637.
- Zhang, H.M., Wang, B.R. and Xu, M.G. (2008). Effects of inorganic fertilizer inputs on grain yields and soil properties in a long term wheat corn cropping system in south China. *Commun. Soil Sci. Plant Anal.* 39: 1583-1599.
- Zhao, Y., Wang, P., Li, J., Chen, Y., Ying, X. and Liu, S. (2009). The effects of two organic manures on soil properties and crop yields on a temperate calcareous soil under a wheat maize cropping system. *Eur. J. Agron.* 31: 36-42.

Metal ions affect the growth and amylase activity of thermotolerant *Bacillus* sp. isolated from 'Atri' hot spring of Odisha (India)

E. Marric, A.K. Dalai and B. Behera^Ψ

Department of Botany (Biotechnology Wing)

Ravenshaw University, Cuttack-753003

(Received : 30 November, 2010; Revision received : 08 December, 2010)

The effect of various metal ions on colony growth of five amylase positive thermotolerant *Bacillus* strains was studied to find the possibility of hydrolyzing raw plant starch. The metal ions used could be grouped into two categories. One group comprised of the metal ions that have less than 30% inhibition (Ca^{++} and Mg^{++}) and the second group consisted of the metal ions that impart more than 30% inhibition (Zn^{++} , Hg^{++} and Cu^{++}). The effect of Mn^{++} was found to be intermediate between the effects of metal ions of these two groups. Considering the lower susceptibility, two strains were selected for further studies to find the effect of metal ions on amylase activity and growth in broth culture. The grouping of the metal ions as per their inhibitory effect on colony growth on nutrient agar was found to hold good for growth in broth culture, except Mg^{++} that enhanced the growth of the selected strains. Calcium enhanced the amylase activity in both the strains showing nearly upto 1.5 fold increase, whereas Mg^{++} enhanced the activity of only one strain upto the relative activity of 109.99. Hg^{++} and Zn^{++} were found to have more inhibitory effect for both the strains with respect to amylase activity.

Key words: 'Atri' hot spring, *Bacillus*, metal ions, amylase activity

Introduction

Plants produce starch which is the important source of carbon and energy for living organisms. Major portion of starch remains as raw starch in the plant body and rest as food starch mainly available in the grains. Moreover, starch is composed of different proportion of amylose and amylopectin, which requires different types of starch hydrolyzing enzymes for their hydrolysis. The important source of amylase which is the major contributor to the starch hydrolyzing enzymes is microbes. For efficient hydrolysis of raw starch and food starch there is a need to search for such amylases amongst the microbes suitable for starch processing industries. The demand of amylases is approximately 25% of the enzyme market (Sidhu *et al.*, 1997; Rao *et al.*, 1998). Thermostable amylases isolated from thermophilic organisms have a number of commercial applications because of their overall inherent stability

(Demirijan *et al.*, 2001). The stability of the amylase is affected by metals. In the enzyme action, metallic cofactors like Ba^{++} , Ca^{++} , Co^{++} , K^{+} , Ni^{++} , Ag^{+++} , Mn^{++} , Hg^{++} , Li^{++} , Cu^{++} , Mg^{++} and Pb^{++} are important because their presence or absence regulates enzyme activity. The presence of specific metallic ions along with nutrient content can inhibit or enhance amylase activity (Dutta *et al.*, 2006). To meet the demand of highly thermostable amylases, a series of attempts has been made to propose thermostabilization mechanisms (Sternner and Liebl, 2001; Vieille and Zeikus, 2001) and to enhance the enzyme thermostability through rational mutagenesis based on the proposed mechanisms (Kim *et al.*, 2005). Such an attempt will also help to hydrolyze raw starch available in the plant body. Among the thermostabilizing factors, the calcium ion is a very important factor in stabilizing enzymes (Kim *et al.*, 2005). It is required to maintain the structural integrity of α -amylase (Vallee *et al.*, 1959) and its

^Ψ Corresponding author; E-mail: bharatibehera@rediffmail.com

removal leads to decreased thermostability and/or enzymatic activity (Violet and Meunier, 1989) or increased susceptibility to proteolytic degradation (Machius *et al.*, 1995). The affinity between divalent metal ions and the α -amylase varies considerably with the source of the enzyme (Saboury *et al.*, 2005). The bacterial strains of hot springs are supposed to have adaptable interactions with metal ions, as the water of hot springs are with higher level of metal ions.

The genus *Bacillus* produces a large variety of extracellular enzymes of which amylases are of significant industrial importance (Reddy *et al.*, 2003). An extremely thermostable α -amylase is available from the mesophile *B. licheniformis* (Morgan *et al.*, 1981). The present investigation aims at finding the effect of metal ions on growth and amylase production of certain *Bacillus* strains isolated from 'Atri' hot spring of Odisha.

Materials and methods

Isolation and culture maintenance of bacterial strains

The amylase positive *Bacillus* strains isolated from the sulfur hot spring at Atri located 42 km away from Bhubaneswar, Capital city of Odisha, India in the month of December, 2007 were used in this investigation. The temperature of the hot spring at the time of sampling was 55 °C. The strains were maintained on nutrient agar slants (5 g/l peptone, 3 g/l beef extract, 15 g/l agar, pH 7.0)/nutrient broth (5 g/l peptone, 3 g/l beef extract, pH 7.0).

To test the effect of metal ions on growth of the strains, the nutrient agar supplemented with different concentrations (1, 3 and 5 mM) of metal ions including Ca^{++} , Mg^{++} , Mn^{++} , Cu^{++} , Hg^{++} and Zn^{++} were prepared. The control plate was without any metal ions. These agar plates were inoculated with 2 μl of 24 h-grown working culture and allowed to grow at 37 ± 1 °C to develop colonies. The plates were observed up to 96 h at an interval of 24 h.

Additionally, nutrient broth cultures supplemented with 1 mM each of metal ions including Ca^{++} , Mg^{++} , Mn^{++} , Cu^{++} , Hg^{++} and Zn^{++} were prepared to assess the effect of metal ions on

growth of the strains and the production of amylase by the strains. The control sample was without any metal ions. The fresh media with or without metal ions, as required, was inoculated with 24 h grown culture in nutrient broth, with inoculum volume of 1%. The culture was grown up to 24 h at 37 ± 1 °C.

Measurement of growth

The growth of the colony was measured by its diameter and the growth of the strains in broth culture was measured by taking the optical density of the culture at 550 nm against the same media.

Amylase assay

The culture was centrifuged at 4000 rpm for 10 min. The cell free supernatant was used as the crude enzyme extract. The enzyme assay was performed following the modification method of Bernfeld (1955). The reaction was carried out in 0.5 ml volume containing 0.25 ml 1% starch in 0.2 M sodium phosphate buffer (pH 8) and 0.25 ml of crude enzyme extract. The reaction was stopped by adding 0.5 ml DNS reagent and heating in boiling water bath for 10 min. An aliquot of 0.5 ml of reaction mixture was diluted to 10 ml and optical density measured at 540 nm. The level of amylase activity was determined by measuring the reducing sugar in terms of maltose released from soluble starch (Nelson, 1944). One unit of amylase activity was defined as the amount of enzyme which liberates 1 μmole of reducing sugar as maltose per minute under the conditions of the assay.

Results and discussion

Effect of metal ions on colony growth

To find out the effect of metal ions on colony growth of five amylase producing *Bacillus* strains, six metal ions were used each at 1, 3 and 5 mM concentrations. All the metal salts used in this experiment were found to have inhibitory effect on colony growth on nutrient agar media (Tables 1-3).

Considering the percentage of inhibition, the metal ions used could be grouped into two categories. One group comprised of the metal ions that have less than 30% inhibition and the second group consisted of the metal ions that impart inhibition

Table-1
Percentage inhibition of colony growth of different *Bacillus* strains at 96 h of culture
by metal ions (1 mM each)

Strain Metal ion	ARBE LICrg	ARBE LICra	ARBE UIC ₂ c ₂	ARBE LIC ₂ c ₂	ARBE UuSs
Ca ⁺⁺	10.25 ± 1.23	1.14 ± 0.065	20.97 ± 4.86	9.9 ± 0.82	14.57 ± 1.99
Mg ⁺⁺	5.13 ± 0.612	11.36 ± 0.097	22.58 ± 5.23	0.99 ± 0.073	4.5 ± 0.88
Mn ⁺⁺	23.07 ± 3.86	3.4 ± 0.023	20.97 ± 2.89	0.99 ± 0.061	34.67 ± 2.76
Hg ⁺⁺	15.38 ± 0.981	100	100	100	100
Zn ⁺⁺	100	64.77 ± 11.18	70.96 ± 9.25	68.32 ± 10.23	91.2 ± 9.62
Cu ⁺⁺	41.02 ± 3.21	100	100	100	100

* Data represent Mean ± S.D. of three repeats of experiment with six replicates each.

Table-2
Percentage inhibition of colony growth of different *Bacillus* strains at 96 h of culture
by metal ions (3 mM each)

Strain Metal ion	ARBE LICrg	ARBE LICra	ARBE UIC ₂ c ₂	ARBE LIC ₂ c ₂	ARBE UuSs
Ca ⁺⁺	11.53 ± 1.01	4.54 ± 0.53	25.00 ± 3.11	18.81 ± 1.72	29.64 ± 1.98
Mg ⁺⁺	11.53 ± 1.10	11.36 ± 1.96	29.03 ± 2.11	4.95 ± 0.18	12.06 ± 2.63
Mn ⁺⁺	53.84 ± 7.24	39.77 ± 6.29	57.26 ± 3.82	48.51 ± 2.95	52.26 ± 9.82
Hg ⁺⁺	100	100	100	100	100
Zn ⁺⁺	100	100	100	100	100
Cu ⁺⁺	100	100	100	100	100

* Data represent Mean ± S.D. of three repeats of experiment with six replicates each.

Table-3
Percentage inhibition of colony growth of different *Bacillus* strains at 96 h of culture
by metal ions (5 mM each)

Strain Metal ion	ARBE LICrg	ARBE LICra	ARBE UIC ₂ c ₂	ARBE LIC ₂ c ₂	ARBE UuSs
Ca ⁺⁺	28.2 ± 2.53	5.68 ± 0.25	29.03 ± 3.22	30.69 ± 1.68	30.15 ± 2.92
Mg ⁺⁺	15.38 ± 0.96	13.63 ± 1.51	29.03 ± 2.51	8.9 ± 0.71	10.52 ± 2.85
Mn ⁺⁺	55.12 ± 8.30	48.86 ± 5.20	62.09 ± 9.22	60.39 ± 11.68	76.38 ± 10.23
Hg ⁺⁺	100	100	100	100	100
Zn ⁺⁺	100	100	100	100	100
Cu ⁺⁺	100	100	100	100	100

* Data represent Mean ± S.D. of three repeats of experiment with six replicates each.

more than 30%. Thus Ca^{++} and Mg^{++} fall into the first category. The percentage of inhibition ranged between 0.99 to 22.58 at 1 mM concentration and from 4.5 to 30.69 at 5 mM concentration. The second group consisted of the metal ions Hg^{++} , Zn^{++} and Cu^{++} . These three metal ions showed 100% inhibition at 3 mM and 5 mM concentration. The percentage of inhibition varied between 41.02 to 100% at 1 mM concentration with an exception for Hg^{++} for strain ARBE LICrg. The Mn^{++} ion could not be placed in either groups, as at 1 mM concentration the percentage of inhibition varied between 0.99 to 34.67 and at 5 mM concentration it ranged from 48.86 to 76.38. So, considering the effect of Mn^{++} ion at 1, 3 and 5 mM concentration, it can be said that its effect was intermediate between the effect of first group metal ions comprising Ca^{++} and Mg^{++} and the second group metal ions comprising Hg^{++} , Zn^{++} and Cu^{++} .

The response of the strains to the first group metals (Ca^{++} and Mg^{++}) at 1 mM concentration was different. The susceptibility of the strains to the metals can be evaluated taking average inhibition of growth by the metals Ca^{++} and Mg^{++} . On the basis of this criterion the strain ARBE LIC₂c₂ was found to be the least susceptible strain followed by ARBE LICra.

The inhibition of growth by metal salts was also reported by Nies and Silver (1995). Nies (1999) observed the influence of metal ions on nutrient uptake mechanism, thereby influencing the colony growth. Sterner and Liebl (2001) have shown that

Ca^{++} , Mg^{++} and Mn^{++} ions used to participate in the process of metabolism and may be essential metal ions for the growth. Olama and Sabry (1989) reported the inhibitory effect of Cu^{++} and Zn^{++} on growth of *Aspergillus lavus* and *Penicillium purpurescence* at 1 mM and 5 mM concentration.

For further experiments, 1 mM concentration of metal ions was selected to find out their effect on growth of the strains in broth culture and amylase production by the strains.

Effect on growth and amylase activity in broth culture

Of a total of five strains employed only two strains ARBE LICra and ARBE LIC₂c₂ were used as per the lower susceptibility to metals to observe the effect of metal ions on growth in broth culture and amylase production. The strains with respect to growth and relative amylase activity under the influence of metal ions showed varied responses (Table-4).

The grouping of the metal ions as per their inhibitory effect on colony growth on nutrient agar was found to hold good for broth culture, except for Mg^{++} ion. It was found that Mg^{++} enhanced the growth of strains ARBE LICra and ARBE LIC₂c₂ by 28.57% and 20.7% respectively. The inhibitory effect of Zn^{++} was found to be more pronounced in broth culture. The inhibitory effect of all other ions was found to be more or less the same as that of in nutrient agar medium.

Table-4
Effect of metal ions on growth and amylase activity of strain ARBE LICra and ARBE LIC₂c₂

Media	Relative growth		Relative activity	
	ARBE LIC ₂ c ₂	ARBE LICra	ARBE LIC ₂ c ₂	ARBE LICra
Nutrient broth (NB)	100	100	100	100
NB + Ca^{++}	87.94	90.5	109.29	144.29
NB + Mg^{++}	128.57	120.7	95.71	109.99
NB + Mn^{++}	65.12	39.2	92.85	82.86
NB + Hg^{++}	2.41	1.76	-	-
NB + Zn^{++}	5.38	4.6	39.28	35.71
NB + Cu^{++}	26.9	5.28	78.57	44.29

* Data represent mean of three repeats of experiment with six replicates each.

All the metal ions used in this experiment either inhibited or enhanced the amylase activity. The relative amylase activity was influenced by Ca^{++} and Mg^{++} for both the strains ranged from 95.71 to 144.29. Calcium enhanced the amylase activity in both the strains showing nearly up to 1.5 times rise for ARBE LICra strain, whereas Mg^{++} enhanced the activity of only ARBE LICra strain up to relative activity 109.99. However, Hg^{++} and Zn^{++} were found to be more inhibitory metal for both the strains with respect to amylase activity.

Effects of metal ions on amylase production were studied by different authors in different microbial strains (Obi and Odibo, 1984; Chung *et al.*, 1995; Shih and Labbe, 1995; Sarikaya and Gurgun, 2000; Asgher *et al.*, 2007; Burhan, 2008). There was no specific effect with respect to increase or decrease in amylase activity by a particular metal ion in all the strains examined. However, most of the studies were in favour of enhancement effect of amylase production by calcium. The present study with *Bacillus* strains was also an corroboration of this common response. The inhibitory effect of Zn^{++} , Hg^{++} and Cu^{++} on amylase production was reported by Chung *et al.* (1995) and Asgher *et al.* (2007). Mg^{++} was shown to enhance amylase activity in some cases as in *B. subtilis* (Sarikaya and Gurgun, 2000), *B. amyloliquefaciens* II (Sarikaya and Gurgun, 2000), and *Thermococcus profundus* (Chung *et al.*, 1995) and contrarily inhibit amylase activity in others, as in *B. subtilis* (Asgher *et al.*, 2007), *B. amyloliquefaciens* I (Sarikaya and Gurgun, 2000). Similarly, Chung *et al.* (1995) and Asgher *et al.* (2007) reported Mn^{++} to be moderately inhibitory.

When the impact of metal ion on growth and amylase activity was compared, it was marked that the increase in growth might not lead to increase in amylase production. By the addition of Ca^{++} , the growth was found to drop for both the strains whereas the amylase activity was considerably enhanced. The reverse was true for Mg^{++} for the strain ARBE LIC₂C₂. Thus it can be concluded that the growth and amylase production may be under different control systems. It gives a clue to manipulate the strains with Ca^{++} and Mg^{++} so that raw starch can be hydrolyzed to produce various products of simple sugars and fermentation of sugars to yield alcohol.

Acknowledgements

The authors are thankful to the Head of the Department of Botany and Vice Chancellor of Ravenshaw University for providing necessary laboratory facilities to undertake this investigation. The authors also thankfully acknowledge the Department of Science and Technology (Biotechnology), Govt. of Orissa for providing financial assistance.

References

- Asgher, M., Asad, M.J., Rahman, S.U. and Legge, R.L. (2007). A thermostable α -amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. *J. Food Eng.* 79: 950-955.
- Bernfeld, P. (1955). Amylases α - and β -methods. *Enzymol.* 1: 149-158.
- Burhan, A. (2008). Highly thermostable, thermophilic, alkaline, SDS and chelator resistant amylase from a thermophilic *Bacillus* sp. isolate A3-15. *Bioresour. Technol.* 99: 3071-3076.
- Chung, Y.C., Kobayashi, T., Kanai, H., Akiba, T. and Kudo, T. (1995). Purification and properties of extracellular amylase from hyperthermophilic archaeon *Thermococcus profundus* DT5432. *Appl. Env. Microbiol.* 61 (4): 1502-1506.
- Demirijan, D., Moris-varas, F. and Cassidy, C. (2001). Enzymes from extremophiles. *Curr. Opin. Chem. Biol.* 5: 144-151.
- Dutta, T.K., Malabendu, J., Pahari, P.R. and Bhattacharya, T. (2006). The effect of temperature, pH and salt on amylase in *Heliodiaptomus viduus* (Gurney) (Crustacea: copepoda: Calanoida). *Turk. J. Zool.* 30: 187-195.
- Kim, Y.W., Kim, D.K., Kim, M.J., Cha, H., Park, C.S., Moon, T.W. and Park, K.H. (2005). Engineering *Thermus* maltogenic amylase with improved thermostability: probing the role of the conserved calcium binding site in cyclodextrin-degrading enzymes. *J. Appl. Glycosci.* 52: 7-13.
- Machius, M., Wiegand, G. and Huber, R. (1995). Crystal structure of calcium-depleted *Bacillus licheniformis* α -amylase at 2.2 Å resolution. *J. Mol. Biol.* 246: 545-559.
- Morgan, F.J. and Priest, F.G. (1981). Characterization of a thermostable α -amylase from *Bacillus licheniformis* NCIB6346. *J. Appl. Bacteriol.* 50: 107-114.
- Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* 153: 375-380.

- Nies, D.H. (1999). Microbial heavy metal resistance. *Appl. Microbiol. Biotechnol.* **51**: 730-750.
- Nies, D.H. and Silver, S. (1995). Ion efflux systems involved in bacterial metal resistances. *J. Ind. Microbiol.* **14**: 186-199.
- Obi, S.K.C. and Odibo, F.J.C. (1984). Partial purification and characterization of a thermostable Actinomycete β -amylase. *Appl. Env. Microbiol.* **47** (3): 571-575.
- Olama, Z.A. and Sabry, S.A. (1989). Extracellular amylase synthesis by *Aspergillus flavus* and *Penicillium purpurescence*. *J. Islamic Acad. Sci.* **2** (4): 272-276.
- Rao, M.B., Tanksale, A.M., Gathe, M.S. and Deshpande, V.V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.* **62**: 597-635.
- Reddy, N.S., Nimmagadda, A. and Sambasivarao, K.R.S. (2003). An overview of the microbial amylase family. *Afr. J. Biotechnol.* **2**(12): 645-648.
- Saboury, A.A., Ghasemi, S. and Dahot, M.U. (2005). Thermodynamic study of magnesium ion binding to α -amylase. *Ind. J. Biochem. Biophys.* **42**: 326-329.
- Sarikaya, E. and Gurgun, V. (2000). Increase of α -amylase yield by some *Bacillus* strains. *Turk. J. Biol.* **24**: 299-308.
- Shih, N. and Labbe, R.G. (1995). Purification and characterization of an extracellular α -amylase from *Clostridium perfringens* Type A. *Appl. Env. Microbiol.* **61**(5): 1776-1779.
- Sidhu, G.S., Sharma, P., Chakrabarti, T. and Gupta, J.K. (1997). Strain improvement for the production of a thermostable α -amylase. *Enz. Microbial. Technol.* **21**: 525-530.
- Sterner, R. and Liebl, W. (2001). Thermophilic adaptation of proteins. *Crit. Rev. Biochem. Mol. Biol.* **36**: 39-106.
- Vallee, B.L., Stein, E.A., Summerwell, W.N. and Fisher, E.H. (1959). Metal content of α -amylases of various origins. *J. Biol. Chem.* **234**: 2901-2929.
- Vieille, C. and Zeikus, G.J. (2001). Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. *Microbiol. Mol. Biol. Rev.* **65**: 1-43.
- Violet, M. and Meunier, J.C. (1989). Kinetic studies of the irreversible thermal inactivation of *Bacillus licheniformis* α -amylase. *J. Biochem.* **263**: 665-670.

Synthesis of biotechnologically relevant amylase from wheat bran using native isolates of *Aspergillus tamarii*

B.K. Sethi¹, P.K. Bhuyan¹, S. Rout¹, P.K. Nanda² and S.L. Sahoo^{1*}

¹Microbiology Research Laboratory, P.G. Department of Botany, Utkal University, Bhubaneswar-751004

²Department of Botany, Shaila Bala Women's College, Cuttack-753001

(Received : 08 November, 2010; Revision received : 14 December, 2010)

Aspergillus tamarii was isolated from soil samples of Ganga Nagar, Bhubaneswar and identified according to its colony morphology, microscopic and biochemical characters. Growth response of *A. tamarii* was studied as its performance was maximum in starch hydrolyzing activity (2.4 cm) followed by other isolates. It was found that growth was maximum at pH 7.0, 30±2°C with an incubation period of 12 days. Supplementation of glucose and peptone to the medium also enhanced the growth. Amylase production was carried out in both submerged fermentation (SmF) and solid-state fermentation (SSF) using wheat bran (10% w/v) as the substrate and maximum activity was observed after 4 days of incubation in SSF (9493.33 U/ml) than that of SmF (8430 U/ml).

Key words: Amylase, *Aspergillus tamarii*, wheat bran, fermentation

Introduction

Many species of microorganisms have already been identified as good amylase producers. Studies of amylases from bacteria and fungi are well documented (Sindhu *et al.*, 2009). Filamentous fungi are microorganisms that secrete large amount of protein in culture medium. It was reported by Saha and Zeikus (1990) that *Aspergillus niger* synthesizes an α -amylase and glucoamylase of different molecular weight in submerged culture respectively.

The α -amylases (endo-1,4- α -D-glucan glucohydrolase, E.C. 3.2.1.1) are enzymes that randomly catalyze the endoamylolytic cleavage of α -1,4-glucosidic chains in polysaccharides with three or more α -1,4-glucosidic starch chains or extracts of similar substrates (amylose and amylopectin). They act by liberating maltose, oligosaccharides and limit dextrins (Mitidieri *et al.*, 2006). During recent years, increasingly growing interests have been focused on the biochemical produced from renewable alternative feedstock and organic waste streams. In these processes, commercial enzymes were often used to promote the bioconversion of substrate to bioproduct. The α -amylases are calcium metalloenzymes, completely unable to function in the absence of

calcium. The α -amylase tends to be faster-acting than β -amylase. For the properties of these enzymes, they have various applications in major areas of food processing, beverage production, animal nutrition, leather, paper, pulp, textiles, detergents, etc. and with the advent of new frontiers in biotechnology, the spectrum of amylase and protease applications has expanded into many new fields such as clinical, medicinal and analytical chemistry (Pandey *et al.*, 1999). This is a class of industrial enzymes that accounts for approximately 25% of the world market (Ikram-ul-Haq *et al.*, 2003).

But for amylase production by a cost effective process, the contents of synthetic media can be replaced with more economically available agricultural by-products. The use of agricultural wastes makes solid-state fermentation (SSF) an attractive alternative method. Baysal *et al.* (2003) have reported α -amylase production in solid-state fermentation with wheat bran and rice husk as substrates. Ramachandran *et al.* (2004) have analyzed the potential of coconut oil cake as substrate for the production of α -amylase using *Aspergillus oryzae*, a GRAS strain. Ikram-ul-Haq *et al.* (2003) have described the selection of a suitable low cost

* Corresponding author; E-mail: santi_bot_uu@yahoo.co.in

fermentation medium for the production of α -amylase by using agricultural by-products.

In view of the wide range of applicability of amylases, the objectives of this study were selection of a suitable strain for the production of amylase, screening of species for maximum enzyme production and optimization of cultural conditions for enzyme production.

Materials and methods

Isolation of microorganisms

Several species of *Aspergillus* were isolated from different soil samples obtained from various locations of Bhubaneswar by Standard Blotter Method (Baki and Anderson, 1973). They were identified on the basis of their morphological, biochemical, micrometric and microscopic characteristics.

Screening of isolates

For each of the *Aspergillus* spp., two growth media viz., Potato Dextrose Agar (PDA) and Starch Agar Medium (SAM), with 6 replicates was laid down in a completely randomized design. The species were cultured on PDA medium and SAM containing soluble starch 10 g/l. The pH of the medium was adjusted to 6.5 before autoclaving. The sterilized medium was cooled and 300 mg of Norfloxacin per 100 ml of medium was used as an antibacterial agent. The plates were incubated at $30\pm 2^\circ\text{C}$ for five to seven days. The extent of growth of representative strains of *Aspergillus* spp. was evaluated qualitatively by visual inspection. Then the hydrolyzing zones were determined using I_2 -KI solution.

Determination of fungal growth under various carbon and nitrogen sources

Czapek Dox broth medium devoid of sucrose and nitrogen source was dispensed into 250 ml sterilized flasks (Borosil made) containing 50 ml of medium. Various carbon and nitrogen sources such as glucose, lactose, sucrose, starch, glycine, peptone, NaNO_3 and urea (1% w/v) were added to the medium and autoclaved. But glucose and glycine were autoclaved separately and then added to the medium. All the flasks containing medium with 1 ml of inoculum were allowed to grow at $30\pm 2^\circ\text{C}$ in static

condition. Growth of the fungal strain was monitored at 24 h interval up to 6 days. At the end of 6th day, the cultures were centrifuged at 8000 rpm for 15 min. The pellets were separated and stored at -20°C for biomass analysis (Xavier, 1998). The dry weight of the mycelia was weighed and calculated separately for C and N-sources.

Submerged fermentation

All the five isolated *Aspergillus* species such as *A. niger*, *A. sydowii*, *A. tamarii*, *A. glaucus*, and *A. ochraceus* were screened for maximum α -amylase activity using starch hydrolysis method. After examining all the species of *Aspergillus*, it was found that *A. tamarii* was the maximum producer of α -amylase. Hence, further *A. tamarii* was grown in minimal salt medium (MSM) along with wheat bran as agro-waste. The wheat bran was grounded to powder and cooked with distilled water for at least 15 min and then allowed to cool. After cooling, the boiled wheat bran was filtered and the extract was used for preparation of medium in submerged fermentation. The sterilized medium was cooled and 300 mg of Ampicillin per 100 ml of medium was used as antibacterial agent prior to addition of 1 ml of inoculum/50 ml of medium. The culture was grown at $30\pm 2^\circ\text{C}$ for 4 days with intermittent observation.

Solid state fermentation

Production of amylase in SSF was carried out according to Prasanthi *et al.* (2008) with some modifications. *Aspergillus tamarii* was grown at $30\pm 2^\circ\text{C}$ in 250 ml flasks containing 5 g (w/w) of wheat bran and 8 ml of Vogel solution was mixed thoroughly to adjust the moisture content up to 80% and autoclaved. Medium was inoculated with 1ml of *A. tamarii* spores for production of amylase by SSF technique. The fungal culture was allowed to grow for 4 days at $30\pm 2^\circ\text{C}$ with an intermittent observation. *A. tamarii* was also grown in SSF medium having different pH i.e. from pH 4.0 to 8.0 (Vidyalakshmi *et al.*, 2009). Rest procedure was same as above.

Enzyme extraction from SSF and SmF

Enzyme was extracted from the fermented media (SSF and SmF) according to Alva *et al.* (2007) method with slight modifications. 50 ml of cold 0.1 M phosphate buffer (pH 6.5) was added to the solid

substrate fermented cultures and the mixtures were shaken manually for 30 min at 25 °C. The mixture was filtered through cheese cloth and centrifuged at 8000 rpm at 4 °C for 15 min. The supernatant obtained after this centrifugation was filtered through Whatman No. 1 filter paper and the filtrate was used as the crude enzyme. Similarly, in case of submerged fermentation, fermented broth was centrifuged at 8000 rpm for 15 min at 4 °C and the supernatant was filtered through Whatman No. 1 filter paper. The filtrate thus obtained was used as the crude enzyme.

Enzyme assay and quantification

α -Amylase assay was performed according to the method of Mishra and Maheswari (1996) with some modifications. α -Amylase was assayed based on the decrease in iodine staining of starch. The enzyme assay mixture consists of (total volume 1 ml) 0.1 ml enzyme solution and 0.1 ml of 0.1% soluble potato starch and 0.3 ml of 0.2 M phosphate buffer (pH 6.5) was incubated for 15 min at 50 °C. Reaction was terminated by adding 0.25 ml of 0.5 M HCl and 0.25 ml of iodine reagent (0.02% I₂ + 0.2% KI in 0.5 M HCl) was added for colour development. After adding distilled water to dilute the colour of the sample, the intensity of the blue colour was measured at 620 nm using a spectrophotometer (Systronics). One unit of α -amylase activity was defined as the amount of enzyme that hydrolyses 1 mg of starch per min at 50 °C.

Statistical analysis

All experiments were carried out in triplicate and repeated three times. The samples collected from each replicate were tested for amylase production and activity. Means of amylase activity, production

and standard deviation (SD) were calculated using Microsoft Excel Program.

Results and discussion

Maximum numbers of fungi were isolated from the soil of Ganga Nagar, Bhubaneswar i.e. 5×10^7 cfu/g followed by garden soil of P.G. Department of Botany, Utkal University, Vani Vihar, Bhubaneswar (Table 1). But confined to *Aspergillus* species, maximum species were obtained from the soil of P.G. Department of Botany, Utkal University, Vani Vihar, Bhubaneswar i.e. 73% of total isolates (Table 1). In all soil samples examined, 5 species of *Aspergillus* were identified basing on their colony morphology, microscopic, micrometric and biochemical characteristics. Besides this, the other isolated fungi were of genus *Penicillium*, *Cladosporium* and *Fusarium*. Identified species of genus *Aspergillus* were *A. niger*, *A. sydowii*, *A. tamarii*, *A. ochraceus* and *A. glaucus*. Among them, the predominant species were *A. niger*, *A. sydowii* and *A. tamarii* (Fig. 1).

For selection of the species having maximum starch hydrolyzing activity on the starch agar plates, screening was carried out for amylase activity using starch as the substrate. On the basis of the zone of clearance, performance of *Aspergillus tamarii* was maximum in starch hydrolyzing activity (2.4 cm) followed by *Aspergillus glaucus* (2.23 cm) and *Aspergillus ochraceus* (2.1 cm). For the above efficiency, *Aspergillus tamarii* was selected as the experimental organism for amylase production. *A. tamarii* exhibited maximum growth in Czapek Dox medium at pH 7.0, 30 ± 2 °C with an incubation period of 12 days.

Carbon and nitrogen sources are required by most of the saprophytes to lead their life processes and to

Table-1
Statistical analytical results of fungi count/g (cfu/g) of the examined soil samples

Collection Area	No. of samples examined	No. of samples having <i>Aspergillus</i> spp.	Percentage of <i>Aspergillus</i> spp.	Minimum (cfu/g)	Maximum (cfu/g)
Ganga Nagar, Bhubaneswar	15	9	60	3×10^2	5×10^7
P.G. Dept of Botany	15	11	73	7×10^2	9×10^5
BDA colony, Bhubaneswar	15	7	46	2×10^1	4×10^4

satisfy their nutritional and energy need. This study explains that supplementation of glucose, the direct carbon source and peptone as nitrogen source to the medium harboured maximum growth of *Aspergillus tamarii*. Supplementation of starch also exhibited almost same result as compared with glucose. All carbon and nitrogen sources including glucose, starch and peptone supported growth in liquid medium with the exception of lactose and urea (Fig. 2 and 3). Geisseler and Horwath (2008) also studied the responses of soil microbes and production of enzyme at different availabilities of C and N sources.

The fungus *Aspergillus tamarii* exhibited maximum amylase synthesis (8430 U/ml) after 4 days of incubation in SmF at 10% wheat bran supplementation. This method was preferred as the macro molecules and the cell debris get settle down forming the pellet in tubes, so that the crude source

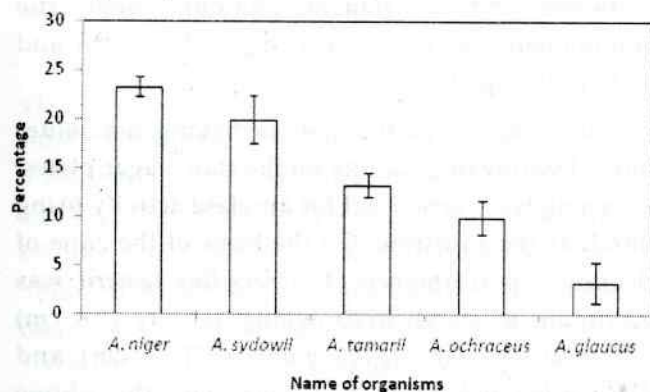


Fig. 1. Frequency distribution of *Aspergillus* species in examined soil sample from garden of P.G. Department of Botany, Utkal University, Vani Vihar, Bhubaneswar (Mean $\pm$ SD)

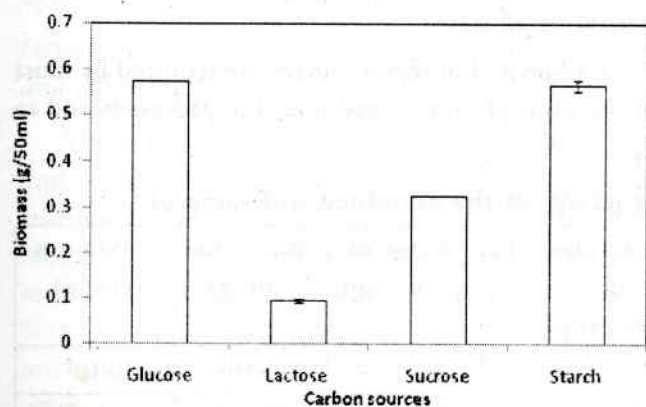


Fig. 2. Effect of carbon sources on growth of *Aspergillus tamarii* (Mean $\pm$ SD)

of the enzyme can be obtained easily (Raj *et al.*, 2009). In solid state fermentation, the production of amylase was 9493.33 U/ml by *Aspergillus tamarii* at 10% wheat bran supplementation. Solid state fermentation (SSF) is widely established for the production of enzymes by filamentous fungi (Wang *et al.*, 2003). Morphology and physiology of these molds enable them to penetrate and colonize various solid substrates. Wheat bran has been a highly reported substrate producing promising results among the various agro-wastes used because of its high carbohydrate content, suitable texture with significant buffering capacity (Kunamneni *et al.*, 2005; Sodhi *et al.*, 2005). Wheat bran has rich morphological characteristic for use as a substrate in large scale solid-state fermentations as a bed of wheat bran has more open structures (Chutmanop *et al.*, 2008). The α -amylase production is also subjected to catabolite repression by glucose and other sugars, like most other inducible enzymes. In this present study, SSF was proved to be superior to SmF as more amount of amylase was produced by SSF (9493.33 U/ml) technique in comparison to SmF (8430 U/ml) (Fig. 4).

After getting maximum synthesis of enzyme in SSF, the effect of pH of the medium for enzyme synthesis was evaluated. It was found that pH has a greater influence on enzyme activity as it works inside a biological system and mostly governed by both pH and temperature. The experiment showed that the production of amylase was maximum in pH 6.0 (Fig. 5). Further increase in pH of the medium resulted in decrease of amylase production by this

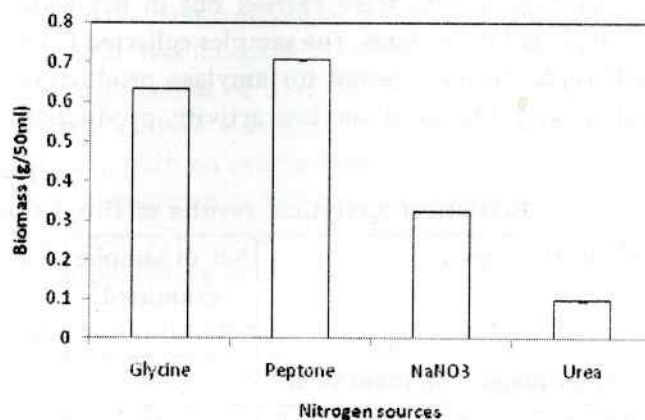


Fig. 3. Effect of nitrogen sources on growth of *Aspergillus tamarii* (Mean $\pm$ SD)

fungus. The peak clearly indicates that this enzyme can be implemented in various industries that work in higher pH environment.

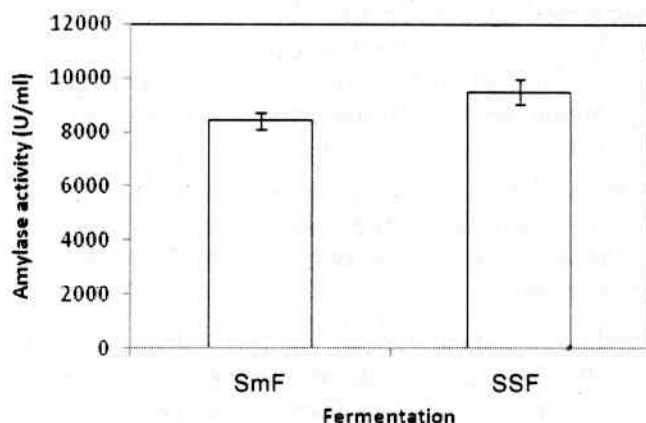


Fig. 4. Comparison of amylase production under solid state fermentation and submerged fermentation (Mean $\pm$ SD)

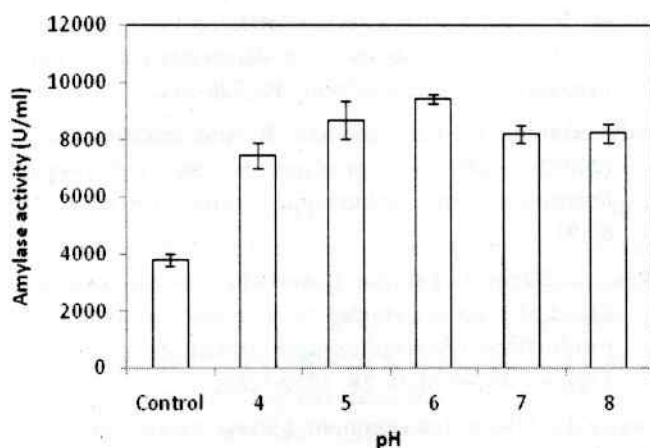


Fig. 5. Amylase production under different pH of fermentation medium (Mean $\pm$ SD)

Isolation of fungi from soil and their rapid screening by plating on starch agar plates led to the finding of a novel strain. This strain was used both for solid-state and submerged fermentation for amylase production. The work has developed a simple and cost-effective protocol for amylase production from agro-wastes under submerged and solid state fermentation. Wheat bran was selected as the substrate due to its very low cost without smash process. The produced amylase is found most suitable for the hydrolysis of agro-wastes. The enzyme production was studied thoroughly and found that the enzyme is very sensitive to pH. Therefore, the

selection of optimum pH of the fermentation medium is essential for the production of α -amylase. *Aspergillus* species, in particular *A. tamarii* can be industrially exploited for the synthesis of α -amylase. In the light of modern biotechnology, α -amylases are now gaining importance in biopharmaceutical application. Their application in food and starch based industries is the major market and thus the demand of α -amylases would always be high in these sectors. But it is necessary to optimize the production of enzyme in media prepared with agro-wastes. Evaluation of different agro-wastes as a substrate, effect of various fermentation parameters such as fermentation period, fermentation temperature, inoculum size, extraction solvent, pH of the production medium, inorganic and organic nitrogen sources and other parameters should be included in further studies. Strain improvement studies also need to be carried out to enhance enzyme production.

Acknowledgement

This work was financially supported by UGC-RGNF, New Delhi which is gratefully acknowledged. The authors are also grateful to the Head of the Department, P.G. Department of Botany, Utkal University, Vani Vihar, Bhubaneswar for providing necessary laboratory facilities.

References

- Alva, S., Anupama, J., Salva, J., Chiu, Y.Y., Vyashali, P., Shruti, M., Yogeetha, B.S., Bhavya, D., Purvi, J., Ruchi, K., Kumudini, B.S. and Varalakshmi, K.N. (2007). Production and characterization of fungal amylase enzyme isolated from *Aspergillus* sp. JGI 12 in solid state culture. *Afr. J. Biotechnol.* 6 (5): 576-581.
- Baki, A. and Anderson, B. (1973). Vigour determination in soyabean seed by multiple criteria. *Crop Sci.* 13: 630-633.
- Baysal, Z., Uyar, F. and Aytekin, C. (2003). Solid-state fermentation for production of alpha-amylase by a thermotolerant *Bacillus subtilis* from hot-spring water. *Process Biochem.* 38: 1665-1668.
- Chutmanop, J., Chuichulcherm, S., Chisti, Y. and Srinophakun, P. (2008). Protease production by *A. oryzae* in solid state fermentation using agro-industrial substrates. *J. Chemical Technol. Biotechnol.* 97(1): 25-32.

- Geisseler, D. and Horwath, W. (2008). Regulation of extracellular protease activity in soil in response to different sources and concentrations of nitrogen and carbon. *Soil Biol. Biochem.* **40**: 3040-3048.
- Ikram-ul-Haq, H., Ashraf, J., Iqbal, M.A. and Qadeer, K. (2003). Production of α -amylase by *Bacillus licheniformis* using an economical medium. *Bioresour. Technol.* **87**: 57-61.
- Kunamneni, A., Kuttanpillai, S.K. and Singh, S. (2005). Response of surface methodological approach to optimize the nutritional parameters for enhanced production of α -amylase in solid state fermentation by *Thermomyces lanuginosus*. *Afr. J. Biotechnol.* **4**: 708-716.
- Mishra, R.S. and Maheshwari, R. (1996). Amylases of the thermophilic fungus *Thermomyces lanuginosus*: Their purification, properties, action on starch and response to heat. *J. Biosci.* **21**: 653-672.
- Mitidieri, S., Martinelli, A.H.S., Schrank, A. and Vainstein, M.H. (2006). Enzymatic detergent formulation containing amylase from *Aspergillus niger*: A comparative study with commercial detergent formulations. *Bioresour. Technol.* **97**: 1217-1224.
- Pandey, A., Selvakumar, P., Soccol, C.R. and Nigam, P. (1999). Solid state fermentation for the production of industrial enzymes. *Curr. Sci.* **77**: 149-162.
- Prasanthi, V., Nikku, M.Y., Vuddaraju, S.P., Nalla, K.K., Raju, C.A.I. and Donthireddy, S.R.I. (2008). Optimization of the fermentation media using statistical approach and artificial neural networks for the production of an alkaline protease from *Bacillus subtilis*. *Int. J. Nat. Eng. Sci.* **2**(3): 51-56.
- Raj, S.V., Raja, A.K., Vimalanathan, B.A., Tyagi, M.G., Johnson, N.A., Justin, M.A., Santhos, B.I. and Sathiyaseelan, K. (2009). Study of starch degrading bacteria from kitchen waste soil in the production of amylase by using paddy straw. *Recent Res. Sci. Technol.* **1**(1): 008-013.
- Ramachandran, S., Patel, A.K., Nampoothiri, K.M., Francis, F., Nagy, V., Szakacs, G. and Pandey, A. (2004). Coconut oil cake - A potential raw material for the production of α -amylase. *Bioresour. Technol.* **93**: 169-174.
- Saha, B.C. and Zeikus, J.G. (1990). Characterization of thermostable cyclodextrinase from *Clostridium thermohydrosulfuricum* 39 E. *Appl. Env. Microbiol.* **56**: 2941-2943.
- Sindhu, R., Suprabha, G.N. and Shashidhar, S. (2009). Optimization of process parameters for the production of α -amylase from *Penicillium janthinellum* (NCIM 4960) under solid state fermentation. *Afr. J. Microbiol. Res.* **3**(9): 498-503.
- Sodhi, H.K., Sharma, K., Gupta, J.K. and Soni, S.K. (2005). Production of thermostable α -amylase from *Bacillus* sp. PS-7 by solid state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol preparation. *Proc. Biochem.* **40**: 525-534.
- Vidyalakshmi, R., Paranthaman, R. and Indhumathi, J. (2009). Amylase production on submerged fermentation by *Bacillus* spp. *World J. Chem.* **4** (1): 89-91.
- Wang, L., Darin, R., Tingyue, G. and Moo-Young, M. (2003). Effect of process parameters on heterologous protein production in *Aspergillus niger* fermentation. *J. Chem. Technol. Biotechnol.* **78**: 1259-1266.
- Xavier, I.J. (1998). Environmental Stress Response of the Hyphomycetous Entomopathogenic Fungi. Ph.D. thesis. University of Saskatchewan, Saskatoon. Canada.

Production of value-added phenolic compounds from wheat bran using isolates of *Staphylococcus aureus*

P.K. Sarangi[✉] and H.P. Sahoo

Department of Botany, Ravenshaw University, Cuttack-753003

(Received : 10 November, 2010; Revision received : 20 December, 2010)

A study was carried out on isolation of some phenolic value-added compounds from wheat bran using *Staphylococcus aureus*. Ferulic acid and vanillin were detected as important phenolic acids from the medium which were identified and quantitated by TLC/HPLC. Ferulic acid was recovered as an intermediate compound which was further biotransformed into vanillin. It was confirmed that about 275 mg of ferulic acid was obtained from 1kg wheat bran after an incubation period of 6 days. Almost 99.9% ferulic acid with an initial concentration of 5 mM was consumed by the organism producing about 9.8 mg/l of vanillin following 6-day incubation.

Key words: Wheat bran, *Staphylococcus*, ferulic acid, vanillin

Introduction

The yearly accumulation of agro-industrial waste-materials generated by the milling, brewing and various food based industries, has led to the consideration of extracting high value residues to offset the cost of treating and disposing of the residues. Most of these by-products are currently used as animal feed; nevertheless, attention towards obtaining high-value compounds from these wastes is being emphasized by various academic as well as industrial researchers. Biological degradation, for both economic and ecological reasons, has become an increasingly popular alternative for the treatment of agricultural, industrial, organic as well as toxic wastes. A high proportion of this waste material is carbohydrate and phenolic in nature. Specific sugar residues can be released from the cell-wall by the action of carbohydrases. On the other hand, phenolics moieties are linked to polysaccharides by ester-bond forming structural cross-linking bridges between various polymers. Some phenolic compounds, such as ferulic acid and p-coumaric acid, are also present in cell wall. Ferulic acid is the most abundant hydroxycinnamic acid in the plant kingdom and occurs mainly in the cell wall of cereal plants, which is covalently linked to lignin with ether bonds (MacAdam & Grabber 2002). Ferulic acid is a very

important component for the structure and the biology of cell-wall as it can cross link polysaccharide chains through dimerisation reaction (Ishii, 1997). It is the precursor of coniferyl alcohol, a monolignol which contributes to lignification in the conifers and angiosperms. It is mainly conjugated with mono- and oligosaccharides, polyamines, lipids and polysaccharides and seldom occurs in a free state in plants. Ferulic acid has been reported to have many physiological functions including antioxidative (Graf, 1992), antimicrobial, anti-inflammatory, anti-thrombosis, anti-cancer activities and antibiotic properties (Beschta *et al.*, 1982). Many species of microorganisms are able to degrade plant aromatic compounds, thus releasing a vast amount of carbon which otherwise would be locked away in plant secondary metabolites such as lignin (Rosazza *et al.*, 1995). Recently, the increasing interest in natural products led to develop flavour production via biotechnological processes involving microorganisms. An important attribute of microbial biocatalysts in bioreactors is the ability to synthesize products in a consistent and predictable manner. Some phenolic compounds, such as ferulic and p-coumaric acids, are also present in cell wall. Due to chemical similarity between ferulic acid and vanillin, the biotransformation of ferulic acid to vanillin was a study of interest. In order to produce 'natural vanillin',

[✉] Corresponding author; E-mail: sarangi77@yahoo.co.in

it is necessary to use 'natural ferulic acid' i.e. released from raw materials by GRAS (Generally Regarded As Safe) enzymes (Bonnin *et al.*, 2001). Hence work is being carried out for the identification, purification and characterization of novel microbial esterases capable of releasing the bioactive phenolic compound, ferulic acid, from cereal cell walls and agro-industrial waste (Faulds *et al.*, 1997). The aim of this paper is to present a mechanistic model for the production of vanillin, the most universally used flavour in the food industry.

Materials and methods

Initiation & maintenance of pure cultures

Staphylococcus aureus was isolated from soil and screened for its ability to grow in ferulic acid containing medium. Pure cultures of these strains were maintained on a mixed medium containing both beef extract and peptone and cultures were incubated at 35°C. In order to obtain high-density cultures, the bacterium was grown in a broth medium containing both beef extract and peptone (pH 7.2) for 5 days.

Collection and destarching wheat bran samples

Wheat bran samples were obtained from common soft wheat (*Triticum aestivum*) cultivars grown in local area for flour production. These materials are normally used as animal feed. The brans were obtained from the market nearby Ravenshaw University campus and stored at 4°C. The brans contained 0.16% of starch (dry matter). Before using as substrates, wheat bran was destarched. The procedure of destarching was described as follows. Certain amount of wheat bran was washed with tap water many times. The bran was recovered by filtration through a glass filter and the water treatment was repeated along with 1.5% Tween 80. The processed wheat bran was soaked into filter paper and dried overnight. Again it was washed with tap water along with 1.5% Tween 80. Then it was dried in oven and autoclaved for 20 minutes at 121 °C to inactivate any endogenous enzymes and proteinaceous inhibitors and stored at -20 °C.

Release of ferulic acid

The microorganism was grown in minimal medium of Muheim & Lerch (1999) which contained

basal inorganic salts, ammonium nitrate (3.0 g/l) as a nitrogen source, hydrated magnesium sulphate (0.2 g/l), sodium chloride (0.2 g/l), potassium dihydrogen phosphate (1.0 g/l), disodium hydrogen phosphate (4.0 g/l), calcium chloride (0.05 g/l) containing wheat bran as sole carbon source. The initial pH of the minimal medium was adjusted to 7.0 before autoclaving for 15 min at 121 °C. The cultures were incubated at 35 °C and analyses were carried out in duplicates on day basis analyses upto 10 days of incubation.

Biotransformation of ferulic acid

For the biotransformation study, 1 ml cell suspension of microorganism was transferred into the 100 ml flask each containing 25 ml minimal medium (Muheim and Lerch, 1999) along with ferulic acid as a sole carbon source. The pH of the media was adjusted to 7.2. The cultures were incubated at 35 °C and analyses were carried out on day-to-day basis upto 8 days of incubation to detect the degradation product of ferulic acid.

Extraction and detection of metabolites from the culture media

For the extraction of ferulic acid and its degradation products from the culture media, culture supernatants were prepared by centrifugation. These were acidified (pH 1-2) and extracted with equal volume of ethyl acetate. The ethyl acetate was evaporated in vacuum and the residue was re-dissolved in 50% methanol. This processed culture filtrate was subjected to thin layer chromatography (TLC) and HPLC. The TLC analysis was performed on Avicel® microcrystalline cellulose plate as described by Dey *et al.* (2003). The plates were developed in 2% aqueous formic acid. The phenolic acids were viewed under a dual-wavelength (254 nm/310 nm) UV-lamp. The bands corresponding to standards were detected on the plate. The band which corresponded to standard was eluted separately with methanol. The extract was centrifuged at 5000 rpm for 10 min. The supernatant free from any cellulose fraction were collected. Ultraviolet scan of the supernatant containing the compound was performed in UV-VIS scanning diode-array spectrophotometer (ELICO BIO BL 200 spectrophotometer, India). HPLC analyses and quantification of ferulic acid and its

degradation products were performed on a Waters Symmetry® C18 column (4.6 x 75 mm; 3.5 m) equipped with a Waters 2487 Dual Absorbance Detector set at 254 nm and 310 nm. Aqueous trifluoroacetic acid (1.0 mM)-methanol (68:32) at a flow rate of 1.0 ml/min for 25 min at room temperature was used for the separation of the phenolic compounds (Sachan *et al.*, 2004). The identification of each phenolic compound was confirmed by comparing retention times and UV-spectra with external standards.

Results and discussion

Staphylococcus aureus was inoculated in minimal medium containing de-starched wheat bran and incubated for 10 days at 35 °C and results obtained are shown in Fig. 1. It was confirmed that about 275 mg of ferulic acid was obtained from 1kg of wheat bran after the 6th day of incubation period. Day basis analyses were performed to detect the products of ferulic acid bioconversion by *Staphylococcus aureus*. Vanillin was detected as the major degradation product. About 9.8 mg/l of vanillin was detected from the fermentation broth after 6 days of incubation period. (Fig. 2). The phenolic acid(s) were identified and quantitated by TLC/HPLC (Fig. 3, Fig. 4). Our result suggested that vanillin was formed as a major product in the pathway and ferulic acid was produced as the intermediate product (Fig. 5).

Wheat bran is a major milling by-product and represents an abundant but under-exploited renewable resource. Starch depleted bran from wheat is rich in arabinoxylans (AX). Enzymatic upgrading of bran is an attractive alternative to environmentally damaging chemical methods currently used for

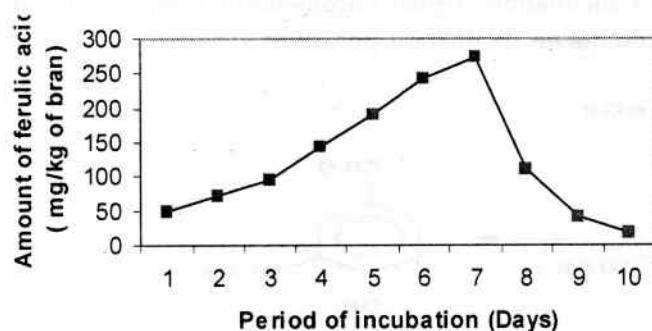


Fig. 1 : Release of ferulic acid from wheat bran by *Staphylococcus aureus*

lignocellulose saccharification. Over the last decade concentration on the isolation and purification of number of microbial esterase which can cleave ferulic acid from sugar residues in agro-industrial waste was emphasized. Wheat bran obtained from market, contained 0.16% of total sugar (dry matter). Before using as substrates, wheat bran was destarched for easy release of ferulic acid during esterase activity. In this case study, it was detected that maximum amount of ferulic acid was found to be 275 mg per kg of wheat bran only after 6 days of incubation which was higher than the amount of ferulic acid isolated from wheat bran by *Streptomyces* isolates earlier (Sarangi *et al.*, 2009a). Microbial modification of phenylpropenoic acids such as cinnamic acid and ferulic acid has been the object of intensive study in the last decades (Brunati *et al.*, 2004). The main goal of these studies was the identification of microbial species able to convert these substrates in commercially valuable products, such as benzoic acid derivatives or aromatic aldehydes (benzaldehyde and vanillin). *Streptomyces* isolate S 10 showed the biotransformation ability by converting ferulic acid to vanillin (Sarangi *et al.*, 2009b) showing more vanillin accumulation than the vanillin production by *Staphylococcus aureus*. Maximum amount of vanillin accumulation into the medium was 9.8 mg/l after day 6 of incubation period. After day 7, a decrease in the amount of vanillin was observed. Probably, after the total degradation of ferulic acid, the isolate utilized vanillin as sole source of carbon for its growth and maintenance.

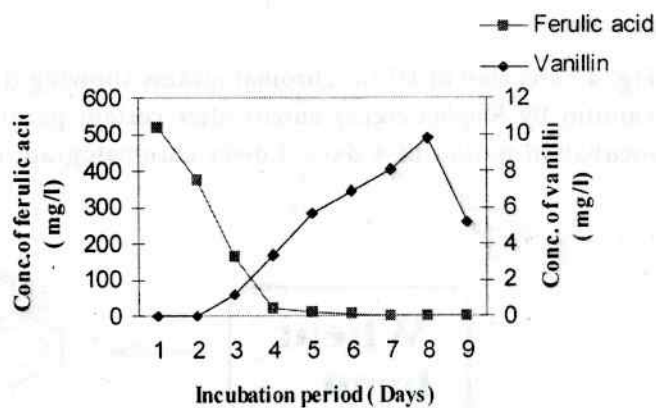


Fig. 2 : Time course degradation of ferulic acid and subsequent formation of vanillin by *Staphylococcus aureus*

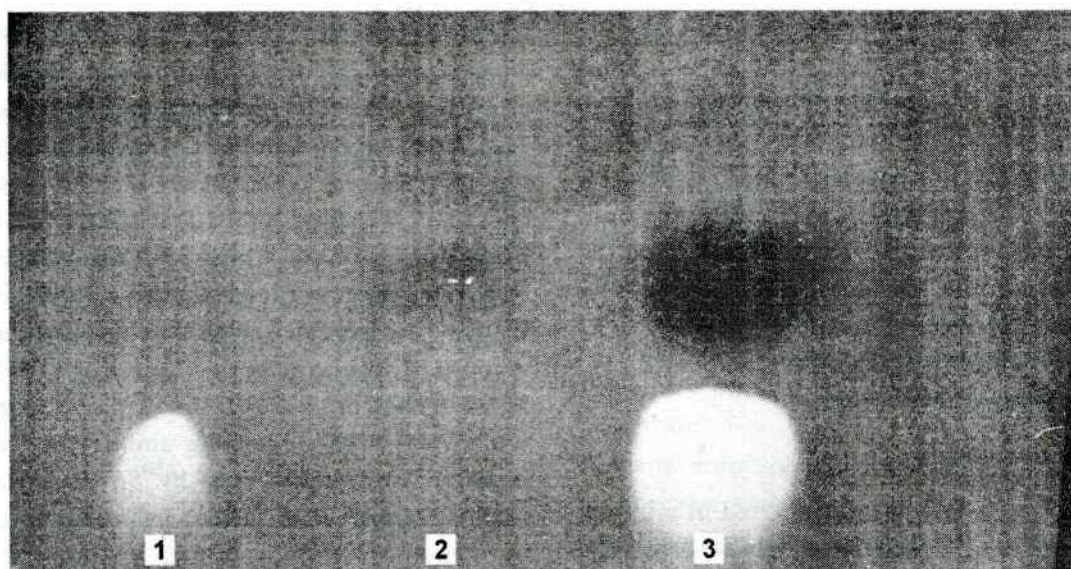


Fig. 3 : TLC chromatogram of processed culture filtrates of *Staphylococcus aureus* grown on minimal medium containing 5.0 mM ferulic acid (Lane 1: Ferulic acid, Lane 2: Vanillin, Lane 3: Processed culture filtrate; lower band = ferulic acid; upper band = vanillin)

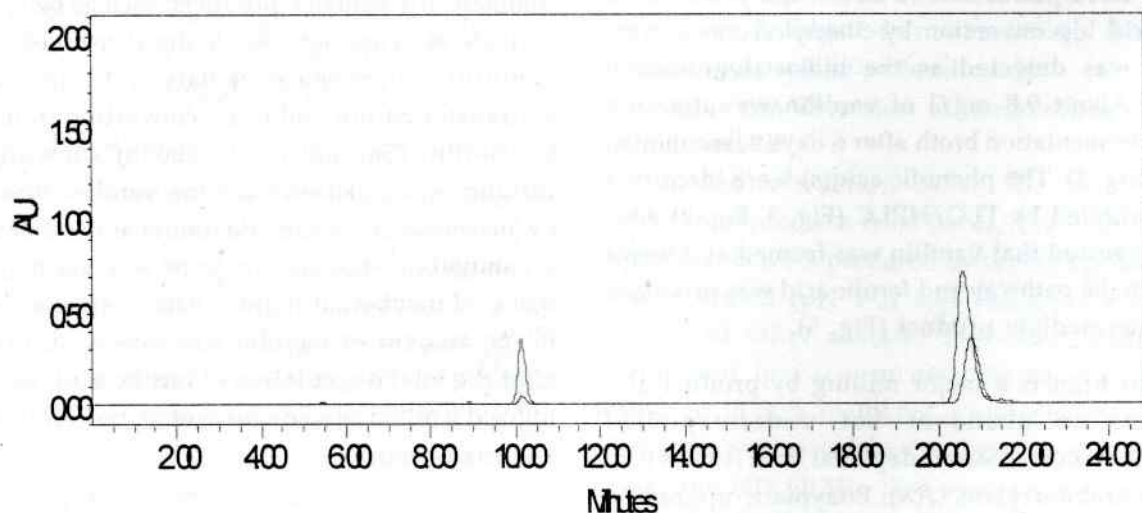


Fig. 4 : Overlay of HPLC chromatograms showing degradation of ferulic acid and subsequent formation of vanillin by *Staphylococcus aureus* after certain periods of incubation. Upper chromatogram represents an incubation period of 4 days. Lower chromatogram represents an incubation period of 7 days

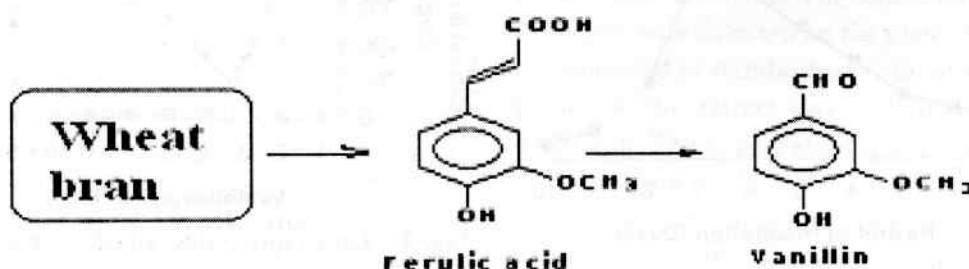


Fig. 5 : Proposed catabolic route of ferulic acid by *Staphylococcus aureus*

References

- Beschia, M., Leonte, A. and Oancea, I. (1982). Phenolic components with biological activity in vegetable extracts. *Bull.Univ.Galati*. 6: 59-63.
- Bonnin, E., Brunel, M., Gouy, Y., Asther, M. and Thibault, J.F. (2001). *Aspergillus niger* I-1472 and *Pycnoporus cinnabarinus* MUCL 39533, selected for the biotransformation of ferulic acid to vanillin, are also able to produce cell wall polysaccharide-degrading enzymes and feruloyl esterases. *Enzyme Microb. Technol.* 28: 70-80.
- Brunati, M., Marinelli, F., Bertolini, C., Gandolfi, R., Daffonchio, D. and Molinari, F. (2004). Biotransformations of cinnamic and ferulic acid with actinomycetes. *Enzyme Microb. Technol.* 34: 3-9.
- Dey, G., Sachan, A., Ghosh, S. and Mitra, A. (2003). Detection of major phenolic acids from dried mesocarpic husk of mature coconut by thin layer chromatography. *Ind. Crops Prod.* 18: 171-176.
- Faulds, C.B., Bartolome, B. and Williamson, G. (1997). Novel biotransformations of agro-industrial cereal waste by ferulic acid esterases. *Ind. Crops Prod.* 6: 367-374.
- Graf, E. (1992). Antioxidant potential of ferulic acid. *Free Rad. Biol. Med.* 13: 435-448.
- Ishii, T. (1997). Structure and functions of feruloylated polysaccharides. *Plant Sci.* 127: 111-127.
- MacAdam, J.W. and Grabber, J.H. (2002). Relationship of growth cessation with the formation of diferulate cross-links and *p*-coumaroylated lignins in tall fescue leaf blades. *Planta* 215: 783-793.
- Muheim, A. and Lerch, K. (1999). Towards a high-yield bioconversion of ferulic acid to vanillin. *Appl. Microbiol. Biotechnol.* 51: 456-461.
- Rosazza, J.P.N., Huang, Z., Dostal, L., Volm, T. and Rousseau, B. (1995). Biocatalytic transformation of ferulic acid: an abundant aromatic natural product. *J. Ind. Microbiol.* 15: 457-471.
- Sachan, A., Ghosh, S. and Mitra, A. (2004). An efficient isocratic separation of hydroxycinnamates and their corresponding benzoates from plant and microbial sources by HPLC. *Biotechnol. Appl. Biochem.* 40: 197-200.
- Sarangi, P.K., Sahoo, H.P., Ghosh, S. and Mitra, A.P. (2009a). Isolation of Ferulic Acid from Wheat Bran Using Streptomyces Isolates. *Intl. J. Appl. Agri. Res.* 4(1): 57-62.
- Sarangi, P.K., Sahoo, H.P., Ghosh, S. and Mitra, A.P. (2009b). Biotransformation of ferulic acid by *Sreptomycies* isolate S 10. *Asian J. Microbiol. Biotechnol. Env. Sci.* 2: 273-278.

Identification of endophytic fungi associated with rice seeds by molecular techniques

U. Dhua^{1*}, B. Bhattacharjee², S.R. Dhua², A. Chhotaray², I. Sahoo¹ and S. Nayak²

¹Plant Pathology, Crop Protection Division, Central Rice Research Institute, Cuttack-753006

²Crop Improvement Division, CRRI, Cuttack-753006

(Received : 15 December, 2009; Revision received : 03 May, 2010)

Molecular characterization of six fungal isolates associated with the de-husked, surface sterilized, healthy seeds of rice cultivars was done. DNA of all the isolates was extracted, ITS region of ribosomal DNA was amplified, and fungus was identified by the homologous sequence analysis in NCBI- Blast algorithms for nucleotide. Multiple alignments of six sequences in Pearson sequence format was done by ClustalW2. Bootstrap test, Interior Branch Test by Neighbor-Joining method Evolutionary Divergence and Disparity Index analysis (Composition distances and Disparity Index) were conducted in MEGA4. All the six isolates of endophytic microbes belonged to Kingdom Fungi, Phylum Ascomycota and Sub Phylum Pezizomycotina. All isolates except EN5 were in Class Dothideomycetes (unclassified Fungi). Isolate EN3 had significant similarity with the sequences of *Cochliobolus*. Isolate EN5 was placed in class Eurotiomycetes, Order Eurotiales, Family Trichocomaceae and had identity with *Neosartorya* which is sexual phase of *Aspergillus*.

Key words : Endophytic fungus, *Cochliobolus*, *Neosartorya glabra*, ITS region

Introduction

Fungal endophytes are fungi that live within plant tissues without causing obvious symptoms of tissue damage either intercellularly or intracellularly (Wilson, 1995). They offer great-untapped potentials, which can be exploited to maintain healthy crops. Some species of endophytic fungi have been identified as sources of anticancer, antidiabetic, insecticidal, fungicidal and thermoprotective compounds (Maheswari, 2006). However, several endophytes, cannot be identified due to the lack of sporulation. Non-sporulating isolates are generally termed 'mycelia sterilia' and grouped as 'morphospecies' based on similarity in cultural characteristics such as, colony surface texture, hyphal pigments, exudates, margin shapes and growth rates (Bills, 1996; Umali *et al.*, 1999; Fröhlich *et al.*, 2000; Lacap *et al.*, 2003). Grouping mycelia sterilia into morphospecies, however, may not reflect species phylogeny, because morphospecies are not real taxonomic entities. Furthermore, interpretation of morphospecies as separate taxonomic entities may have implications

on fungal diversity measurement and fungal species composition of a particular plant species (Rollo *et al.*, 1995; Zhang *et al.*, 1997; Guo *et al.*, 2000, 2001; Lacap *et al.*, 2003). To resolve this problem, alternative techniques specially molecular techniques have been developed to identify these fungi to family or genus level and also to assess the validity of the current morpho-species concept.

Fungal growth was observed from the healthy looking dehusked, surface sterilized seeds of rice cultivars on MS media at CRRI, Cuttack. The associated fungus could not be identified as it did not produce spores but only mycelial growth was observed. Molecular techniques have been used successfully for phylogenetic placement and segregation of endophytic mycelia sterilia (Promputtha, *et al.*, 2005). Internal Transcribed Spacer (ITS) region of Ribosomal DNA is now perhaps the most widely sequenced DNA region in fungi and was found to be useful for the identification of non sporulating endophytes Hence present investigations were carried out to identify the associated fungus by these molecular techniques.

* Corresponding author; E-mail: urmila.dhua@gmail.com

Materials and methods

Six fungal isolates associated with the healthy seeds of rice cultivars were studied. Fungal DNA of all the isolates was extracted, ITS region was amplified, and fungus was identified by molecular techniques.

DNA extraction

Isolates were grown on CZ (sucrose 30 g, NaNO₃ 2 g, K₂HPO₄ 1 g, MgSO₄·7H₂O 0.5 g, KCl 0.5 g, FeSO₄ 0.01 g) broth. Fifty mg blotter-dried-mycelia of each isolate, 50 mg sand and 600 µl extraction buffer (400 mM Tris-HCl (pH 8.0), 60 mM EDTA, 150 mM NaCl and 1% sodium dodecyl sulfate) were taken in to a sterilized mortar and ground with a pestle to get a thick paste. Extraction buffer (400 µl) was added to this paste followed by addition of 100 µl of Potassium Acetate after 15 minutes. It was centrifuged at 10,000 rpm for two minutes and supernatant was collected, and again centrifuged at 10,000 rpm for 10 minutes after adding 500 µl of Chloroform : Isoamyl Alcohol (24:1). Equal volume of isopropanol was added and incubated at -20 °C overnight. Tubes were brought to normal temperature and centrifuged at 10000 rpm for 3 minutes. The supernatant was discarded and the pellet was washed by adding 300 µl of 70% Ethanol and centrifuged at 10000 rpm for 3 minute. Air dried pellet was dissolved in 50 µl of 1X TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer and then stored at -20 °C.

PCR amplification

Following primers (Operon) were included in this study:

ITS-1 (TCCGTAGGTGAACCTGCGG),
ITS-2 (GCTGCGTTCTTCATCGATGC),
ITS-3 (GCATCGATGAAGAACGCAGC)
ITS-4 (TCCTCCGCTTATTGATATGC)

Three combinations i.e. ITS-1 & ITS-2; ITS-3 & ITS-4; ITS-1 & ITS-4 of these ITS primers were utilized for the PCR reaction.

PCR was done using 50 µl reaction mixture containing 1X Taq buffer (Biotools Spain), 2 mM MgCl₂, 10 picco moles of each primer, 0.1 mM each dNTP and 1U of Taq polymerase. The PCR reaction

was performed in a programmable DNA Thermocycler (PTC-100, M.J. Research INC). Forty cycles of amplification were performed after initial denaturation of DNA at 95 °C for 4.5 min. Each cycle consisted of a denaturation step at 95 °C for 30 s, an annealing step at 50 °C for 30 s, and an extension step at 72 °C for 1 min, with a final extension at 72 °C for 3 min following the last cycle (Henry *et al.*, 2000). After amplification, the products were stored at 4 °C until use. The PCR product was outsourced to Geni for DNA sequencing.

Analysis and prediction

Sequence alignment was done for identifying the microbes. The sequences were analyzed with the algorithms afforded by free bioinformatics software on Internet i.e. Blast algorithms for nucleotide or polypeptide homologous sequence analysis in NCBI (Zhang *et al.*, 2000), BLAST computes a pair-wise alignment between a query and the database sequences searched. For purposes of this sequence tree presentation an implicit alignment between the database sequences is constructed, based upon the alignment of those (database) sequences to the query. An expected value is calculated and indicates the number of alignments one expects to find with a score greater than or equal to the observed alignments score in a search against a random database. This means that an expected value close to 0 indicates great similarity to the analyzed sequence (Gibas and Jambeck, 2001). Multiple sequence alignment of all the isolates included in this study was done using the multiple sequence alignment program ClustalW2. Phylogram tree showing the distances was constructed.

Boot strap test (UPGMA, Maximum Parsimony and Neighbor-Joining), Interior Branch Test (Neighbor-Joining) were conducted in MEGA4. Disparity Index per site and difference in base composition were also analyzed in MEGA4.

Results

Similarity was observed among ITS amplicons of isolate EN1, EN2, EN4 and EN6 (Fig. 1). It was further confirmed by the homologous sequence analysis in NCBI- Mega Blast algorithms for nucleotide. The database sequences of "Fungal

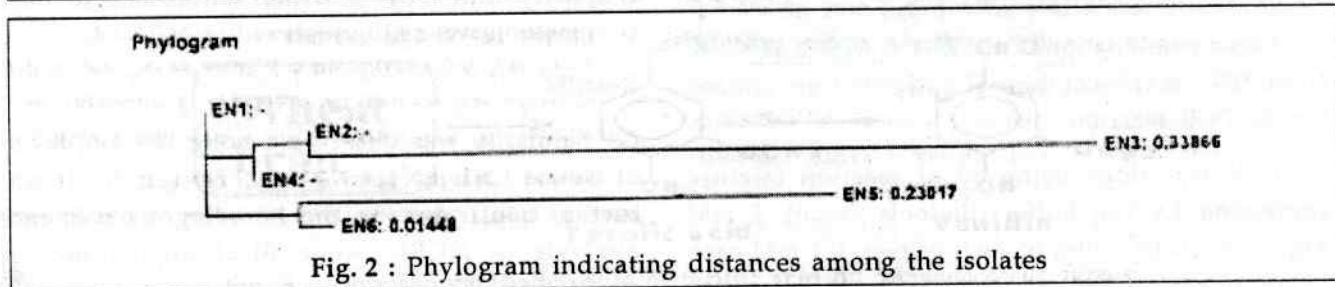
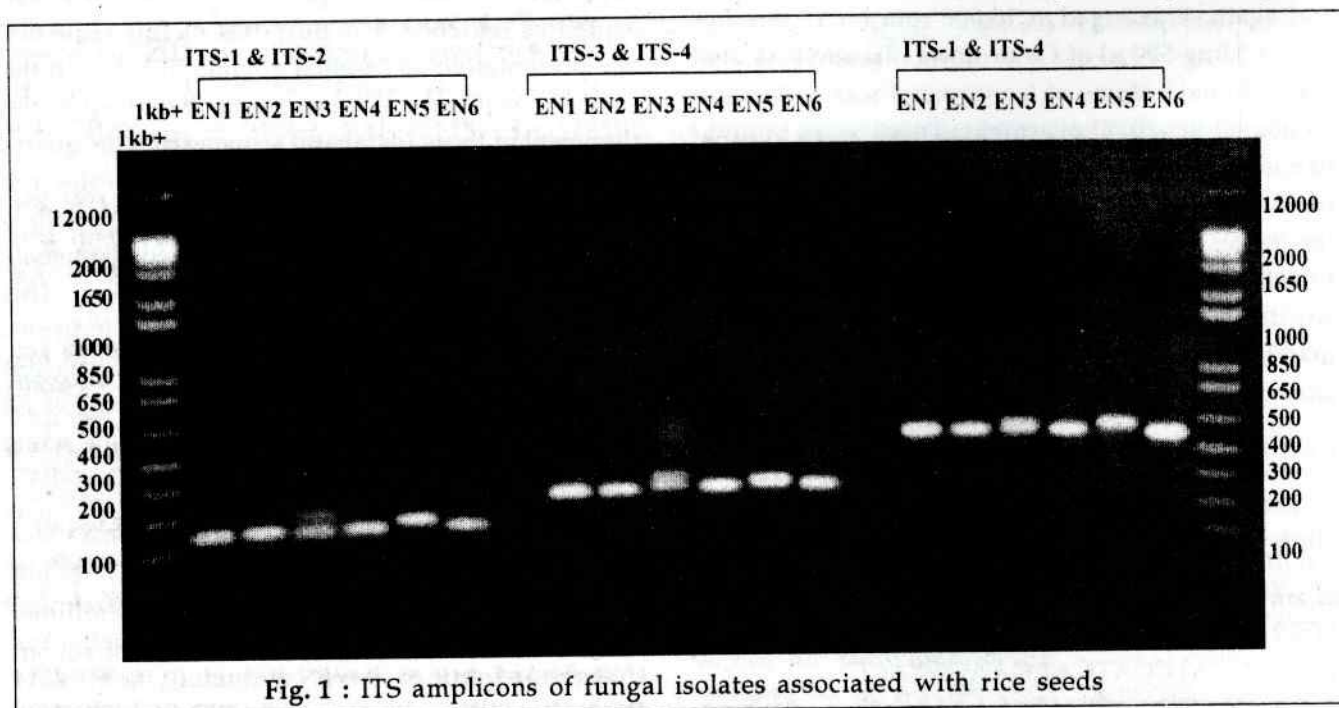
endophyte isolate 9058" with max. score had 95% identity and E value 0, with isolate EN1, EN2 and EN4. Isolate EN3 had 90% identity with the database sequences of *Cochliobolus* in Dothideomycetes but E value was 7e-141. Ninety five percent identity was found between isolate EN6 and database sequences of 'Fungal endophyte isolate 9058' as well as of 'uncultured soil fungus' but E value was 5e-146 and 8e-149 respectively. Isolate EN5 had shown 98% identity with *Neosartorya glabra*, however E value was 2e-164 (Table-1).

Multiple alignments of six sequences in Pearson sequence format was done by using ClustalW2. The alignment score was 29595 and this analysis reconfirmed the earlier findings. The multiple alignment Scores were 100% for EN1 & EN4, 99% for EN1 & EN2, 97% for EN1 & EN6, 72% for EN1 & EN5 and 66% for EN1 & EN3 (Table-3). This trend was also reflected in phylogram. EN5 had 0.23017

distance from EN1 whereas EN3 showed 0.33866 distances (Fig. 2).

The evolutionary history was inferred using the UPGMA (Sneath and Sokal, 1973), Maximum Parsimony (Eck and Dayhoff, 1966) and Neighbor-Joining (Saitou and Nei, 1987) methods. Interior Branch Test by using the Neighbor-Joining was also done. Disparity Index per site and difference in base composition were also analysed. Codon positions included in above mentioned analyses were, 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 327 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

By UPGMA method (Sneath and Sokal, 1973) the optimal tree (Fig. 3) with the sum of branch length = 92.00486257 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 (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site.

The most parsimonious tree with length = 943 is shown. The consistency index is (0.743083), the

retention index is (0.329897), and the composite index is 0.261679 (0.245141) for all sites and parsimony-informative sites (in parentheses). 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 MP tree was obtained (Fig. 4), using the Close-Neighbor-Interchange algorithm with search level 3 (Felsenstein, 1985; Nei and Kumar, 2000) in which the initial trees were obtained with the random addition of sequences (10 replicates). The most parsimonious ancestral states assignments for site #1 are shown next to each node.

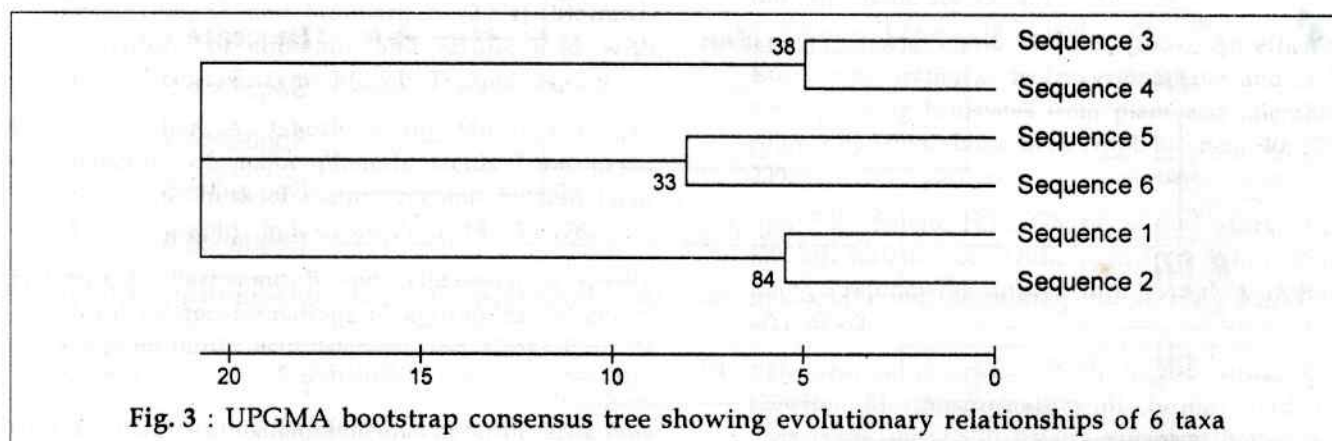


Fig. 3 : UPGMA bootstrap consensus tree showing evolutionary relationships of 6 taxa

Table-1

Database sequences searched by Mega BLAST and showing pair-wise similarities with fungal isolates associated with rice cultivars

Isolate ID	Database sequences with max. score showing similarity		Max. Identity	Max. score	Query coverage	E-value
	Accession	Description				
EN1	EF420016.1	Fungal endophyte isolate 9058	95%	767	85%	0
EN2	EF419930.1					
	EF419982.1					
EN3	EU680523	unclassified Dothideomycetes	90%	508	85%	7e-141
	EU680521	Sordariomycete sp. 7658	90%	508	85%	7e-141
	DQ836799.1	Dothideomycetes <i>Cochliobolus</i>	90%	508	85%	7e-141
EN4	EF420016	Fungal endophyte isolate 9058	95%	767	86%	0
	EF419930	Fungal endophyte isolate 9059	95%	767	86%	0
	EF419982	Fungal endophyte isolate 9057	95%	765	85%	
EN5	EF669964	<i>Neosartorya glabra</i>	98%	586	99%	2e-164
	EF669948					
EN6	DQ420930	uncultured soil fungus	97%	534	96%	8e-149
	EF420016.1	Fungal endophyte isolate 9058	95%	525	97%	5e-146

The evolutionary history was also studied (Fig. 5) using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 85.17031097 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 (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were

computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site.

Interior Branch Test by using the Neighbor-Joining method was done (Fig. 6). The optimal tree with the sum of branch length = 85.77840269 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to

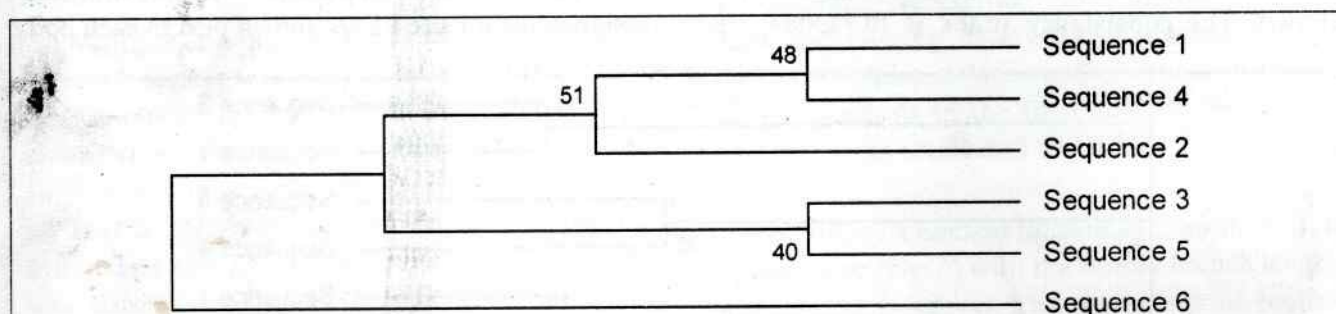


Fig. 4 : Maximum parsimony bootstrap consensus tree showing evolutionary relationships of 6 taxa

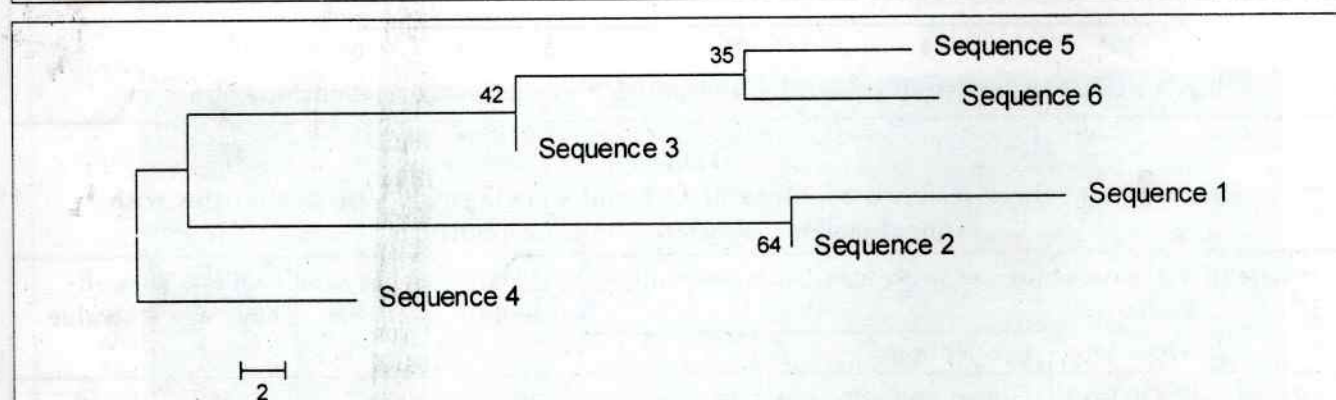


Fig. 5 : Neighbor-Joining bootstrap consensus tree showing evolutionary relationships of 6 taxa

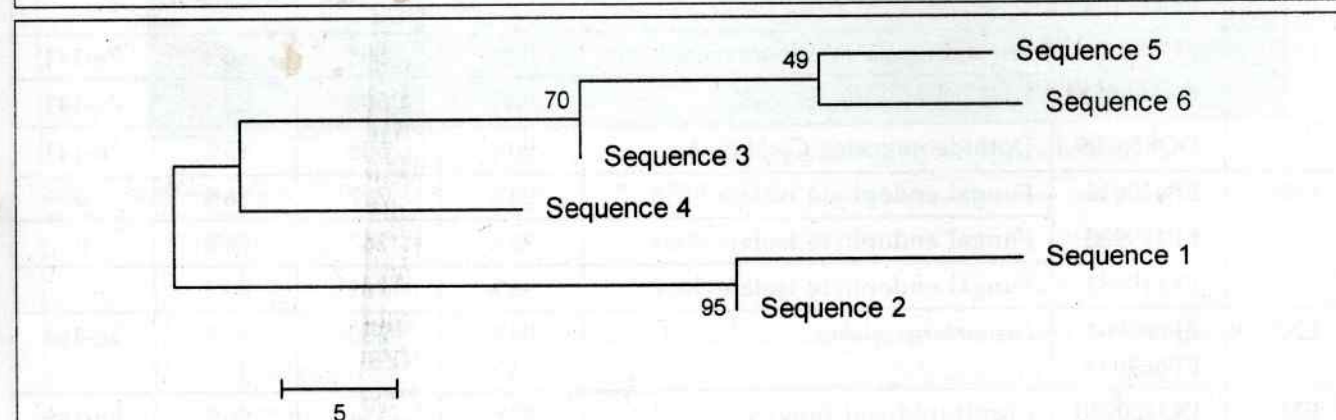


Fig. 6 : Interior branch test by neighbor-joining method for evolutionary relationships of six taxa

Table.-2
Taxonomy report of endophytic fungi by homologous sequence analysis in
NCBI- Mega Blast algorithms for nucleotide

Isolate ID	Taxonomic position						
	Kingdom	Phyla	Sub Phyla	Class	Order	Family	Geneus
EN1	Fungi	Ascomycota	Pezizomycotina	Dothideomycetes	1.Pleosporales 2.unclassified Pleosporales	mitosporic Pleosporaceae	unclassified Fungi/uncultured/ environmental samples
EN2	Fungi	Ascomycota uncultured Ascomycota	Pezizomycotina Unclassified Pezizomycotina	Dothideomycetes Unclassified Dothideomycetes	1.Pleosporales 2.unclassified Pleosporales	mitosporic Pleosporaceae	unclassified Fungi/ environmental samples
EN3	Fungi	Ascomycota	Pezizomycotina	1.Dothiseomycetes 2. unclassified Dothiseomycetes	Pleosporales	Pleosporaceae	1. <i>Cochliobolus</i> 2.mitosporic <i>Cochliobolus</i> 3.unclassified Fungi/ environmental samples
EN4	Fungi	Ascomycota	Pezizomycotina	1.Dothiseomycetes 2. unclassified Dothiseomycetes	1.Pleosporales 2.Uncultured, unclassified Pleosporales	mitosporic Pleosporaceae	unclassified Fungi/ uncultured/ environmental samples
EN5	Fungi	Ascomycota	Pezizomycotina	Eurotiomycetes	Eurotiales	Trichocomaceae	1. <i>Neosartorya</i> 2. <i>Aspergillus</i>
EN6	Fungi	Ascomycota	Pezizomycotina	1.Dothiseomycetes 2.unclassified Dothiseomycetes	1.Pleosporales 2.unclassified Pleosporales	mitosporic Pleosporaceae	unclassified Fungi/ uncultured/ environmental samples/34hits

Table-3
ClustalW2 Multiple alignment scores of fungal isolates associated with rice cultivars

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	EN1	559	2	EN2	559	99
1	EN1	559	3	EN3	442	66
1	EN1	559	4	EN4	555	100
1	EN1	559	5	EN5	333	72
1	EN1	559	6	EN6	327	97
2	EN2	559	3	EN3	442	68
2	EN2	559	4	EN4	555	99
2	EN2	559	5	EN5	333	72
2	EN2	559	6	EN6	327	97
3	EN3	442	4	EN4	555	68
3	EN3	442	5	EN5	333	33
3	EN3	442	6	EN6	327	45
4	EN4	555	5	EN5	333	72
4	EN4	555	6	EN6	327	97
5	EN5	333	6	EN6	327	75

scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method

In Table-4, the difference in base composition bias per site is shown (Kumar and Gadagkar, 2001). It was noted that even when the substitution patterns are homogeneous among lineages, the compositional distance correlate with the number of differences between sequences. Disparity Index per site is shown for all sequence pairs (Table-5). Values, greater than 0 indicate the larger differences in base composition biases than expected based on evolutionary divergence between sequences and by chance alone.

Evolutionary Divergence between 6 Sequences was estimated. The number of base substitutions per site from analysis between sequences is shown. All results are based on the pairwise analysis of 6 sequences. Analyses were conducted using the Maximum Composite Likelihood method in MEGA4 (Tamura *et al.*, 2004, Tamura *et al.*, 2007) and EN1 was found to be very close to EN2 but its distance with EN4, EN5 and EN6 was significant (Table-6).

Discussion

The ITS region of ribosomal DNA is now perhaps the most widely sequenced region in fungi. It has typically been most useful for molecular systematic at the species level, and even within species. ITS primers ITS1 as the forward primer situated at the 3' end of SSU and ITS4-R as the reverse primer in the 5' end of LSU amplified the entire ITS region. The Tax BLAST Report in NCBI- Blast algorithms indicated that all the six isolates of endophytic microbes belonged to Kingdom Fungi, Phylum Ascomycota and Sub Phylum Pezizomycotina. All isolates except EN5 were in class Dothideomycetes. Sequences of EN1, EN2, EN4, and EN6 had similarity with unclassified Fungi/uncultured/environmental samples.

Isolate EN5 was in class Eurotiomycetes, order Eurotiales, family Trichocomaceae and had identity with *Neosartorya* and *Aspergillus* spp. *Neosartorya* species are the sexual states of *Aspergillus* species (Table-2).

EN3 may be placed in family Pleosporaceae and it had significant similarity with the sequences of *Cochliobolus*/unclassified fungi/environmental samples.

Interior Branch Test by Neighbor-Joining method conducted in MEGA4, indicated similarity between EN1 and EN2 and both the isolates had identity with "Fungal endophyte isolate 9058" included in Database sequences searched of Mega BLAST. The multiple

Table-4
Composition distances of base composition bias difference between sequences

Isolate ID	Composition distances of isolates				
	EN1	EN2	EN3	EN4	EN5
EN2	0.028				
EN3	0.131	0.052			
EN4	0.006	0.028	0.15		
EN5	4.81	4.976	5.024	4.823	
EN6	0.771	0.853	1.08	0.716	2.205

Table-5
Disparity index of net base composition bias disparity between six sequences

Isolate ID	Composition distances of isolates				
	EN1	EN2	EN3	EN4	EN5
EN2	0.000				
EN3	0.000	0.000			
EN4	0.000	0.000	0.000		
EN5	4.021	4.193	4.272	4.055	
EN6	0.021	0.107	0.367	0.000	1.446

Table-6
Estimates of evolutionary divergence between six sequence based on pairwise analysis by maximum composite likelihood method in MEGA4

Isolate ID	Pairwise distance between isolates				
	EN1	EN2	EN3	EN4	EN5
EN2	9.493				
EN3	22.88	71.147			
EN4	73.014	15.222	12.697		
EN5	74.02	76.217	15.5	21.552	
EN6	77.249	23.486	12.512	79.084	16.339

alignment scores, of four endophytic fungal isolates, EN1, EN2, EN4 and EN6 by ClustalW2 Multiple alignment analysis was 97-100%. "Disparity index" of Net Base Composition Bias Disparity between Six Sequences which was used to measure and test the homogeneity was zero for isolate EN1, EN2, EN3 and EN4. Composition distances of base composition bias difference between sequences analysis indicated that EN1 was closely related to EN2 and EN4. It had some similarities with EN3 (Composition distance 0.131) followed by EN6 (Composition distance 0.771) but EN5 was distinctly different from EN1 (Composition distance 4.81) and also from all other isolates (Composition distance 2.25-5.024). These findings support the Taxonomy report in NCBI-Mega Blast algorithms.

The potential of endophytic fungus isolated and identified during this investigation is to be explored and exploited for the benefit of mankind.

Acknowledgement

We thank Director CRRI, Cuttack for providing the facilities required for carrying out this study.

References

- Bills, G.F. (1996). Isolation and analysis of endophytic fungal communities from woody plants. In: Redlin, S.C., Carris L.M. and St. Paul, M.N. (eds.) *Endophytic Fungi in Grasses and Woody Plants*, APS Press. USA: 31-65.
- Eck, R.V. and Dayhoff, M.O. (1966). *Atlas of Protein Sequence and Structure*. National Biomedical Research Foundation, Silver Springs, Maryland, U.S.A.
- Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**:783-791.
- Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**:783-791.
- Fisher, P.J. and Petrini, O. (2006). Fungal saprobes and pathogens as endophytes of rice *Oryza sativa* L. *New Phytologist* **120**: 137-143.
- Fröhlich, J., Hyde, K.D. and Petrini, O. (2000). Endophytic fungi associated with palms. *Mycol. Res.* **104**: 1202-1212.
- Gibas, L. and Jambeck, P. (2001). Tools for Bioinformatics. In: *Developing Bioinformatics Computer Skills*. Sebastopol, California: O'Reilly and Associates Inc., CA, USA. 131-214.
- Guo, L.D., Hyde, K.D. and Liew, E.C.Y. (2000). Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. *New Phyto.* **147**: 617-630.
- Guo, L.D., Hyde, K.D. and Liew, E.C.Y. (2001). Detection and taxonomic placement of endophytic fungi within from tissues of *Livistona chinensis* based on rDNA sequences. *Mol. Phylogenet. Evol.* **20**: 1-13.
- Henry, T., Iwen, P.C. and Hinrichs, S.H. (2000). Identification of species using Internal Transcribed Spacer Region 1 and 2. *J. Clin. Microbiol.* **38**(4): 1510-1515.
- Kumar, S. and Gadagkar, S.R. (2001). Disparity Index: A simple statistic to measure and test the homogeneity of substitution patterns between molecular sequences. *Genetics* **158**: 1321-1327.
- Lacap, D.C., Hyde, K.D. and Liew, E.C.Y. (2003). An evaluation of the fungal 'morphotype' concept based on ribosomal DNA sequences. *Fung. Diver.* **12**: 53-66.
- Maheswari, R. (2006). What is an endophytic fungus? *Curr. Sci.* **90**: 1309.
- Nei, M. and Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. Oxford University Press, New York.
- Promptutha, I., Jeewon, R., Lumyong, S., McKenzie, E.H.C. and Hyde, K.D. (2005). Ribosomal DNA fingerprinting in the identification of non sporulating endophytes from *Magnolia liliifera* (Magnoliaceae). *Fung. Diver.* **20**: 167-186.
- Rollo, F., Sassaroli, S. and Ubaldi, M. (1995). Molecular phylogeny of the fungi of the Iceman's grass clothing. *Curr. Genet.* **28**: 289-297.
- Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406-425.
- Sneath, P.H.A. and Sokal, R.R. (1973). *Numerical Taxonomy*. Freeman, San Francisco, U.S.A.
- Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**: 1596-1599.
- Tamura, K., Nei, M. and Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. National Academy of Sci. (USA)* **101**: 11030-11035.

- Umali, T.E., Quimio, T.H. and Hyde, K.D. (1999). Endophytic fungi in leaves of *Bambusa tuldoidea*. *Fungal Sci.* 14: 11-18.
- Wilson, D. (1995). Endophyte the evolution of a term, and clarification of its use and definition. *Oikos* 73: 274-276.
- Zhang, Z., Schwartz, S., Wagner, L. and Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *J Comp. Biol.* 7(1-2): 203-14.
- Zhang, W., Wendel, J.E. and Clark, L.G. (1997). Bamboozled again! inadvertent isolation of fungal rDNA sequences from bamboos (Poaceae: Bambusoideae). *Mol. Phylogenet. Evol.* 8: 205-217.

Orange peel based fermented plant beverage against antibiotic resistant human pathogens

P. Pradhan¹, R.C. Mohanty² and A.K. Mishra^{1*}

¹Centre for Applied Microbiology and Biotechnology (AMIFEM), Laxmi Vihar,
Bhubaneswar- 751005

²P.G.Department of Botany, Utkal University, Bhubaneswar-751004

(Received : 02 November, 2010; Revision received : 24 December, 2010)

Orange peel is an important source of pectin (insoluble carbohydrate) and enzymatic hydrolysis of pectin releases free sugars, which can be utilized by probiotic bacteria for their growth and also for producing many antimicrobial agents. *In vitro* antibacterial activity of orange peel extracts were tested against antibiotic resistant *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus agalctiae*. Fermented plant beverage was prepared by enzymatic hydrolysis of orange peel with pectinase and fermented with 5 strains of probiotic bacteria to study their growth effect on probiotic *Lactobacillus* and inhibitory effect against test organisms. Ethanolic extracts of orange peel showed broad zone of inhibition against all the test organisms. Fermentation products were observed to have low pH (3.4 - 4.3) and high amount of titratable acid. Fermented orange peel products inhibited the growth of test organisms. An increase in number of viable cells of probiotic bacteria was also observed in all the fermented products. The antibacterial activity of fermented plant beverage was attributed to some bioactive compounds derived from plant extracts and antimicrobial agents produced by probiotic bacteria.

Key words: Orange peel, probiotics, *Lactobacillus*, inulin, antibiotic sensitivity

Introduction

Fermented plant beverages (FPBs) are non-alcoholic beverages produced from different kinds of plants such as cereals, fruits and vegetables. These healthy beverages are believed to cure some diseases. They have a high nutritional value and contain bioactive compounds derived from the raw materials, particularly the plant as well as products from the fermentation reactions.

Fermented plant beverage is derived from lactic acid fermentation catalyzed by lactic acid bacteria (LAB) (Kantachote *et al.*, 2005). The lactic acid fermentation has long been recognized and used for producing a variety of foodstuffs (Battcock and Azam-Ali, 1998; Jay, 2000; Ray, 2003). LAB produce a range of compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocins (bactericidal proteins) during lactic acid fermentations and these compounds extend the shelf life of the fermented foods by controlling spoilage organisms and also

potential human pathogens (Adams and Moss, 2000; Oyedayo *et al.*, 2003; Savadogo *et al.*, 2004). Some persons are allergic to dairy products. Lactose intolerance and the cholesterol content are two major drawbacks related to the fermented dairy products. Traditions and economic reasons that limit the use of dairy fermented products in some developing countries promote the idea of reduction of milk components as vehicles for the probiotic agents (healthy beneficial microorganisms). Therefore, non-dairy products are now studied as the carrier of probiotic bacteria in lactic acid fermentation to produce healthy beverages.

Orange peel is a source of probiotic substance (pectin) and can be a potential medium to develop non-dairy probiotic juice. Probiotics are nondigestible foods that stimulate the growth of beneficial probiotic bacteria and fermentation in the large intestine. Orange Peel has been shown to aid in the prevention of indigestion, lower cholesterol and help in the digestion of fatty foods. Orange peels contain both

* Corresponding author; E-mail : arunakmisra@yahoo.co.in

soluble and insoluble carbohydrates. The latter are present in the cell walls of the peels, particularly in the form of pectin, cellulose and hemicellulose. These polymers can be hydrolyzed enzymatically by cellulase, β -glucosidase and pectinase to their corresponding soluble carbohydrates. *In vitro* tests revealed that pectin acts as a probiotic, preventing pathogens from binding to the intestine and increasing the growth of probiotic bacteria in the large intestine (Hotchkiss, 2003 and Manderson *et al.*, 2005).

Orange peel has nearly the same amount of vitamin C as the flesh. However, little work has been done on antibacterial properties of orange peel against antibiotic resistant human pathogens so far. Orange peel based probiotic juice can be a healthy way to increase vitamin C intake, thus improving overall health in regard to increased immunity to cold and flu as well as aiding digestion and preventing food-related illnesses. This juice can also be useful to inhibit pathogens causing gastrointestinal and urogenital diseases as the probiotic bacteria produces antimicrobial substance during fermentation. Antibacterial activity of orange peel against multidrug resistant *Echerichia coli*, *Staphylococcus aureus* and *Streptococcus agalactiae* have already been tested by the authors (Pradhan *et al.*, 2008).

The present study was set out to develop fermented non-dairy beverages containing probiotic bacteria and orange peel with and without inulin.

Materials and methods

Probiotic *Lactobacillus* strains and media

Probiotic bacterial strains used in the study included *Lactobacillus fermentum*, *L. plantarum*, *L. acidophilus*, *L. rhamnosus* and *L. gasseri* which were isolated from yoghurts, stools and vaginal samples. These strains were maintained as a frozen stock at -20 °C in Man Rogosa Sharpe (MRS) broth plus 20% (v/v) glycerol and propagated twice in MRS broth at 30 °C before use.

Test organisms

Multi-drug resistant gram negative bacteria (*E. coli*) and gram positive bacteria *S. aureus* and *S. agalctiae*) were used as test organisms. These strains

were isolated from urine, vaginal swabs and stool samples from patients with urinary tract infections, bacterial vaginosis and diarrhoeal diseases respectively and these strains were identified at microbiological laboratory of AMIFEM- Centre for Applied Microbiology and Biotechnology, Bhubaneswar, Orissa, India.

Lactic acid fermentation set up

Four kilograms of oranges were bought from market and these were peeled off. Then 100 g grinded orange peel were poured into 1000 ml conical flask containing 500 ml of 95% ethanol (1:5). All the flasks were kept at 30 °C for 24 h. Then 0.15% of pectinase (Sigma) was added into each flask and kept for 24 h at 30 °C. The extracts were boiled at 100 °C for 15-30 min. The juice was extracted by pressing the orange peel extracts by cheese cotton cloth. After that ethanolic extracts were diluted with distilled water to make the final volume 600 ml. An inoculum (35 ml) of starter culture was inoculated into 600 ml of prepared extract and then potassium metabisulphite was added (60 mg/60 ml of extract). The conical flask containing orange peel and water without enzymatic treatment served as control. Fermentation of orange peel extracts and *Lactobacillus* cultures with 2% sterilized inulin were also prepared. Orange peel extracts were allowed to ferment for 2 days at 30 °C. After 2 days the fermented juice was poured into the sterile glass bottles using a filter and funnel. The bottles were filled up in the mouth so that there is no air left out.

Biochemical analysis of fermented products

Fermented products were tested for several physical (pH) and biochemical constituents such as total sugar, reducing sugar and titratable acidity. All the fermented products were taken into consideration for pH analysis. pH of the fermented product was detected by portable digital pH meter (Hanna Instruments). Titratable acidity was determined by phenolphthalein indicator and then calculated as stated in A.O.A.C (1980). Total sugar and reducing sugar of fermented products were estimated by methods given by Dubois *et al.*, (1956) and Somoyogi (1952) respectively.

Microbiological analysis of fermented products

The enumeration of *Lactobacillus* from fermented product was done by using MRS medium. Briefly, the homogenized fermented product was serially diluted and 0.1 ml of 10^4 , 10^5 and 10^6 dilutions were transferred into sterile Petri dishes. About 18-20 ml of sterile MRS agar was poured on the Petri dishes. The dishes were allowed to set and were incubated under aerobic environment at 37 °C for 48 h. Thereafter, colony forming unit per millilitre was calculated at 10^6 dilution.

For detection of antagonistic activities, well diffusion assay method was used. An overnight culture of the indicator strains (*E. coli*, *S. aureus* and *S. agalactiae*) were used to pre-inoculated nutrient agar media. Wells of 5 mm diam were cut into pre-inoculated agar plates and 100 µl of fermented product containing antibacterial activity were added to each well. Once solidified, the dishes were stored for 2 h in a refrigerator. The inoculated plates were incubated for 24 h at 37 °C and the diameter of the inhibition zone was measured.

Results and discussion

Biochemical analysis of fermented product

The rapid decrease of the pH values of fermented products during lactic acid fermentation is essential

for their microbiological stability. In the present study the initial pH of orange peel medium was measured as 5.0. pH values decreased after 48 h of fermentation and the values are represented in Table-1. The difference in pH values of fermented products with and without inulin were not so significant (Table-1). pH of fermented products with inulin after 48 h were found be high in *L. plantarum* (3.4), followed by *L. fermentum* (3.5), *L. acidophilus* (3.7), *L. gasseri* (3.8) and *L. rhamnosus* (4.0), which indicates that the probiotic inulin has accelerated effect on the acidification. Numerous authors have indicated the pH value 4.2 as being a parameter that guaranteed a shelf life of the fermented juices (Buruleanu *et al.*, 2009). It seems that the addition of polysaccharide was exerted a positive influence on the time to reach pH 4.2 in comparison with the control (without inulin); pH values decreased due to acid production.

Fermentation of hexoses occurs in the genus *Lactobacillus* resulting acids without evolution of CO₂. Total titratable acid in fermented products with and without inulin is represented in Table-1. After 48 h of fermentation amount of titratable acid produced in orange peel fermentation without inulin was found to be less than in fermentation products with inulin. Addition of inulin results in production of more acid as high amount of metabolizable sugar is available in the fermentation medium (Buruleanu *et al.*, 2009).

Table-1

Changes in pH, titratable acidity (g/l), total sugar (g/l) and reducing sugar (g/l) after 48 h of orange peel fermentation with Op+I or without inulin (Op)

Parameter	Samples	Value at 0 h	After 48 h				
			Lb1	Lb2	Lb6	Lb7	Lb11
pH	Op	5	3.8	3.7	4.1	4.3	4.2
	Op + I	5	3.5	3.4	3.7	3.8	4
Titratable acidity	Op	NS	7.1	8.9	5.4	3.7	1.8
	Op + I	NS	12.7	14.5	9.5	7.2	5.4
Total sugar	Op	32	22	28	22.5	23.5	23.8
	Op + I	34	24.5	26.8	25	25.8	26
Reducing sugar	Op	12	13	13.2	12.8	12.5	10.2
	Op + I	12	17	18	17.4	18.2	18.5

Op - Orange peel, I - Inulin, Lb1 - *L. fermentum*, Lb2 - *L. plantarum*, Lb6 - *L. acidophilus*, Lb7 - *L. rhamnosus*, Lb11 - *L. gasseri*.

But *L. rhamnosus* and *L. gasseri* produced less amount of titratable acid than of other strains. The reason for the low production of acid may be due to slow fermentation capacity of these strains in orange peel substrate. Yoon *et al.* (2006) reported a similar increase in lactic acid concentration and simultaneous decrease in pH during production of fermented plant beverages by lactic acid bacteria. The increase in concentration of titratable acidity was due to bioconversion of metabolizable sugar and finally to lactic acid which resulted in further decrease in sugar concentration in the fermented medium (Tamang *et al.*, 2005). Hence, titratable acidity was increased after addition of inulin to 12.7, 14.5, 9.5, 7.2 and 5.4 g/l in *L. fermentum*, *L. plantarum*, *L. acidophilus*, *L. rhamnosus* and *L. gasseri* respectively. In the present study it was observed that *L. plantarum* produced high amount of lactic acid as *Lactobacillus plantarum* can produce extracellular polygalacturonase (pectinase) which dissolve pectin and releases glucose, which is utilized by the *lactobacillus* strains to produce lactic acid (Sakellaris *et al.*, 1989).

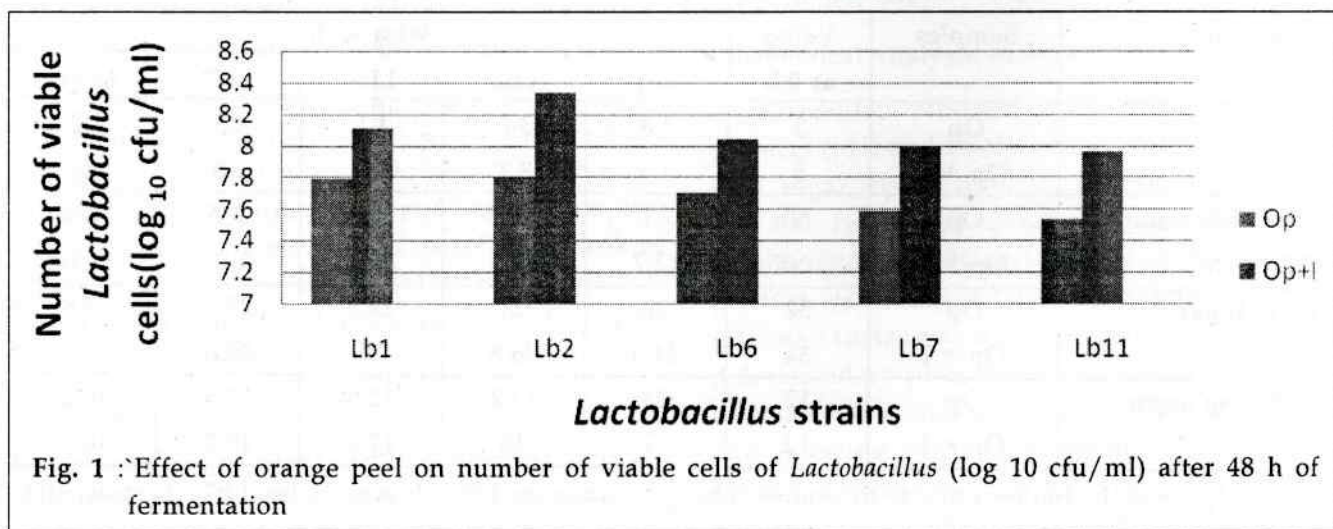
Enzymatic hydrolysis of orange peel resulted in high amount of total sugar (Table-1). Enzymatic hydrolysis of orange peel have been studied by some scientists. Telebina *et al.*, (2008) observed the enzymatic hydrolysis of orange peel released 72% of total solid containing glucose, fructose, arabinose, galactose and galactouronic acid. The same result was obtained by enzymatic hydrolysis of argetina orange peel (Pourbrafrani *et al.*, 2007). Because inulin

consists of linear chains of 2,1-linked D-fructofuranose molecules terminated at the reducing end by a glucose residue attached through a sucrose-type linkage, the addition of this probiotic on juices influenced the amount of their reducing sugars during fermentation. After 48 h of fermentation total sugar concentration decreased in both the cases as sugar is utilized to produce organic acids. Reducing sugar present in the medium before fermentation was measured to be 12 g/l. The reducing sugar value after 48 h is shown in Table-1.

L. plantarum utilized the soluble sugar and also degrade pectin to release reducing sugars such as glucose, which is attributed to the high amount of reducing sugar in the medium. From Table-1 it is clearly seen that reducing sugar in fermented products produced by *Lactobacillus* strains like *L. fermentum*, *L. acidophilus*, *L. rhamnosus* and *L. gasseri* in presence of inulin were found to be 17, 17.4, 18.2 and 18.5 g/l respectively.

Growth of *Lactobacillus* in fermented products

Number of viable cells survived during fermentation is given in Fig. 1. After 48 h number of *Lactobacillus* was found to be more in fermented products containing inulin than the control. Among the *Lactobacillus* strains used, *L. plantarum* was observed to grow well indicating their survival capacity at low pH. Francesca *et al.* (2006) studied the survival capacity of probiotic strains of *L. plantarum* and *L. paracasei* in the brine containing



artichokes, which is a potential source of probiotic. They observed no significant reduction in the bacterial population.

Presence of inulin in the medium increased the number of viable *Lactobacillus*. Similar results were obtained by Cebeci and Gurakan (2003) who demonstrated that *in vitro* batch culture experiments with *Lactobacilli*, were able to grow well in culture medium with inulin as the single source of carbon. *L. plantarum* population was found to be highest than the other strains.

Antibacterial activity of fermented products

In vitro antibacterial activity of fermented product against resistant bacteria is represented in Table-2. All the samples (with and without inulin) were observed to be effective against the test organisms. It has been established that factors contributing to microbial inhibition by fermentation products are pH and anti-microbial substances such as organic acids, hydrogen peroxide, bacteriocin and diacetyl produced by *Lactobacillus* strains. Bioactive compounds of plant extracts is also an important factor for the antibacterial activity of fermented plant beverages. Kantachote *et al.* (2008) reported that the antibacterial activity of plant beverages caused increased inhibition

to gram positive and gram negative bacteria. In our previous study we found that orange peel inhibited all the test organism (Pradhan *et al.*, 2008). All the *Lactobacillus* strains used in the study with orange peel showed inhibition zone against the multi-drug pathogens. It was due to the presences of bioactive compounds derived from orange peel present in fermented products. Hence, it can be said that orange peel fermented product contains bioactive compounds derived from plant source and also the antimicrobial agents released from fermentation of *Lactobacillus* strains. Presence of inulin did not show any significant difference in the size of the inhibition zone.

The present study revealed the antibacterial activity of orange peel against multi-drug resistant *E. coli*, *S. aureus* and *S. agalactiae*. These results indicate that orange peel extract with inulin can be a suitable substrate for producing fermented health drink 'fortified' with selected probiotic *Lactobacillus* strains and act as a food vector by sustaining adequate population of viable bacteria, which is an essential factor in the formulation of a nondairy fermented plant beverages. Orange peel with probiotic bacteria can be a healthy beverage that can help cure diseases like diarrhoea, urinary tract infection and bacterial vaginosis. However, more extensive research on

Table-2
Antibacterial activity of fermented products against test organisms

Fermented products	Zone of inhibition against test organisms (mm)						
	E1	E2	E3	S1	S2	S3	GBS
Op+Lb1	31	32	31	16	15	16	13
Op+Lb2	30	31	30	16	17	14	18
Op+Lb6	31	30	31	15	18	14	13
Op+Lb7	29	32	29	18	17	18	13
Op+Lb11	31	32	31	17	12	12	13
Op+Lb1+I	26	30	32	17	17	14	13
Op+Lb2+I	31	34	29	17	16	13	18
Op+Lb6+I	27	25	30	13	13	13	13
Op+Lb7+I	29	22	24	13	12	14	13
Op+Lb11+I	30	26	20	14	11	13	13

E1, E2, E3 are *E. coli* and S1, S2 and S3 are *S. aureus* isolated from urine, vagina and stool samples respectively.
GBS - *S. agalactiae* (Group B *Streptococcus*)

orange peel with probiotic bacteria needs to be done to confirm the health benefits of orange peel based fermented plant beverages.

References

- A.O.A.C. (1980). Official Methods of Analysis, 13th Edn. Association of Official Analytical Chemists. Washington, D.C., U.S.A. pp. 376-384.
- Adams, M.R. and Moss, M.O. (2000). Food Microbiology, 2nd Edn. Royal Society of Chemistry, University of Surrey, Guildfield, UK.
- Battcock, M. and Azam-Ali, A. (1998). Fermented fruits and vegetables: A global prospective. Food and Agriculture Organization of the United Nations Rome. FAO Agric. Serv. Bull. 14: 130-134.
- Buruleanu, L., Manea, I., Gabriela, M., Avram, D. and Nicolescu, L. (2009). Effects of prebiotics on the quality of lactic acid fermented vegetable juices. Ovidius Univ. Ann. Chem. 20: 102-107.
- Cebeci, A and Gurakan, C. (2003). Properties of potential probiotic *Lactobacillus plantarum* strains. Food Microbiol. 20: 511-518.
- Champagne, C.P., Roy, D. and Gardner, N.J. (2005). Challenges in the addition of probiotic cultures to foods. Crit. Rev. Food Sci. Nutr, 45: 61-84.
- Dubois, M., Gilles, K.A.; Hamilton, J.K.; Rebers, P.A. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350-358.
- Francesca, V., Palmira, D.B., Stella, L.L., Lorenzo, M., Angelo, V. and Paola, L. (2006). *In vitro* and *In vivo* survival and transit tolerance of potentially probiotic strains carried by artichokes in the gastrointestinal tract. Appl. Env. Microbiol. 72: 3042-3045.
- Hotchkiss, A.T. (2003). Pectin carries probiotic properties. Nutra ingredients.com.
- Jay, J.M. (2000). Modern Food Microbiology. 6th Edn. Aspen Publishers Inc., Maryland, U.S.A.
- Kantachote, D., Charenjiratrakul, W and Aussawaringpipop, N. (2005). Characteristics of fermented plant beverages in southern Thailand. Songklanakarin J. Sci. Technol. 27: 601-615.
- Manderson, K., Pinnart, M., Tuohy, K.M., Grace, W.E., Hoychkiss, A.T., Widemer, W., Yadav, P., Gibson, G.R. and Rastall, R.A. (2005). *In Vitro* Determination of Prebiotic Properties of Oligosaccharides Derived from an Orange Juice Manufacturing By-Product Stream. Appl. Environ. Microbiol. 71: 8383-8389.
- Oyetayo, V.O., Adneyi, F.C. and Akinyosoye, F.A. (2003). Safety and protective effect of *Lactobacillus acidophilus* and *Lactobacillus casei* used as probiotic agents *in vivo*. Afr. J. Biotech. 2: 448-452.
- Pourbrafrani, M. and Telebina F. (2007). Protective effect of encapsulation in limonene contained media and orange peel hydrolyzate. Int. J. Mol. Sci. 2: 777-787.
- Pradhan, P., Mohanty, R.C. and Mishra, A. (2008). Prospects of control of multidrug resistant pathogenic strains of *E.coli* and *Staphylococcus aureus* through edible plant extracts. Plant Sci. Res. 30: 103-111.
- Ray, B. (2003). Fundamental Microbiology. 3rd Edn. CRC Press, Florida, U.S.A.
- Savado, A. and Traore, A.S. (2004). Antimicrobial activities of lactic acid bacteria strains isolated from Burkina Faso fermented milk. Pak. J. Nutr. 3: 174-179.
- Sakellaris, G., Nikolaropoulos, S. and Evangelopoulos, A.E. (1989). Purification and characterization of an extracellular polygalacturonase from *Lactobacillus plantarum* strain BA 11. J. Appl. Microbiol. 67: 77-85.
- Somogyi, M. (1952). Notes on sugar determination. J. Biol. Chem. 195: 19-23.
- Tamang, J.P., Tamang, B., Schillinger, U., Franz, C.M., Gores, M. and Holzapfel, W.H. (2005). Identification of predominant lactic acid bacteria isolated from traditionally fermented vegetable products of the Eastern Himalayas. Int. J. Food Microbiol. 105: 347-356.
- Talebina, F., Pourbrafrani, M., Lundin, M., Taherzadeh, M.J. (2008). Optimization study of Citrus waste saccharification by dilute-acid hydrolysis, Bioresources. 3: 108-122.
- Yoon, K.Y., Woodams, E.E. and Hang, Y.D. (2006). Production of probiotic cabbage juice by lactic acid bacteria. Bioresour. Technol. 97: 1427-1430.

Influence of seasonal variation and HCH pre-treatment on survival and longevity of HCH degrading aerobic bacteria in soil

S.K. Sahu^{1*}, J. Chakrabarty², S. Panda¹, L. Choudhury¹, S. Biswas¹, N.K. Mohapatra¹
and N. Sethunathan³

¹Department of Botany, Christ College, Cuttack-753008

²Department of Botany, Cotton College, Guwahati

³Plot No. 103, S. V Officer's Colony, Ramakrishnapuram, Secunderabad-500056

(Received : 19 November, 2010; Revision received : 18 December, 2010).

Soil suspension from HCH-treated plots (unplanted or planted with sugarcane) from Ravenshaw Campus caused a rapid degradation of γ -HCH in a mineral salts medium under aerobic conditions. The HCH degrading bacteria in treated plots were influenced by temperature according to seasonal variation of the year. Thus aerobic bacteria were enriched in the soil after single treatment of commercial HCH from month of July to January of the subsequent year. The rate of degradation of γ -HCH became slower in February to April, and consequently the ability to degrade γ -HCH was completely lost during May, June, July and onwards. Temperature affected the degradation of γ -HCH by the HCH treated sugarcane rhizosphere soil. Loss of HCH degrading bacteria at 40 °C suggested that the elimination of these bacteria from HCH treated soil during summer (May - June) was due to higher atmospheric temperature beyond 40 °C and can recur only during rainy season in July - August after receding of the atmospheric temperature and re-treatment of HCH.

Key words: Sugarcane rhizosphere soil, γ -HCH degrading bacteria, seasonal impact

Introduction

Hexachlorocyclohexane (HCH) or benzene-hexachloride (BHC) in commercial form known as Gammaxine, is a broad spectrum insecticide widely used in agriculture. Domestic and public health accounts for nearly 47% of total pesticides used in India (David, 1992). This insecticide contained mainly four isomers namely α -, β -, γ - and δ - in the commercial formulations. Gamma isomer (γ -HCH) has the target insecticidal property presently available as Lindane, whereas β - and δ - being more thermodynamically persistent isomers unlike earlier practice are eliminated during manufacture of commercial BHC.

Persistence of HCH in upland soil has been remarkably well established (Lichenstein and Polivka, 1959). About 41% was still recovered even after 11 years of application. But it undergoes moderately rapid degradation in predominantly flooded soil and other anaerobic ecosystems (Raghu and MacRae, 1966; Sethunathan *et al.*, 1983). Anaerobic bacteria, obligate

or facultative, have been implicated in degradation of HCH isomers, α -, γ - in particular, in anaerobic soil environment (MacRae *et al.*, 1969; Jagnow *et al.*, 1977; Heritage and MacRae, 1977a; 1977b; 1979). Aerobic bio-mineralization of α -HCH, (Bachmann *et al.*, 1988a; 1988b) and γ -HCH, (Wada *et al.*, 1989) has been reported in acclimatized soil environment. A *Pseudomonas paucimobilis* isolated from upland rice field after repeated application of γ -HCH, readily degraded γ -HCH under aerobic conditions (Senoo and Wada, 1989; 1990a; 1990b). A *Pseudomonas* sp. isolated from γ -HCH treated sugarcane rhizosphere soil rapidly degraded not only α - and γ - but also thermodynamically more stable β - and δ - isomers of HCH in a mineral salts medium as sole source of carbon under aerobic conditions (Sahu *et al.*, 1990b; 1992).

Accumulation of transitory metabolites during degradation of all the isomers by *Pseudomonas* sp., and eventual stoichiometric release of covalently linked chlorine as chloride, suggesting complete metabolism of HCH isomers by the bacterium. The

* Corresponding author; E-mail : sahu.siddhartha.k@gmail.com

metabolites were identified to be PCH for α -, γ - and δ - isomers of HCH respectively. β -HCH showed intermediate metabolite as β -Pentachlorocyclohexanol and β -tetrachlorocyclohexane diol (Sahu *et al.*, 1995). In a complex soil environment α - and γ - HCH were degraded by the *Pseudomonas* sp., but β -HCH showed recalcitrance in soil (Sahu *et al.*, 1993).

Degradation of otherwise persistent β - and δ -HCH was enhanced several folds in soil in presence of α - and γ -HCH by a *Pseudomonas aeruginosa* ITRC-5 (Kumar *et al.*, 2005). Mineralization of HCH isomers occurred more frequently under oxic than anoxic system and in soil at moderate temperature, near neutral pH, where soil texture and organic matter influence degradation by sorption mechanism (Phillips *et al.*, 2005). Degradation of HCH isomers in liquid and soil slurry by *Pandora* sp. was demonstrated (Siddique *et al.*, 2007), where 51-60% γ -HCH was degraded in pH 8.0 - 9.0 and in optimum temperature 30 °C after 4 weeks of incubation. Lin A and Lin B genes of dehydrochlorinase and hydrolytic dechlorinase enzymes respectively play key role in degradation of isomers of HCH (Lal *et al.*, 2010). In the last decade several efforts have been taken to isolate bacteria and characterize Lin genes responsible for degradation of lindane (HCH) (Kumari *et al.*, 2002 and Nagata *et al.*, 2007), but regarding longevity of HCH degrading bacteria in soil, associated with seasonal temperature not much work has been done. In present study we tried to find out the effect of environmental temperature and longevity of HCH degrading consortium in soil.

Materials and methods

Commercial dust formulations of HCH containing 50% active ingredient (a.i.) used for soil application and treating sugarcane setts was obtained from Das Enterprise, Kolkata. Authentic technical γ -HCH, 99.1% purity used in studying degradation by soil suspension and bacterial culture was obtained from Lachat Chemicals, Mequon, Wisconsin, USA.

The characteristics of Ravenshaw soil used in this experiment was loamy texture, pH 7.1; Clay 7.06%, silt 4.3%, sand 88.64% (Bouyoucos hydrometric method), organic matter 0.91% (Walkey-Black

method), total nitrogen, 0.29% (Kjeldahl method), Cation Exchange Capacity 11.2 m.e./100 g soil (ammonium acetate method).

In order to determine whether the exceptionally rapid degradation of HCH by sugarcane rhizosphere soil suspension under aerobic conditions (Sahu *et al.*, 1990a), was induced by prior treatment of sugarcane setts with HCH, this experiment was conducted. Experimental plots (1 sq. m), were planted with sugarcane under non-flooded condition. The commercial dust formulations of HCH was applied to the planted and unplanted soil in duplicate plots at 2 g a.i./sq.m. After 15 days of HCH application, 2 g soil from HCH-treated and untreated plots were shaken for 1 h with 100 ml of sterile distilled water in 250 ml Erlenmeyer flasks to make the soil suspension. To determine the ability of this soil suspension to degrade γ -HCH, 10 ml volume of aqueous mineral salts medium [(NH₄)₂HPO₄, 0.5 g; MgSO₄ · 7 H₂O, 0.2 g; FeSO₄ · 7 H₂O, 0.001g; K₂HPO₄, 0.1 g; Ca(NO₃)₂, 0.01g and distilled water 1 liter, pH 7] was equilibrated with technical γ -HCH (c.a. 7 µg/ml) for 24 h and then sterilized by filtration through a Millipore filter (0.3 µm); were inoculated with 0.1 ml of soil suspension from HCH treated and untreated plots (unplanted and planted). The uninoculated medium served as control. At 0, 5 and 10 days after incubation at room temperature (30 ± 2°C) under aerobic conditions, 1 ml portions of the inoculated and un-inoculated medium were withdrawn from duplicate flasks under aseptic condition and analyzed for γ -HCH by gas liquid chromatography (GLC) after extraction with hexane and 100 mg of sodium sulphate.

A follow up experiment was carried out to test the longevity of HCH degrading factor developed in HCH treated soil unplanted and planted with sugarcane. A commercial formulation of HCH (2g a.i.) was applied to plots of 1 sq. m with and without sugarcane plants in the month of July. Soil samples were collected from HCH treated and untreated plots (unplanted and planted with sugarcane) at monthly intervals from August to July next year. Two gram soil shaken with 100 ml of sterile distilled water from duplicate plots of each treatment. One ml soil suspension was tested for their ability to degrade

γ -HCH added to the 10 ml mineral salts medium in duplicate flasks as described in previous experiment. Un-inoculated ones served as control. At 0, 5 and 10 days after incubation at room temperature, one ml aliquots were withdrawn from each of duplicate flasks and analyzed for γ -HCH by GLC after extraction with hexane and 100 mg of sodium sulphate.

Effect of temperature on degradation of γ -HCH in a mineral salt medium as sole source of carbon was tested. The soil suspension (0.1ml) from HCH treated plots were inoculated in 10 ml sterile mineral salts medium with 6-8 $\mu\text{g ml}^{-1}$ technical γ -HCH and incubated in BOD incubators maintained at different temperatures (10 - 40 °C). At 0, 5 and 10 days intervals one ml sample from un-inoculated and flasks inoculated with soil suspension from treated plots were withdrawn from duplicate flasks and analyzed for γ -HCH by GLC after extraction in hexane fraction.

Gas liquid chromatography

The hexane layer after appropriate dilution, was injected into a gas chromatograph (Perkin Elmer model 3920) equipped with a ^{60}Ni detector and a glass column (6.25 mm o.d.; 2m length) packed with 5% QF-1 Chromosorb W, 60/80 mesh. The column, injector and detector were maintained at 180 °, 210 ° and 250 °C respectively with a flow rate of carrier gas (95% argon in methane) at 13 ml/min. Under these conditions the retention time was 2.42 min for γ -HCH. The recovery of γ -HCH by the extraction analytical procedures used in this study ranged from 95 to 100% from mineral salts medium. All the data of this study are based on duplicate estimations.

Results and discussion

Development of HCH degrading factor in plots by treatment of commercial HCH

Degradation of γ -HCH by soil suspension from HCH treated and untreated plots with and without sugarcane plants was studied. Exceptionally rapid degradation of α - and γ -HCH by sugarcane rhizosphere soil suspension under aerobic conditions as noted in earlier studies (Sahu *et al.*, 1990a) was of great interest in view of the reported recalcitrance of HCH isomers in aerobic environment. It must be

mentioned here that sugarcane plants used in the earlier experiments for preparation of rhizosphere soil suspension were raised from sugarcane setts those had been treated with commercial HCH formulations. It is a usual practice to treat the sugarcane setts with HCH before planting to prevent the attack of termites and other common pests. It was, therefore, not clear whether the HCH degrading factor (bacteria) developed in response to presence of HCH used for treatment of sugarcane setts.

Plots planted with sugarcane and unplanted plots were treated with the commercial formulation of HCH @ 2g a.i./m². After 15 days of HCH application mineral salts medium containing γ -HCH as a sole source of carbon was inoculated with suspension of untreated and HCH treated soil samples from planted and unplanted plots as described in methods. Interestingly, γ -HCH disappeared completely within 5 days after inoculation of the medium with soil suspensions from HCH treated plots with or without sugarcane plants (Table-1. Similar trend of result from July to next year January represented by September data). During that period loss of γ -HCH from un-inoculated medium or from the medium inoculated with soil suspensions (planted and unplanted) from untreated plots was negligible. The HCH degrading bacteria developed only in soil (irrespective of the presence or absence of sugarcane plants) treated with HCH and not in untreated soils. Evidently development of HCH degrading factor was not governed by presence of sugarcane plants, since it developed in both planted and unplanted soil, but only in response to HCH application. According to the data of this study, (treatment of HCH in sugarcane and Bhuyan *et al.*, 1992), with treatment of HCH in flooded and non-flooded plots probably led to the development of an enrichment of aerobically mediated HCH degrading bacteria.

It may be mentioned here that rice rhizosphere soil used in earlier experiments (Sahu *et al.*, 1990a) was collected from lowland rice fields not treated with HCH. Later it was observed that rhizosphere soil collected from low land rice fields (Bhuyan *et al.*, 1992), after two applications of HCH, effected complete disappearance of γ -HCH under aerobic

conditions. Wada *et al.* (1989) have reported accelerated degradation of γ -HCH from an upland rice field after multiple application of HCH. In the present study γ -HCH degraded in a mineral salts medium within 5 days of inoculation with rhizosphere soil suspension from treated and untreated plots after single application of commercial BHC. There was no appreciable loss of γ -HCH even after 10 days in mineral salts medium inoculated with rhizosphere soil from untreated rice fields. Evidently aerobically mediated HCH degrading factor developed also in low land rice fields, as well as in sugarcane fields or in an unplanted plot following application of HCH. A flooded soil is predominantly anaerobic, but a flooded soil planted to rice need not be as anaerobic as a flooded unplanted soil, because of the capacity of rice plants to transport molecular oxygen from the foliage to the roots (Van Raalt, 1941; Barber *et al.*, 1962).

Longevity of HCH degrading factor developed in HCH treated soil

Previous experiments using soil samples collected at 15 days after HCH application showed distinct development of HCH degrading factor in planted (to sugarcane) and unplanted soil treated with HCH. It is important to determine how long this HCH degrading factor lasted in the soil after HCH application. In this experiment HCH was applied in

plots planted with sugarcane and unplanted soil in the month of July and soil samples collected at monthly intervals from August to subsequent year August were tested for their ability to degrade γ -HCH in a mineral salts medium.

Data in Table-1 shows that soil sample collected from HCH treated plots (both unplanted and planted to sugarcane) at monthly intervals till January next year (Table-1) exhibited an exceptional capacity to degrade all the added γ -HCH in a mineral salts medium within 5 days after inoculation. Soil samples collected from HCH treated plots in February, March and April (represented by April data, Table-1) were also effective in degrading γ -HCH, but only 50% of γ -HCH added to mineral salts medium was degraded in 5 days followed by complete disappearance in 10 days. However, soil samples collected from HCH treated plots in May, June and July (represented by June data, Table-1), did not show any γ -HCH degrading ability. In experiments conducted in first week of all the months throughout the year (Table-2), 100% degradation of γ -HCH occurred within 5 to 10 days period by the soil suspensions from HCH treated plots, irrespective of unplanted or planted to sugarcane. It is evident from unpublished data that there was no degradation of γ -HCH in uninoculated medium or in the medium inoculated with soil suspension from plots (planted or unplanted)

Table-1

Degradation of γ -hexachlorocyclohexane (γ -HCH) in mineral salts medium inoculated with soil samples collected from HCH treated and untreated plots with and without sugarcane at monthly intervals from August to next July

Month	Days	Untreated			Treated	
		Un-inoculated	Unplanted	Planted	Unplanted	Planted
September	0	7.8	7.6	7.7	7.8	7.7
	5	7.0	7.3	6.4	0	0
	10	6.6	6.7	6.2	0	0
April	0	8.0	7.8	8.0	7.7	8.4
	5	7.6	7.5	7.6	3.9	4.1
	10	6.4	6.3	6.6	0	0
June	0	8.14	7.9	7.9	8.0	8.0
	5	7.2	7.0	7.1	6.8	6.7
	10	6.0	5.6	6.3	5.4	6.1

not treated with HCH. This suggested that the γ -HCH degrading factor (bacteria) developed even after one application of HCH in August survived for 8 to 9 months (until April next year) in HCH treated planted and unplanted plots.

Effect of temperature on survival and longevity of HCH degrading factor

Results similar to the above field experiment could be verified in laboratory in separate incubators under different temperatures. Degradation of γ -HCH by soil suspensions from HCH treated plots was affected by different temperatures (Table-3). Lower temperature (10 °C) affected the degradation of γ -HCH in slower rate. At 20 °C γ -HCH declined to 50% in 5 days and up to 20% after 10 days. Most ideal temperature was found to be 30 °C, when γ -

HCH disappeared completely in 5 days. The rate of degradation of γ -HCH; however, increased with increase in temperature from 10 - 30 °C. But further rise in the temperature appeared to slow down the degradation, while simultaneously accelerating the volatilization loss. Similar trend of results as in the above field experiment could be further confirmed in this laboratory experiment. Thus when minimum diurnal variation of temperature was up to 20 to 35 °C during February to April, gradually the population of HCH degrading bacteria started declining, but still were enough to degrade γ -HCH in mineral medium using as sole source of carbon within 10 days. Interestingly during May, June and July when the diurnal variation of temperature increased from 30 to 40 °C or even 45 °C, population of HCH degrading microorganisms were totally

Table-2.

Degradation of γ -hexachlorocyclohexane (γ -HCH) in a mineral salts medium inoculated with a suspension of soil samples collected from HCH-treated^b plots (planted with and without sugarcane) at monthly intervals

Soil sample : % of γ -HCH recovered^a

Month of sample collection	5 days		10 days	
	Un-planted treated	Planted treated	Un-planted treated	Planted treated
August	02	01	0	0
September	0	0	1	0
October	0	22	0	0
November	0	0	0	0
December	0	0	0	0
January	0	31	0	0
February	23	38	0	0
March	02	38	0	0
April	49	51	0	0
May	91	87	80	74
June	85	85	66	76
July	87	93	78	84

^a γ -HCH was introduced to the mineral medium in aqueous solution

^b Commercial formulation of HCH was applied to the duplicate plots unplanted or planted to sugarcane at 2 g active ingredient/sq. m plots

^c Soil samples were collected from duplicate plots with and without sugarcane at monthly intervals from August and the mineral salts medium was inoculated with soil suspension

There was no loss of γ -HCH from un-inoculated and from medium inoculated with suspension from untreated plots.

Table-3

Hexachlorocyclohexane (γ -HCH) recovered from mineral salts inoculated with sugarcane rhizosphere soil suspension at different temperature under aerobic condition

γ -HCH recovered $\mu\text{g/ml}$ of medium

Incubation	10 °C		20 °C		30 °C		40 °C	
(days)	UI	I	UI	I	UI	I	UI	I
0	7.1 $\pm$ 0.2 ^b	6.8 $\pm$ 0.3	6.8 $\pm$ 0.1	6.8 $\pm$ 0.1	7.0 $\pm$ 0.2	7.0 $\pm$ 0.2	7.1 $\pm$ 0.1	7.1 $\pm$ 0.1
5	7.0 $\pm$ 0.05	6.1 $\pm$ 0.4	6.8 $\pm$ 0.2	3.2 $\pm$ 0.2	6.7 $\pm$ 0.2	0	5.5 $\pm$ 0.4	5.2 $\pm$ 0.5
10	7.0 $\pm$ 0.1	5.5 $\pm$ 0.2	6.8 $\pm$ 0.3	1.2 $\pm$ 0.5	6.8 $\pm$ 0.2	0	5.0 $\pm$ 0.3	4.8 $\pm$ 0.3

^a γ -HCH was incorporated to mineral salts medium in aqueous solution

^b Mean of duplicate estimation $\pm$ Standard deviation.

UI - Uninoculated I- Inoculated with sugarcane rhizosphere soil suspension

eliminated from the soil. Present study shows that the HCH degrading microorganisms in HCH treated soil, later isolated as a *Pseudomonas* sp. (Sahu *et al.*, 1990b) appeared to have inducible thermo-labile enzyme system for degrading HCH isomers.

Acknowledgements

The authors are thankful to the authorities of Ravenshaw College in extending the plots for the experiments to be conducted and to the Director CRRI, Cuttack for the laboratory facilities for conducting analysis in the Laboratory of Microbiology.

References

- Bachmann, A., Walet, P., Wijnen, P., deBruin, W., Huntjens, J.L.M., Roelofsen, W. and Zehnder, A.J.B. (1988a). Biodegradation of alpha and beta-hexachlorocyclohexane in a soil slurry under different redox conditions. *Appl. Env. Microbiol.* **54**: 143-149.
- Bachmann, A., deBruin, W., Jumlet, J.C., Rijnearns, H.H.N. and Zehnder, A.J.B. (1988b). Aerobic bio-mineralization of alpha-hexachlorocyclohexane in contaminated soils. *Appl. Environ. Microbiol.* **54**: 548-554.
- Barber, D.A., Elbert, M. and Evans, N.T.S. (1962). The movement of $^{15}\text{O}_2$ through barley and rice plants. *J. Exp. Bot.* **13**: 397-403.
- Bhuyan, S., Sahu, S.K., Adhya, T.K., Sethunathan, N. (1992). Accelerated aerobic degradation of Hexachlorocyclohexane in suspension of flooded and non-flooded soils pretreated with hexachlorocyclohexane. *Biol. Fert. Soil.* **12**: 279-284.
- David, B.V. (1992). Pest Management and Pesticides. Indian Scenario, Namrutha Publication. Madras, India, 225 p.
- Heritage, A.D. and MacRae, I.C. (1977a). Identification of Intermediate formed during degradation of HCH by *Clostridium sphenoides*. *Appl. Env. Microbiol.* **33**: 1295-1297.
- Heritage, A.D. and MacRae, I.C. (1977b). Degradation of lindane by cell free preparations of *Clostridium sphenoides*. *Appl. Env. Microbiol.* **34**: 222-224.
- Heritage, A.D. and MacRae, I.C. (1979). Degradation of hexachlorocyclohexane and structurally related substances by *Clostridium sphenoides*. *Aust. J. Biol. Sci.* **32**: 493-500.
- Jagnow, G., Haider, K. and Ellwardt, P.C. (1977). Anaerobic dechlorination and degradation of hexachlorocyclohexane by anaerobic and facultative anaerobic bacteria. *Arch. Microbiol.* **115**: 285-292.
- Kumar, M., Choudhary, P., Dwivedi, M., Kumar, R., Paul, D., Jain, R.K. and Garg, S.K. (2005). Enhanced biodegradation of β - and δ -hexachlorocyclohexane in the presence of α - and γ -isomers in contaminated soils. *Env. Sci. Technol.* **39**(1): 4005-4011.
- Kumari, R., Subudhi, S., Suar, M., Dhingra, G., Raina, V., Dogra, C., Lal, S., Vander Meer R.J., Holliger, C., and Lal, R. (2002). Cloning and characterization of lin genes responsible for the degradation of hexachlorocyclohexane isomers by *Sphingomonas paucimobilis* strain B90. *Appl. Env. Microbiol.* **68**(12): 6021-6028.
- Lal, R., Pandey, G., Sharma, P., Kumari, K., Malhotra, S., Pandey, R., Raina, V., Kohler, H.E., Holliger, C., Jackson, C. and Oakeshott, J.G. (2010). *ASM Microbiol. Mol. Bio. Rev.* **74**(1): 58-80.

- Lichenstein, E.P. and Polivka, J.B. (1959). Persistence of some chlorinated hydrocarbon insecticides in turf soil. *J. Econ. Entomol.* **52**: 289-293.
- MacRae, I.C., Raghu, K. and Bautista, E.M. (1969). Anaerobic degradation of lindane by *Clostridium* sp. *Nature* **221**: 859-860.
- Nagata, Y., Endo, R., Ito, M., Ohtsubo, Y. and Tsuda M. (2007). Aerobic degradation of lindane (gamma-hexachlorocyclohexane) in bacteria and its biochemical and molecular basis. *Appl. Microbiol. Biotechnol.* **76**(4): 741-752.
- Phillips, T.M., Seech, A.G., Lee, H. and Trevors, J.T. (2005). Biodegradation of hexachlorocyclohexane (HCH) by microorganisms. *Biodegradation*. **16**(4): 363-392.
- Raghu, K. and MacRae, I.C. (1966). Biodegradation of gamma isomers of benzene-hexachloride in submerged soil. *Science* **154**: 263-264.
- Sahu, S.K., Patnaik, K.K., Sethunathan, N. (1990a). Degradation of Alpha- Beta and Gamma- isomers of Hexachlorocyclohexane by Rhizosphere soil Suspension from Sugarcane. *Ind. Acad. Sci. Plant Sci.* **100**: 165-172.
- Sahu, S.K., Patnaik, K.K., Sharmila, M., Sethunathan, N. (1990b). Aerobic Degradation of Alpha- Beta and Gamma- Hexachlorocyclohexane by a soil bacterium. *Appl. and Env. Microbiol.* **56**: 3620-3622.
- Sahu, S.K., Patnaik, K.K., and Sethunathan, N. (1992). Dehydrochlorination of isomers of hexachlorocyclohexane by a soil bacterium *Pseudomonas* sp.. *Bull. Env. Contam. Toxicol.* **48**: 265-268.
- Sahu, S.K., Patnaik, K.K., Bhuyan, S., Sreedharan, Bindu, Kurihara, N., Adhya, T.K. and Sethunathan, N. (1993). Degradation of soil-applied isomers of hexachlorocyclohexane by a soil bacterium *Pseudomonas* sp. *Soil Biol. Biochem.* **25**(3) : 387-391.
- Sahu, S.K., Patnaik, K.K., Bhuyan, S. and Sethunathan, N. (1995). Mineralization of and isomers of hexachlorocyclohexane by a soil bacterium under aerobic conditions. *Agric. Food. Chem.* **43**(3): 833-837.
- Senoo, K. and Wada, H. (1990a). Accurate and sensitive method of enumeration of specific microorganism, *Pseudomonas paucimobilis* Ss-86 in non-sterile soil. *Soil Sci. Plant Nutri.* **36**: 389-395.
- Senoo, K. and Wada, H. (1990b). Fate of a bacterium *Pseudomonas paucimobilis* Ss-86 in upland field. *Soil Sci. and Plant Nutri.* **36**: 593-598.
- Sethunathan, N., Rao, V.R., Adhya, T.K. and Raghu, K. (1983). Microbiology of rice soil *CRC Critical Rev. Microbiol.* **10**: 125-172.
- Siddique, T., Okeke, B.C., Arshad, M. and Frankenberger, Jr. (2002). Temperature and pH effects on biodegradation of hexachlorocyclohexane isomers in water and a soil slurry. *J. Agric. Food Chem.* **50**(18): 5070-5076.
- Van Raalt, M.H. (1941). On the Oxygen supply of rice plants. *Ann. Bot. Gardens Buitanzorg.* **51**: 43-57.
- Wada, H., Senoo, K. and Takai, Y. (1989). Rapid degradation of γ -HCH in upland soil under multiple applications. *Soil Sci. Plant Nutri.* **35**: 71-77.

Effect of soils of varied pH on the growth and N₂ fixation of the water fern *Azolla*

K.B. Satapathy[†]

P.G. Department of Botany, Utkal University, Bhubaneswar-751004

(Received : 12 October, 2010; Revision received : 12 November, 2010)

The pot culture studies revealed that all the three tested *Azolla* species (*A. pinnata*, *A. filiculoides*, *A. mexicana*) grew better in slightly acidic soil of Cuttack (pH 6.5) than in the acidic soil of Bhubaneswar (pH 5.5), or alkaline soil of Hyderabad (pH 8.1), whereas all these species grew very poorly in alkaline-saline soil of Kernal (pH 8.4). Growth of *A. pinnata* and *A. mexicana* was better than that of *A. filiculoides* in the acidic soils of Cuttack and Bhubaneswar, whereas *A. mexicana* grew well in alkaline saline soil of Kernal. There was no significant difference between *Azolla* species with respect to growth in the alkaline soil of Hyderabad. Among the three species, the growth performance of *A. mexicana* was comparatively better in alkaline soil of Hyderabad and alkaline saline soil of Kernal, preferably in the latter, exhibiting its higher tolerance to high pH and high salt concentrations than that of *A. pinnata* and *A. filiculoides*.

Key words: Soil pH, *Azolla* growth, N₂ fixation

Introduction

The importance of biological N₂ fixation using free-living cyanobacteria or in consortium with *Azolla* fern is immensely realized in rice cultivation as biofertilizers. This is so because rice crop is harvested year after year without depleting soil-N despite denial of chemical N fertilizers. Tropical rice fields offer most suitable habitat for the luxuriant growth of a large number of free-living cyanobacteria and *Azolla-Anabaena* complex. *Azolla* has drawn considerable attention for its utilization in agriculture especially in rice cultivation (Becking, 1979; Moore, 1969; Satapathy, 1993, 1995, 1999, 2000; Satapathy and Singh, 1985; Satapathy and Chand, 1984, 1992; Singh 1977, 1979; Watanabe, 1977). It is a small, aquatic leptosporangiate pteridophyte which harbours a heterocystous nitrogen-fixing cyanobacteria viz. *Anabaena azollae* residing inside a cavity in the dorsal lobe of the frond. Although *Azolla-Anabaena* consortium in rice fields has been found to contribute significantly (Kanayian *et al.*, 1982; Satapathy, 1995; Satapathy and Chand, 2008; Satapathy and Singh, 1985; Satapathy *et al.*, 2005; Singh, 1977, 1979), there are serious ecological limitations in achieving the desired growth of native or inoculated *Azolla* species.

In this context the study of the effect of environmental factors especially soil pH on the growth and nitrogen contribution of this water fern is interesting and essential.

This paper addresses to the soil pH influence on the growth, N₂ fixation and the survival potential of *Azolla-Anabaena* consortium in the soil-water system of selected rice soils of varied pH.

Materials and methods

Plant materials and soils

One tropical *Azolla* species (*A. pinnata*) and two temperate species of *Azolla* viz. *A. filiculoides* and *A. mexicana* maintained in the breeding plots of the Biofertilizer Division, Orissa University of Agriculture and Technology (O.U.A.T.), Bhubaneswar were used in the experiment. Soils of different pH (Cuttack - pH 6.5; Bhubaneswar - pH 5.5; alkaline soil of Hyderabad - pH 8.1; alkaline saline soil of Kernal - pH 8.4) were procured from the Division of Soil Science, CRRI, Cuttack.

Pot culture

Earthenware pots of 500 ml capacity with thin polyethylene sheet inner lining were used for this

[†] Corresponding author; E-mail: kbs_bot@rediffmail.com

study. After thorough washing and drying of pots 200g of dried and sieved (2.0 mm mesh) soils of different pH (CRRI, pH 6.2; Bhubaneswar, pH 5.5; Hyderabad, pH 8.1; Kernal pH 8.4) were taken in triplicate. Each pot was then filled with 400 ml demineralised water to maintain 5.0 cm water level above the soil surface. Exponentially grown *Azolla* plants (*A. pinnata*, *A. filiculoides* and *A. mexicana*) were washed repeatedly with distilled water, blotted and fresh inoculums (0.5 g) were cultured separately in each individual pot. Single super phosphate solution at the rate of 8.0 kg P₂O₅/ha on soil basis was sprinkled over the tested plants in two splits in every seven days interval (first at the time of inoculation and second after 7 days of inoculation). After 15 days of incubation its growth, chlorophyll, anthocyanin and nitrogen content were estimated following standard methods. Initial and final pH of the soil was also observed.

Plant analysis

Chlorophyll pigments were extracted with 80% cold acetone and its absorbance measured at 652 nm using Bausch and Lomb Spectronic-20 Spectrophotometer. Anthocyanin pigments were extracted with methanolic 0.01% hydrochloric acid and then pigment absorbance was measured at 500 nm. Total nitrogen was analysed by the modified Kjeldahl's method (Jackson, 1967) and its fixation was computed after estimating the N% and dry matter yield of the fern. The dried plant materials were digested with tri-acids and the aliquots were used for the determination of phosphorus colorimetrically by sulphomolybdate blue-colouration method (Jackson, 1967)

Physico-chemical measurements of water and soil

Hydrogen ion concentration of both water and soil was measured using Elico combined electrode pH meter. Available phosphorous of the soil was estimated by chlorostannous reduced molybdophosphoric blue-colour method in sulfuric acid system (Jackson, 1967). The data were statistically analyzed for critical difference (C.D.) up to 1% level of significance (Gomez and Gomez, 1984).

Results

All the species of *Azolla* were multiplied in pots under green house condition during February, 2005 using different soils of variable pH to study their growth performance. The data (Table-1) showed that after 15 days *A. pinnata* (India) produced significantly higher fresh matter of 3.8 g/pot than *A. filiculoides* and *A. mexicana* which yielded 3.21 and 3.5 g/pot fresh matter respectively in slightly acidic soil of Cuttack. In acidic soil of Bhubaneswar *A. pinnata* (India) and *A. mexicana* (USA) produced statistically higher yields than the *A. filiculoides*. However, there was no significant difference in the fresh matter yield among three species tested in alkaline Hyderabad soil. The alkaline saline soil of Kernal did not support good growth of all the three species. But *A. mexicana* had comparatively higher yield than *A. pinnata* and *A. filiculoides*. Considering the type of soils, the productivity of all the three species of *Azolla* varied and were in the order of Cuttack soil > Bhubaneswar soil > Hyderabad soil > Kernal soil (Table-1). Again among the three species, *A. pinnata* grew comparatively better in Cuttack and Bhubaneswar soil and poorly in Hyderabad and Kernal soil, whereas *A. mexicana* produced more biomass in Hyderabad as well as Kernal soil. Dry matter production also followed similar trend as the fresh matter yield (Table-1). Maximum dry matter yield of 190 and 166 mg/pot/15 d by *A. pinnata* (India) in Cuttack and Bhubaneswar soil were observed whereas *A. filiculoides* recorded minimum of 154 and 144 mg/pot/15 d of dry matter in these two soils respectively. On the other hand *A. mexicana* showed highest dry matter yield of 118 and 80 mg/pot/15 d in Hyderabad and Kernal soil respectively whereas minimum dry matter was gained by *A. pinnata* (India) in these two soils.

Wide fluctuations in the chlorophyll and anthocyanin contents of the *Azolla* species were also noticed in these four types of soils. In Cuttack and Bhubaneswar soil, *A. filiculoides* showed significantly higher chlorophyll content (0.5 and 0.48 O.D.) than the other two species whereas *A. mexicana* contained highest chlorophyll content in the Hyderabad and Kernal soil (Table-1). All the three species produced chlorophyll differently in the four types of soils and

were in the order of Cuttack > Bhubaneswar > Hyderabad > Kernal soil. However the anthocyanin pigments exhibited a reverse order to that of the chlorophyll pigment (Table-1).

The N content of the tested *Azolla* species in the four different soils also followed a similar trend as the biomass production. *A. pinnata* and *A. mexicana* showed significantly higher total nitrogen content of 9.13 and 8.99 mg/pot than *A. filiculoides* (7.85 mg/pot) in Cuttack soil (Table-1). In contrast high nitrogen content (3.92 and 4.45 mg/pot) was observed in case of *A. filiculoides* and *A. mexicana*, respectively in Hyderabad soil and 2.57 mg/pot in case of *A. mexicana* in Kernal soil. However *A. pinnata* (India) showed a minimum N content i.e. 3.17 and 1.39 mg/pot in Hyderabad and Kernal soil, respectively.

Discussion

Among the various soil properties, pH is one of the most important factors that influences growth and development of plants, as availability of most of the essential nutrients would depend upon its pH. The pot culture studies on *Azolla* species showed that the slightly acidic nature of the Cuttack soil (pH 6.5) supported an appreciably good growth while little more acidic soil of Bhubaneswar (pH 5.5) slightly reduced the growth of the fern. The alkaline soil of Hyderabad (pH 8.1) and alkaline-saline soil of Kernal (pH 8.4) failed to support good growth irrespective of the type of *Azolla* species inoculated. This implied that slightly acidic soil pH was favourable for better growth of *Azolla*. Similar findings were reported by Le Van and Sobachkin (1963), Satapathy (1995) and Singh (1977) who concluded from their field as well

Table-1
Effect of soils of different pH on the growth of various species of *Azolla*

Treatment	Fresh matter (g/pot)	Dry matter (mg/pot)	Total N (mg/pot)	Chlorophyll (O.D. ₆₅₂)	Anthocyanin (O.D. ₅₀₀)
CRR I Soil (pH 6.5)					
T ₁ <i>A. pinnata</i> (India)	3.81	190.0	9.13	0.48	0.45
T ₂ <i>A. filiculoides</i> (U.S.A.)	3.21	154.0	7.85	0.5	0.41
T ₃ <i>A. mexicana</i> (U.S.A.)	3.5	182.0	8.99	0.46	0.44
Bhubaneswar soil (pH 5.5)					
T ₄ <i>A. pinnata</i> (India)	3.2	166.0	7.9	0.44	0.48
T ₅ <i>A. filiculoides</i> (U.S.A.)	2.66	144.0	6.31	0.48	0.44
T ₆ <i>A. mexicana</i> (U.S.A.)	2.94	165.0	7.94	0.42	0.49
Hyderabad soil (pH 8.1)					
T ₇ <i>A. pinnata</i> (India)	1.8	99.0	3.17	0.32	0.5
T ₈ <i>A. filiculoides</i> (U.S.A.)	1.95	109.0	3.92	0.41	0.48
T ₉ <i>A. mexicana</i> (U.S.A.)	2.1	118.0	4.45	0.42	0.52
Kernal soil (pH 8.4)					
T ₁₀ <i>A. pinnata</i> (India)	0.8	44.0	1.39	0.28	0.54
T ₁₁ <i>A. filiculoides</i> (U.S.A.)	1.0	55.0	1.71	0.3	0.5
T ₁₂ <i>A. mexicana</i> (U.S.A.)	1.4	80.0	2.57	0.34	0.52
C.D. at 5%	0.296	9.028	0.555	0.034	0.034
1%	0.403	12.267	0.754	0.046	0.046

Azolla inoculation 0.5 g/pot; Incubation period - 15 days; P₂O₅ dose - 8 kg/ha on soil basis (in two splits).

as laboratory studies that a pH range of 5-7 was ideal for producing maximum growth of *A. filiculoides* and *A. pinnata*. Among the three species examined, growth performance of *A. filiculoides* and *A. mexicana* was comparatively better in alkaline soil of Hyderabad and alkaline-saline soil of Kernal respectively, thereby exhibiting their better tolerance to high pH and high salt concentrations than that of *A. pinnata*. A relatively high anthocyanin content was observed in *Azolla* growing in alkaline-saline soils which was due to higher pH and salt concentration. Lumpkin and Plucknett (1980) and Satapathy and Chand (1992) suggested that anthocyanin production could be more due to stress factors including high pH, strong sunlight and P deficiency which might limit photosynthesis. The present study indicates that all the three *Azolla* species had a preference for slightly acidic soil-water environment, while *A. filiculoides* and *A. mexicana* exhibited a comparatively high adaptability to alkaline and saline situations. Soil pH has a selective effect on the distribution and predominance of both indigenous and introduced *Azolla*. This fern generally, has a very wide range of pH tolerance but some of them are acid forms. This shows the existence of variation at inter-specific level. Lumpkin and Plucknet (1980) suggested that salinity factor should be considered wherever the *Azolla* is introduced. It was reported that the growth of *Azolla* ceased at about 1.3% salt (33% of sea water) and higher salinity levels resulted death of the species (Haller *et al.*, 1974). The results from pot culture studies also revealed the suitability of two exotic *Azolla* species namely *A. filiculoides* and *A. mexicana* for their exploitation particularly in soils of higher alkalinity and salinity respectively. Being temperate in nature these two species should be more suitable for utilization in low land rice cultivation during the 'Rabi' season.

Acknowledgement

The author is thankful to the Head, P.G. Department of Botany, Utkal University, Vani Vihar, Bhubaneswar for providing necessary facilities to conduct the experiment.

References

- Becking, J.H. (1979). Environmental requirements of *Azolla* for use in tropical rice production. Nitrogen and Rice. IRRI, Los Banos, Laguna, The Philippines, pp.344-373.
- Gomez, K.A. and Gomez, A.A. (1984). Statistical procedures for Agricultural Research. John Wiley & Sons, New York, U.S.A.
- Haller, W.T., Sutton, D.L. and Barlowe, W.C. (1974). Effects of salinity on the growth of several aquatic macrophytes. *Ecol.* 55: 891-894.
- Jackson, M.L. (1967). Soil Chemical Analysis. Prentice Hall of India, New Delhi, India.
- Kannaiyan, S., Thangaraju, M. and Oblisami, G. (1982). Studies on the multiplication and utilization of *Azolla* biofertilizer for rice crop. In: Thomas, J. (ed.) Proc. Natl. Symp. Biological nitrogen fixation. IARI, New Delhi, India, pp. 451-460.
- Le Van Kan and Sobachkin, A.A. (1963). The problem in the utilization of *Azolla* as green manure in Democratic Republic of Vietnam. In proc. for Timuyazev Moscow Agric. Acad. 94: 93-97.
- Lumpkin, T.A and Plucknett, D.L. (1980). *Azolla* botany, physiology and use as a green manure. *Econ. Bot.* 34(2): 111-153.
- Moore, A.W. (1969). *Azolla* : Biology and agronomical significance. *Bot. Rev.* 35(1): 17-34.
- Satapathy, K.B. (1993). Effect of plant spacing pattern on the growth of *Azolla* and rice. *Indian J. Plant Physiol.* 36(2): 101-105.
- Satapathy, K.B. (1995). Comparative efficiency of *Azolla* and chemical-N fertilizer in increasing rice yield. *Curr. Agric. Res.* 8(2): 49-57.
- Satapathy, K.B. (1999). Comparative efficiency of blue green algae, *Azolla* and other biofertilizers on the growth of rice. *Ind. J. Plant Physiol.* 4(2) : 100-104.
- Satapathy, K.B. (2000). *Azolla-Anabaena* consortium - a potential organic nitrogen source for rice farming. In: Chandra, K. (ed.) Organic Farming in the New Millennium. Regional Bio-fertilizer Development Center, Bhubaneswar, India, pp.65-76.
- Satapathy, K.B. and Chand, P.K. (1984). Studies on the ecology of *Azolla pinnata* R. Br. of Orissa. *J. Ind. Bot. Soc.* 63: 44-52.
- Satapathy, K.B. and Chand, P.K. (1992). Correlation between heterocyst frequency of *Anabaena azollae* and its nitrogen fixation at varied climatic conditions. In: Kausik, B.D. (ed.) Proc. Natl. Symp. Cyanobacterial

Nitrogen Fixation. Associated Publishing Company, New Delhi, India, pp. 325-333.

Satapathy, K.B. and Chand, P.K. (2008). Growth and nitrogen content of symbiont-free water-fern *Azolla* regenerated from *in vitro* culture of frond primordia. *Cytologia*. **73**(4): 445-452.

Satapathy, K.B. and Singh, P.K. (1985). Control of weeds by *Azolla* in rice. *J. Aquat. Plant. Manage.* **23**: 40-42.

Satapathy, K.B., Chand, P.K. and Mohanty, R.C. (2005) Symbiotic cyanobacterium *Anabaena azollae* and organic rice farming. In : Mohanty, R.C. and Chand, P.K (eds.)

Microbes in Our Lives, Post-Graduate Department of Botany, Utkal University, Bhubaneswar, India, pp. 37-59.

Singh, P.K. (1977). Multiplication and utilization of fern 'Azolla' containing nitrogen fixing algal symbiont as green manure in rice cultivation. *RISO*. **26**: 125-136.

Singh, P.K. (1979). Use of *Azolla* in rice production in India. Nitrogen and Rice. IRRI, Los Banos, Laguna, The Philippines, pp.407-418.

Watanabe, I. (1977). *Azolla* utilization in rice culture. *Int. Rice Res. Newslett.* **2**: 3.

Changes in biochemical constituents in betel leaf (*Piper betle* L.) during storage using banana leaves

N.P. Das¹, J.R. Rout², S.L. Sahoo^{2*} and P.K. Mishra³

¹Department of Botany, Stewart Science College, Cuttack-753001

²Biochemistry and Molecular Biology Laboratory, P.G. Department of Botany, Utkal University, Bhubaneswar-751004

³Bimala Nivas, Professor Pada, Cuttack-753004

(Received : 23 November, 2009; Revision received : 13 December, 2010)

Betel leaf (*Piper betle* L.) is one of the important plant species in the Asiatic region which ranks second to coffee and tea in terms of daily consumption. Though the plant is known for abuse, in recent years several reports have been published on the effects of the leaf extract and chemical constituents on different biological activities *in vitro* and *in vivo*. The present study was undertaken for its qualitative storage by banana leaves. Here eight common varieties of *Piper betle* viz. 'Ambadi', 'Ghanghate Bangla', 'Kaker', 'Kali', 'Nabkar', 'Parua', 'Ravesi' and 'Tellaku' were studied under *in vivo* condition. Harvested leaves were stored and screened for biochemical parameters to check their quality during storage up to three weeks. Biochemical constituents (total sugar, amino acid, protein content, phenol), photosynthetic pigment content (chlorophyll a,b) and mineral content (nitrogen, phosphorous) were analyzed after first, second and third weeks of storage. During the storage some changes was observed in each set of experiment; phosphorous, vitamin A, total sugar, phenol content increased but nitrogen, vitamin C and chlorophyll content decreased.

Key words: *Piper betle* L., banana leaves, biochemical constituents, mineral content, photosynthetic pigments

Introduction

The deep green heart shaped leaves of *Piper betle* L. are popularly known as 'Pan', 'Nagaballi', 'Nagurvel', 'Saptaseera', 'Sompatra', 'Tamalapaku', 'Tambuli', 'Tambuli', 'Vaksha Patra', 'Vettilai', 'Voojanganalata' in different parts of the country. The plant species belongs to the Family Piperaceae. It is a slender, aromatic creeper rooting at the nodes. The branches of the plant are swollen at the nodes. The plant has alternate, heart shaped, smooth, shining and long stalked leaves with pointed apex. Use of betel leaf have been known for centuries for its curative properties such as to reduce body odour, bad breath, throat/lung problems, offensive vaginal discharge and to prevent itching caused by fungus or bacteria. Medicinally betel leaves are aromatic, carminative, stimulant, astringent and used as a preventive for worms and in snake bite (CSIR, 1992). Juice of the leaves is dropped into eyes in painful

infection and night blindness. Essential oil from leaves of this plant has been used for the treatment of respiratory problems and as antiseptic and the fruit is employed with honey as a remedy for cough (Chandra *et al.*, 1987).

Betel leaf is cultivated using straight bole trees or shrubs as a support or host plant. The trees provide partial shade. *P. betle* is raised by vegetative propagation from the cuttings under partially shaded and humid environment inside the *Boroj*, which is a small hut like structure of approximately 2 m in height and 0.02 ha in area. It is constructed with the locally available materials like bamboo stems, jute sticks, paddy straw, petioles and leaves of banana etc. It is a compatible integration of biological and social systems (Alam and Mahiuddin, 1992). This agro-forestry practice provides an ideal environment for optimum harvesting of leaves.

After post-harvesting, the betel leaf becomes

* Corresponding author; E-mail: santi_bot_uu@yahoo.co.in

perishable commodity and therefore, always subject to wastage by quick spoilage due to dehydration, fungal infection, dechlorophyllation etc. This may cause a post-harvest loss ranging from 35% to 70% during transport and storage (Rao and Narasimham, 1977). Even if the most conservative estimate of 10% loss were considered that too would reveal a menacing loss worth about Rs 900 million every year to the country. Not only this, particularly in the rainy season a large portion of the leaves remain unsold or sold at a throw-away price. However, a strikingly contradictory situation may be observed during the peak demand period in the winter season when a single piece of good quality betle leaf may cost exorbitantly high (Guha, 1997). In view of the alarming losses, attempts are being made to minimize the wastage by chemical treatments, manipulation of storage temperature, or adoption of better packaging materials and methods. Due to application of some chemical treatment and use of paddy straw for long term storage, the medicinal properties of leaves were reportedly affected (Guha, 2004; Rao and Narasimham, 1977). Therefore, the present study was undertaken to investigate the biochemical properties of *P. betle* during the storage by banana leaves as a viable alternative measure.

Materials and methods

Plant material and treatment

Freshly harvested betel leaves of different varieties of *Piper betle* such as 'Ambadi', 'Ghanghate Bangla', 'Kaker', 'Kali', 'Nabkar', 'Parua', 'Ravesi', 'Tellaku' were collected from different parts of the State of Odisha and maintained in the pots in the botanical garden of P.G. Department of Botany, Utkal University, Bhubaneswar. The healthy selected leaves were taken and covered with fresh banana leaves. For control experiments the betel leaves were covered by paddy straw. Experiments were conducted under *in vivo* simulated condition having proper aeration and drainage facility. The biochemical properties of leaves were studied after first, second and third week of storage in each variety of leaf. Each treatment, including control, was replicated 3 times.

Estimation of total protein

Total leaf protein was extracted by the polyvinyl polypyrrolidone (PVP) precipitation method (Ferreira

et al., 2002). Fresh leaf tissues (0.5 g) were homogenized in 50 mM sodium phosphate buffer containing 10% (w/v) insoluble PVP using a prechilled mortar and pestle and incubated overnight at 4 °C. The homogenates were centrifuged at 17000 rpm for 20 min at 4 °C (Remi Instruments, India). The supernatant was kept under -20 °C for protein estimation and enzyme assay. The protein estimation was done by the method of Lowry *et al.* (1951). Protein in the unknown sample was estimated by measuring the absorbance at 750 nm using bovine serum albumin as standard and expressed as mg per gram fresh weight basis (mg/g f. wt.).

Estimation of total amino acid

Fresh tissue (0.5 g) was weighed and homogenized using a homogenizer in 5 ml of 80% ethanol and the supernatant collected after centrifugation at 20000 rpm for 10 min. The resulting pellet was re-extracted with another 5 ml of 80% ethanol, centrifuged and the supernatants were combined. The combined supernatant was centrifuged for an additional 15 min at 20000 rpm to remove any insoluble material. Total free amino acid content of the ethanol-extracts was determined following the ninhydrin method (Yemm and Cocking, 1955). An aliquot (200 µl) of ethanol-extract were transferred into a 30 ml glass test tube, to which 100 µl ascorbic acid (0.25% solution) and 1 ml solution of ninhydrin (3% solution in piersolve) were added. The mixture was incubated in a boiling water bath for 10 min, cooled immediately, 3 ml of 80% ethyl alcohol were added and the absorbance was measured at 570 nm. Leucine solution (2.5-50 µg) in 80% ethanol was used as a standard to determine the amino acid concentrations. The values were expressed as mg of total free amino acids per gram of fresh tissue (mg/g f. wt.).

Estimation of total soluble sugar

The amount of total soluble sugar was estimated by the phenol sulphuric acid reagent method (Dubios *et al.*, 1951). Fresh normal and galled plant material (500 mg each) was homogenized with 10 ml of 80% ethanol. Each sample was centrifuged at 20000 rpm for 20 min. The supernatants were collected separately in tubes each containing 1.0 ml of alcoholic extract,

1.0 ml of 5% phenol solution and mixed. Then 5.0 ml of 96% sulphuric acid was added rapidly. Each tube was gently agitated during the addition of the acid and then allowed to stand in a water bath at 26-30 °C for 20 min. The OD of the characteristic yellow orange colour thus developed was measured at 490 nm in a spectrophotometer after setting for 100% transmission against the blank. A standard curve was prepared by using known concentration of glucose. The quantity of sugar was expressed as mg/g fresh weight of leaf tissue.

Estimation of total phenolic compound

The total phenolic compound (TPC) from the leaves of different types of *P. betle* was determined by the Folin-Ciocalteu reagent (Iqbal *et al.*, 2005). The reaction was initiated by mixing 0.2 ml of appropriate diluted leaf extracts, 0.8 ml of freshly prepared diluted Folin-Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The final mixture was diluted to 7 ml with deionized water. The mixture was kept in the dark at an ambient temperature for 2 h and the absorbance was measured at 765 nm. The results of the total phenolic compounds were expressed as mg of gallic acid equivalents (GAE) per gram fresh weight of leaf tissue.

Estimation of chlorophyll

For measurement of chlorophyll a & b, 0.1 gm of fresh leaves were cut and immersed in 80% cold acetone for 3 days in the dark at 4 °C following the procedure described by Lichtenthaler and Wellburn (1983). The absorbance at 645 and 663 nm was measured.

Estimation of total nitrogen

The estimation of nitrogen was carried out by the method derived by Peach and Tracey, 1956. Dried leaf materials (100 mg) were digested in 5 ml of salicylic-sulphuric acid mixture (5 g salicylic acid in 100 ml concentrated sulphuric acid). After 30 min, approximately 0.3 g sodium thiosulphate was added and heated gently until fumes appeared. Then 5 ml of concentrated sulphuric acid and approximately 0.1 g of catalyst mixture (copper sulphate, potassium sulphate and selenium dioxide mixed in the ratio of 1:8:1) were added. Digestion was continued for at

least 3 h, till the digest became colourless. On completion of digestion, the flask was cooled and the content diluted to 50 ml in volumetric flask using distilled water.

Distillation of digested content was continued until 30 ml distillate had been collected. Then the whole distillate was titrated against standard 1/28 hydrochloric acid solution until the pink colour just reappeared. Blank digestion, distillation and titration were made using all the reagents without the plant sample.

The percentage of total nitrogen was calculated by the following formula:

$$\text{Percentage of nitrogen} = (T - B) \times 5 \times N \times 1.4/S$$

Where

T = Sample titrated (ml)

B = Blank titrated (ml)

N = Normality of hydrochloric acid
(1/28 = 0.0357142)

S = Weight of plant material (g)

Aliquot factor = 5

Estimation of phosphorus

Phosphorus content was measured using the method of Yoshida *et al.*, 1972. One gram of dried and ground leaf tissue was digested with 10 ml acid mixture (750 ml nitric acid, 150 ml sulfuric acid, and 300 ml 60% perchloric acid). The digest was cooled and made up to a volume of 50 ml with water and filtered through Whatman No. 1 filter paper. One milliliter of digest was mixed with 2 ml of 2 N nitric acid and diluted to 8 ml. One milliliter of molybdovanadate reagent (25 g of ammonium molybdate in 500 ml of water and 1.25 g of ammonium vanadate in 500 ml of 1 N nitric acid; both were mixed in equal volumes) was added, made up to 10 ml, shaken, and the absorbance measured at 420 nm in a spectrophotometer after standing for 20 min. A standard graph was prepared using potassium dihydrogen phosphate.

Results and discussion

Total protein was calculated in every week of treatment and it was found that the protein content decreased over the period in all varieties of *P. betle*

leaves (Table-1). Protein content was maximum in all stages of experiment under banana leaf storage compared to that using paddy straw, which may indicate that the pathogen utilized them as a source of energy for survival. Mehrotra (1980) also reported that the contamination by microorganism was maximum after long time storage of betel leaves. Similar results were reported during senescence in betel leaves (Mishra and Gaur, 1970) and in bean leaves (Chiunall, 1954).

Free amino acid content of leaf tissue varied significantly at different week intervals. The maximum content was found in 'Nabkar' variety of leaf samples (8.46 ± 0.64) after the first week of treatment (Table-1). All the eight varieties of betel leaf gave better response towards increment of amino acid content. In treated (wrapped up with banana leaves) and control leaves (stored in paddy straw) amino acid content was significantly increased up to the third week of storage. The soluble amino acids in plants can be categorized into two groups. The major amino acids (glutamate, glutamine, aspartate, asparagine) are normally present in high concentrations and usually include those most closely linked to primary carbon metabolism and nitrogen assimilation, while the minor amino acids (glycine, proline, lysine, isoleucine) are generally less abundant. There was no report regarding the quantity of amino acid during the storage of any leaves. But according to Sengupta (1996), the amino acid content of *P. betle* leaves was maximum after few days of storage.

In general, high amount of total soluble sugars were present in the treated tissues as compared to control (Table-2). Betel leaf tissues treated with banana leaves showed a slightly higher amount of total soluble sugar as compared to paddy straw. But in case of 'Nabkar' variety of leaves the amount of soluble sugar was slightly decreased. The quantity of total soluble sugar was considerably high in treated tissue as compared to that stored in paddy straw. According to Mehrotra and Agarwal (2003), sugar has a large numbers of stereo-isomers, because they contain several asymmetric carbon atoms (Lindhroost and Thisbe, 2003). Increase in sugar content might be due to accumulation of these substances. This accumulation may involve the translocation of soluble

sugars from the neighboring healthy tissues to the physiological sinks. This view is supported by the finding of Wasnikar *et al.* (1993).

Total phenols expressed as mg/g fresh leaf material varied significantly among different types of betel leaves (Table-2). In 'Parua' type, the highest phenol content (5.34 ± 0.37) was reported in first week for control leaves, whereas 'Tellaku' variety leaves had a considerably low levels of phenol (2.21 ± 0.55) after the third week of storage. In most of the cases treated leaves produced less phenolic compounds than untreated leaves. Young treated leaves of *P. betle* had low levels of phenolics, it may be due to low amount of secondary metabolites (Wasnikar *et al.*, 1993). Further studies on the phenolic content and the role of external factors is essential.

The chlorophyll level declined with longer storage period (Table-4), the rate of decline being faster in control than in treated leaves. The chlorophyll content was enhanced in betel leaves after storage treatment with banana leaves. Quantitative differences between the chlorophyll content of treated leaves and that of paddy straw after different time duration of senescence varied from 20 to 60%. The differences between the two treatments were significant beyond the first week. The chlorophyll content seemed to attain a definite minimal level in each group of treated leaves after three weeks of storage. Chlorophyll breakdown is an event closely associated with the process of aging and senescence (Beevass, 1968; Peterson and Huffaker, 1975) which is supported by the present studies (Table-4). This has been attributed to the structural and functional degeneration of chloroplasts (Woolhouse, 1974). Even though we do not have direct evidence, such changes in chloroplasts might have been slowed down in adequately treated leaves during the progression of post-harvest storage period.

Nitrogen and phosphorus content in the leaves of *P. betle* are presented in Table-3. Nitrogen content dropped during the three week period of storage but there was an increase in the phosphorus content. In 'Ghanghate' type of betel leaf the nitrogen amount was maximum (18.93 ± 0.65) at the first week of storage by banana leaves. Kaker variety of leaves gave higher

Table-1
Estimation of total soluble protein and amino acid from different varieties of betel leaf (*P. betle*) during storage

<i>P. betle</i> varieties	Total soluble protein (mg/g f.wt.)						Total amino acid (mg/g f.wt.)					
	1st week		2nd week		3rd week		1st week		2nd week		3rd week	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Ambadi	12.20±0.99	13.05±0.65	11.56±0.78	12.09±0.44	9.77±1.02	10.99±0.98	4.21±0.61	4.92±0.49	5.35±0.92	5.80±0.55	3.76±0.78	4.42±0.67
Ghanghate Bangla	8.98±0.67	8.55±0.70	8.42±0.95	8.98±1.05	7.60±0.54	7.88±0.55	3.48±0.53	5.29±0.63	3.07±0.56	4.93±0.75	2.85±0.44	4.18±0.71
Kaker	10.23±1.04	11.45±0.93	9.45±0.56	11.07±0.99	8.55±0.76	9.13±0.73	5.60±0.66	5.95±0.51	5.17±1.01	6.25±0.77	4.77±0.67	5.39±0.99
Kali	8.88±0.82	9.33±0.76	8.09±0.55	9.11±1.21	7.44±0.34	8.69±0.66	7.55±0.85	7.90±0.79	6.65±1.04	7.16±1.10	5.98±1.09	6.55±0.45
Nabkar	11.88±0.69	11.50±0.54	10.60±0.88	11.00±0.81	9.81±0.65	10.77±0.99	6.51±0.55	8.46±0.64	5.71±0.76	7.34±1.05	4.77±0.82	7.21±1.01
Parua	9.02±1.32	11.22±0.96	8.44±0.54	10.72±0.87	8.32±0.98	10.21±1.02	3.56±0.91	5.45±0.76	3.11±0.99	5.22±0.99	2.33±0.55	4.77±0.77
Ravesi	10.55±0.66	11.58±0.45	10.08±0.67	11.22±0.93	9.34±0.77	10.50±1.19	5.56±0.56	5.98±0.77	4.80±0.57	5.67±0.67	4.45±0.68	4.80±1.03
Tellaku	11.92±1.55	11.87±1.09	11.26±0.70	11.19±1.11	10.35±0.72	10.82±0.89	4.21±0.44	4.83±0.56	3.43±0.67	4.22±0.92	2.60±0.88	3.71±0.56

Data pooled from 3 separate experiments each with at least 3 replicates. Values denote Mean ±S.E.
(Control: betel leaves stored in paddy straw; Treated: betel leaves wrapped up with banana leaves)

Table-2
Estimation of total soluble sugar and phenolic compound from different varieties of betel leaf (*P. betle*) during storage

<i>P. betle</i> varieties	Total soluble sugar (mg/g f.wt.)						Total phenolic compound (mg/g f.wt.)					
	1st week		2nd week		3rd week		1st week		2nd week		3rd week	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Ambadi	2.91±0.22	3.22±0.34	3.02±0.37	3.43±0.23	2.72±0.65	3.14±0.32	2.50±0.34	3.71±0.66	3.91±0.91	3.55±0.55	3.02±0.58	2.67±0.32
Ghanghate Bangla	3.53±0.31	3.73±0.54	2.72±0.76	3.54±0.22	2.42±0.54	3.25±0.55	5.22±0.41	4.90±0.85	4.34±0.54	4.24±0.41	3.89±0.88	3.66±0.66
Kaker	3.10±0.54	3.85±0.12	3.54±0.32	4.02±0.66	3.23±0.45	3.74±0.44	4.86±0.77	4.59±0.90	4.23±0.99	4.01±0.52	3.45±0.54	3.60±0.65
Kali	2.44±0.54	2.42±0.74	2.15±0.98	2.72±0.42	2.07±0.27	2.65±0.52	3.66±0.54	3.11±0.44	2.89±0.22	2.90±0.44	2.67±0.33	2.34±0.31
Nabkar	2.81±0.27	3.24±0.33	2.44±0.54	3.45±0.37	2.96±0.29	3.37±0.51	4.09±0.66	4.23±0.33	3.81±0.39	3.45±0.79	3.44±0.42	3.10±0.88
Parua	3.39±0.61	3.85±0.31	3.15±0.54	3.48±0.59	2.85±0.78	3.15±0.69	5.34±0.37	4.74±0.58	4.90±0.44	4.22±0.67	4.16±0.75	3.67±0.61
Ravesi	2.10±0.22	2.60±0.41	1.74±0.28	2.58±0.61	1.95±0.72	2.47±0.55	4.48±0.98	4.33±0.77	3.38±0.32	3.44±0.66	2.88±0.48	2.64±0.45
Tellaku	3.22±0.45	3.82±0.36	3.53±0.51	4.49±0.76	3.17±0.27	4.16±0.44	3.09±0.54	3.11±0.49	2.92±0.35	2.55±0.58	2.86±0.21	2.21±0.55

Data pooled from 3 separate experiments each with at least 3 replicates. Values denote Mean ±S.E.
(Control: betel leaves stored in paddy straw; Treated: betel leaves wrapped up with banana leaves)

Table-3
Estimation of total nitrogen and phosphorus from different varieties of betel leaf (*P. betle*) during storage

<i>P. betle</i> varieties	Total nitrogen estimation (mg/g f.wt.)						Total phosphorus estimation (mg/g f.wt.)					
	1st week		2nd week		3rd week		1st week		2nd week		3rd week	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Ambadi	16.20±0.87	16.83±0.55	14.61±0.43	14.90±0.56	13.56±0.96	13.99±0.64	13.88±0.43	14.21±0.56	13.92±0.34	15.01±0.45	14.87±0.32	15.62±0.39
Ghanghate Bangla	18.77±0.88	18.93±0.65	17.67±0.66	18.22±0.22	16.97±0.65	17.45±0.34	11.45±0.33	12.09±0.20	12.55±0.22	13.92±0.41	12.88±0.43	14.27±0.40
Kaker	15.32±0.66	15.76±0.45	14.50±0.55	15.45±0.45	13.83±0.74	14.72±0.76	15.66±0.75	15.60±0.43	15.87±0.54	16.56±0.61	16.20±0.51	17.55±0.55
Kali	17.97±0.54	19.35±0.77	17.22±0.64	18.34±0.76	15.44±0.56	16.29±0.65	12.90±0.23	14.7±0.33	13.77±0.72	14.98±0.55	14.66±0.66	16.01±0.41
Nabkar	15.88±0.66	16.35±0.45	14.67±0.88	15.78±0.95	14.21±0.57	15.33±0.76	12.02±0.21	13.35±0.19	12.65±0.29	14.88±0.41	12.97±0.41	14.91±0.54
Parua	16.56±0.78	18.10±	15.59±0.65	16.76±0.61	14.45±0.77	15.09±0.82	11.77±0.19	13.21±0.56	12.45±0.41	13.78±0.86	12.88±0.21	15.03±0.33
Ravesi	18.92±0.91	21.45±1.01	17.42±0.23	20.77±1.02	16.06±0.86	18.66±0.71	14.05±0.55	15.22±0.44	14.63±0.43	15.92±0.56	15.11±0.49	16.71±0.71
Tellaku	14.12±0.99	16.44±0.65	14.01±0.52	15.49±0.44	13.25±0.32	14.57±0.21	13.90±0.54	14.45±0.32	14.22±0.55	14.85±0.21	14.56±0.81	15.45±0.37

Data pooled from 3 separate experiments each with at least 3 replicates. Values denote Mean $\pm$ S.E.
(Control: betel leaves stored in paddy straw; Treated: betel leaves wrapped up with banana leaves)

Table-4
Estimation of total chlorophyll from different varieties of betel leaf (*P. betle*) during storage

<i>P. betle</i> varieties	Total chlorophyll (mg/g f.wt.)											
	1st week				2nd week				3rd week			
	Control		Treated		Control		Treated		Control		Treated	
	Chl a	Chl b	Chl a	Chl b	Chl a	Chl b	Chl a	Chl b	Chl a	Chl b	Chl a	Chl b
Ambadi	2.34±0.32	0.95±0.15	3.20±0.52	1.11±0.32	2.21±0.55	0.82±0.29	2.55±0.34	0.99±0.21	2.01±0.35	0.56±0.16	1.91±0.45	0.69±0.24
Ghanghate Bangla	2.51±0.37	0.82±0.18	3.67±0.57	0.88±0.22	2.33±0.43	0.72±0.18	3.44±0.43	0.82±0.17	2.12±0.54	0.64±0.17	2.78±0.48	0.67±0.22
Kaker	3.01±0.52	1.22±0.21	3.90±0.77	1.29±0.26	2.89±0.54	0.96±0.21	3.45±0.67	1.06±0.19	2.67±0.66	0.84±0.21	3.01±0.65	0.96±0.334
Kali	2.15±0.23	0.77±0.22	2.56±0.84	0.72±0.18	2.01±0.34	0.67±0.22	2.21±0.61	0.54±0.22	1.67±0.45	0.42±0.19	2.11±0.69	0.31±0.11
Nabkar	2.88±0.31	0.63±0.14	3.01±0.45	0.81±0.24	2.80±0.39	0.51±0.18	2.80±0.23	0.69±0.28	2.44±0.56	0.35±0.23	2.38±0.86	0.39±0.16
Parua	3.33±0.65	1.32±0.29	3.42±0.44	1.15±0.32	3.16±0.41	1.08±0.23	3.22±0.66	0.77±0.21	2.78±0.65	0.71±0.31	2.66±0.47	0.52±0.19
Ravesi	2.28±0.44	0.88±0.23	2.44±0.49	0.94±0.18	2.22±0.59	0.68±0.16	2.02±0.89	0.65±0.13	1.91±0.46	0.31±0.13	1.50±0.39	0.41±0.23
Tellaku	2.67±0.33	0.56±0.19	3.21±0.67	0.71±0.14	2.43±0.55	0.49±0.13	3.02±0.63	0.62±0.15	2.13±0.33	0.30±0.14	2.66±0.44	0.43±0.10

Data pooled from 3 separate experiments each with at least 3 replicates. Values denote Mean $\pm$ S.E.
(Control: betel leaves stored in paddy straw; Treated: betel leaves wrapped up with banana leaves)

amount (17.55 ± 0.55) of phosphorus in all sets of designed experiment. It is well known that more amount of phosphorus increases disease resistance indirectly, by encouraging potassium uptake by the plant (Shinde *et al.*, 1982).

Standardization is an urgent need of the modern era for the purpose of safety and quality assurance measures so as to ensure supply of medicinal plant/leaf materials of good quality. Also, the post-harvest handling, processing and storage of the betel leaf have not been scientifically explored well. Consequently, betel leaves worth millions of rupees are sold at a throw-away price and sometimes remain even completely unsold and get wasted due to lack of proper storage. In this context, our study holds significance as it gives an idea for proper preservation of post-harvested betel leaf with better biochemical properties so as to qualify as the 'green gold'.

Acknowledgement

We are grateful to the Head, Department of Botany, Utkal University for his encouragement and providing laboratory facilities. We also wish to acknowledge UGC, New Delhi for providing financial support for this work through SAP-DRS programme.

References

- Alam, M.K. and Mahiuddin, M. (1992). Some potential multipurpose trees for homestead in Bangladesh. Agroforestry Information Series-2. Bangladesh Agricultural Research Council- WINROCK, Dhaka, Bangladesh. pp 170-176.
- Beevass, L. (1968). Growth regulator control of senescence in leaf disks of nasturtium (*Tropaeolum majus*). In: Wightman, F. and Setterfield, G. (eds.) Biochemistry and Physiology of Plant Growth Substances, Runge Press, Ottawa, Canada, pp. 1417-1435.
- Bhowmick, S. (1997). Paan: Anadrita Laxmi (In Bengali). "Betle leaf: The Neglected Goddess of Wealth." Moyna Prakashani, Calcutta, India.
- Chandra, T., Sadigue, J. and Somasundaram, S. (1987). Effect of *Eclipta alba* on inflammation and liver injury. Fitoterapia 58: 23-31.
- Chiunall, A.C. (1954). Protein metabolism in rooted runner-bean leaves. New Phytol. 53: 31- 37.
- CSIR (Council of Scientific and Industrial Research, New Delhi), (1992). The Wealth of India, The Dictionary of Indian Raw Materials and Industrial Products. Raw Material Vol. 5, Revised ed. New Delhi: Publication and information directorate, CSIR; New Delhi.
- Dubios M.K., Gilles J.K., Robers P.A. and Smith F. (1951): Calorimetric determination of sugar and related substance. Anal. Chem. 26: 351-356.
- Ferreira, R.R, Fornazier, R.F, Vitoria A.P, Lea, P.J. and Azevedo, R.A. (2002). Changes in antioxidant enzyme activities in soybean under cadmium stress. J. Plant Nutri. 25(2): 327-342.
- Guha, P. (1997). "Paan Theke Kutir Silpa Sambhabana" (In Bengali). "Exploring Betle Leaves for Cottage Industry", In: Krishi, Khadya-O- Gramin Bikash Mela - A Booklet published by the Agricultural and Food Engineering Department, IIT, Kharagpur, India. pp. 15-19.
- Guha, P. (2004). Development of technology for enhancing shelf life of betle leaves (*Piper betle* L.), In: Annual Report of All India Coordinated Research Project on Post Harvest Technology (ICAR). IIT, Kharagpur (Ed.). Food and Agricultural Engineering Department, IIT, Kharagpur, India. pp. 39-56.
- Iqbal, S., Bhanger, M.I. and Anwar, F. (2005). Antioxidant properties and components of bran extracts from selected wheat varieties commercially available in Pakistan. LWT-Food Sci. Technol. 40(2): 361-367.
- Lichtenthaler, H.K. and Wellburn, R.R. (1983). Determination of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem. Soc. Transact. 11: 591-592.
- Lindhorst T.K. and Thisbe K. (2003). Essentials of carbohydrate chemistry and biochemistry. Wiley Eastern Ltd., New Delhi, India.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin-phenol reagent. J. Biol. Chem. 193: 265-275.
- Mehrotra, R.S. (1980). Plant Pathology. Tata-McGraw Hill Publishing Co. Ltd. New Delhi, India.
- Mehrotra, R.S. and Agarwal, A. (2003). Plant Pathology. Tata-McGraw Hill Publ. Co. Ltd., New Delhi, India.
- Mishra, S.D. and Gaur, B.K. (1970). Senescence in detached betel leaves: role of the petiole. Science. 167: 387-388.
- Peach, K. and Tracey, M.V. (1956). Modern Methods of Plant Analysis, Vol. I. Springer Verlag, Berlin, Germany.
- Peterson, L.W. and Huffaker, R.C. (1975). Loss of ribulose 1, 5-diphosphate carboxylase and increase in

- photosynthetic activity during senescence of detached primary leaves. *Plant Physiol.* **55**: 1009-1015.
- Rao, V.M. and Narasimham, B. (1977). Prolonging storage life of betle leaves, *Ind. J. Hort.* **34** (2): 175-182.
- Sengupta, K. (1996). Pan Patar Sangrakkhan (In Bengali). "Preservation of Betel Leaves." *Nabanna Bharati*. **28**(12): 580-582.
- Shinde, A.D., Sawarkar, N.J., Sachidanand, B. and Soni, B.K. (1982). Major nutrient status of soybean at different stages of growth as affected by P and S application. I. Content and uptake of nutrients. *Jawaharlal Nehru Krishi Vishwa Vidyalaya, Res. J.* **16**(1): 33-37.
- Wasnikar, A.R., Khatik, S.K., Nayak, M.L., Vishwakarma, S.K. and Puneekar, L.K. (1993). Effect of Phosphorus on Bacterial Leaf Spot Disease Incidence, and Chemical Composition and Storage Quality of Piper betle Leaves. *Phytoparasitica*. **21**(1): 75-78.
- Woolhouse, H.W. (1974) Longevity and senescence in plants. *Sci. Progr. Oxf.* **61**: 123-147.
- Yemm, E.W. and Cocking, E.C. (1955). The determination of amino acids with ninhydrin. *Analyst*. **80**: 209-213.
- Yoshida, S., Forno, D.A. and Cock, J. (1972). Laboratory manual for physiological studies of rice. IRRI. The Philippines.

Current vegetation status of mangroves and their associates of Bhitarkanika National Park, India

S.K. Pattnaik¹, P.C. Panda² and H.K. Patra^{1*}

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

²Regional Plant Resource Centre, Nayapalli, Bhubaneswar-751015

(Received : 4 December, 2010; Revision received : 21 December, 2010)

The up-to-date status of flora of Bhitarkanika mangrove ecosystem of Odisha was assessed in the present study. The true mangroves, mangrove associates and other plants of the sanctuary were surveyed with special emphasis on flora of more diversified localities like Gupti, Khola, Dangamala, Kalibhanjadia Island, Bhitarkanika and Ekakula. A total of 168 angiospermic plant species belonging to 131 genera under 66 families have been collected during the study besides three pteridophytes. Out of these, 25 species belong to true mangrove category, 19 are mangrove associates and 18 plant species are classified under back mangroves. Included in these are 2 plant species characteristic of coastal sand-dunes and 107 non-mangrove taxa. The rare and endangered plant species occurring in Bhitarkanika mangrove forests have been identified and their conservation measures suggested. The enumeration of all the species collected from different localities has been provided in this paper.

Key words: Bhitarkanika National Park, true mangroves, mangrove associates, back mangroves

Introduction

In India, about 4461 sq.km area is covered under mangroves, which is 0.14% of the country's total geographic area and approximately 5% of the world's mangrove vegetation. Nearly 57% of the Indian mangroves are found along the east coast. Bhitarkanika mangrove ecosystem lies in the deltaic region of north-eastern coastal plain of Kendrapara district of Orissa along the East coast of India. Located between 20°4'-20°8' N latitude and 86°45'-87°5' E longitude, it occupies an area of 672 sq km, of which mangrove forests extend over nearly 130 sq km (Mishra *et al.*, 2005). It is surrounded by the Bay of Bengal on the east, the villages of Kendrapara district on the west, Baitarani and Dhamra rivers on the north and the Mahanadi river on the south. Bhitarkanika Mangrove ecosystem of Orissa was notified as Wildlife Sanctuary on 22nd April, 1975 and National Park in 1988. In recent past, this ecosystem was subjected to severe anthropogenic pressure and natural threats like cyclone, flood etc. causing considerable damage to mangrove plants and wildlife inhabiting such habitats.

In the past, Bhitarkanika possessed very rich and diverse mangrove vegetation because of its peculiar

geographic location and edaphic and climatic variables. However, the diversity of mangroves remain unexplored till the floristic work of Haines (1921-25) and Mooney (1950), the two British forest officers and pioneer plant explorers for Bihar and Orissa. However, Mooney's (1950) survey was confined to Mahanadi delta alone. Rao and Banerjee (1967 & 1982), Rao *et al.* (1970), Banerjee and Das (1972), Rao and Shastry (1974) and Banerjee (1986) have studied the flora and vegetation of coastal Orissa including tidal swamp forests and added a number of species to the flora of Orissa. Thereafter, Banerjee (1984), Choudhury (1984, 1986, 1990), Samal and Patnaik (1989), Biswal and Choudhury (1993) and Reddy *et al.* (2006) have conducted floristic studies in the Bhitarkanika Wildlife Sanctuary with special emphasis on mangroves as well as their socio-economic importance. Choudhury *et al.* (1991) and Choudhury (1994, 2001) have discussed the biological diversity and conservation of the mangroves of the Bhitarkanika.

The plan for conservation and management of mangroves taken up by the Ministry of Environment & Forests, Govt. of India during the current five year plan period covers the recent study of floristic status of Bhitarkanika mangrove ecosystem which

* Corresponding author; E-mail: drhkpatra@yahoo.co.in

is described below. All plants belonging to different ecological categories have been enumerated in this paper.

Description of the study area

The current survey was conducted to reassess the floristic diversity of the Bhitarkanika mangrove ecosystem by undertaking intensive survey in floristically diversifies localities. The major areas include Gupti (Krisnapriyapur, Gupti Check Gate, Sunei Rupei, Patsala Nala and Baunsagarh Nala), Khola (Khola Check Gate, Barhapita, Khola Nala), Dangamala: Talachua Port, Salendra Sarai and Sanjog Nala to Mundha Jhora (which spreads over an area of 636.00 hectare), Kalibhanjadia Island (extends over an area of 819.00 hectares and covers Dhamra river, Sapua river and Chandbali), Bhitarkanika (spreads over an area of 1712.40 hectares covering Sanjog Nala and Bhitarkanika Nala) and Ekakula (located close to the Bay of Bengal which is mainly composed of sandy and muddy beach). The map showing the location of different study sites is given below (Fig. 1).

Bhitarkanika experiences three distinct seasons namely summer, rainy and winter. Summer starts from mid February and extends up to mid June. Summer is followed by rainy season, which continues till end of October. November-February constitute the winter months characterized by low temperature accompanied by low rainfall. The average annual rainfall is about 1642 mm, bulk of which is received during the period June to October (Mishra *et al.*, 2005). Wind velocity is about 40 km per hour during the monsoon, which is recorded in the ranges of 15-25 km/hr during winter. Sometime the depressions in the Bay of Bengal accelerate the wind velocity to a considerable extent. From the meteorological data of last ten years it is revealed that the maximum and minimum relative humidity ranges from 83% to 49.25% at 8.30 am and from 76% to 46.5% at 5.30 pm respectively.

Methodology

Regular surveys were made in different localities spread across the entire area throughout the year

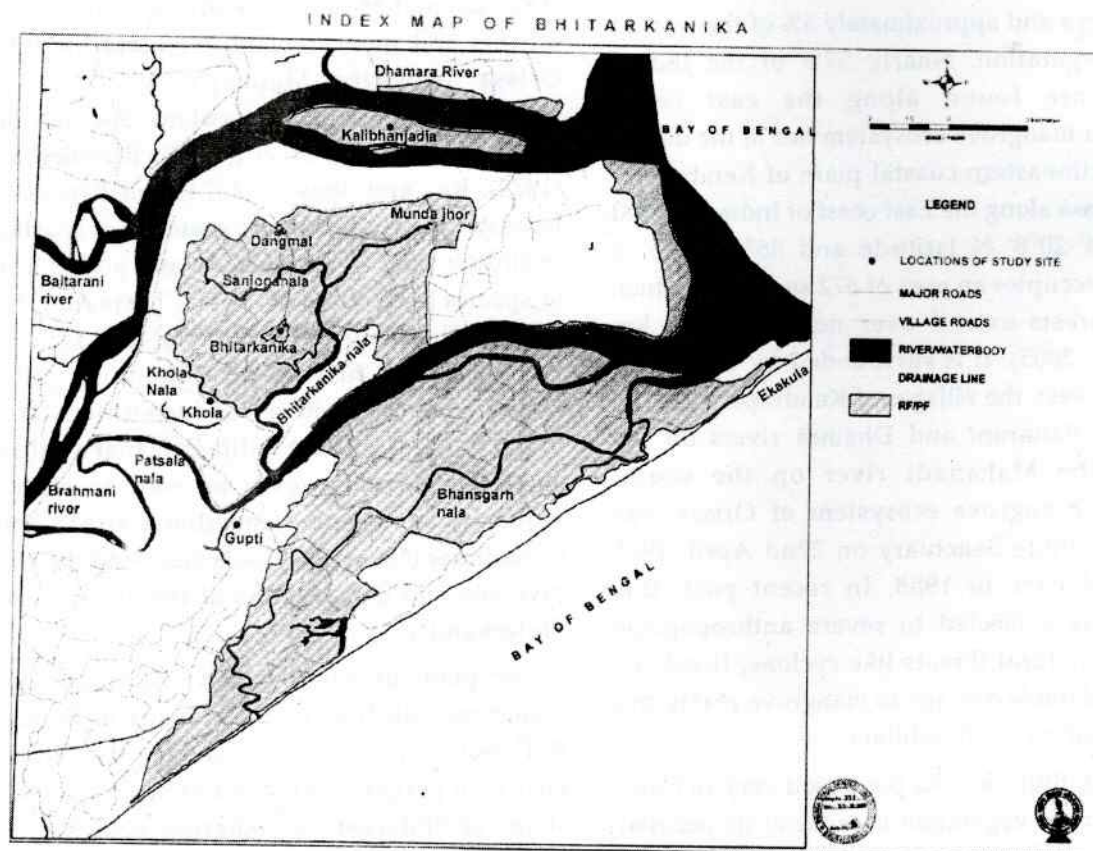


Fig. 1 : Map showing the location of different study sites of Bhitarkanika mangrove ecosystem

with more emphasis on collection from vegetationally diversified habitats. During each field trip, besides ecological studies, plants were collected and detailed field notes recorded on spot which includes field number, date of collection, collection locality, habit, habitat, phenology, plant association, pattern of distribution, abundance/rarity and plant characters those can not be detected from dried specimens. In case of small herbaceous plant, the whole plant was uprooted but for shrubs and trees, the branches, preferably flowering or fruiting ones, were cut out and preserved for subsequent identification or herbarium preparation. The specimens were identified with the help of regional floras i.e., 'The Botany of Bihar and Orissa' by Haines (1921-25) and its supplement by Mooney (1950), 'Flora of Orissa' (Saxena & Brahmam, 1994-96), other floras, monographs and revisionary works. The collected specimens were either preserved in closed polythene bags or pressed among blotters in the field. To avoid fungal infection or rotting of specimens, the blotters were frequently changed till the specimens were completely dry. The dried specimens were dipped in a solution of $HgCl_2$ (4%) in absolute alcohol for poisoning and allowed to dry within blotters under pressure for about a week. The specimens were then mounted onto standard acid-free herbarium mount boards (42 x 28cm) by application of glue and all field data transferred to the herbarium labels to be pasted on the lower right hand corner of the mount board.

Enumeration

A total of 168 angiospermic plant species belonging to 131 genera under 66 families have been collected during the present survey (From March, 2008 to April, 2010) excluding three pteridophytes. Of these, only 24 species belong to monocots and rest 144 to dicots i.e. in the ratio of 1:6. The details of genera and species under these two broad groups are presented in Table 1.

Out of 168 angiospermic species, 25 plant species are classified as true mangroves, 19 species as mangrove associates, 18 plant species as back mangroves and 02 species characteristic to sand dunes and beach flora which are enumerated below.

TRUE MANGROVES

Aegialitis rotundifolia Roxb. Fl. Ind.2:111. 1832; Saxena & Brahmam, Fl. Orissa 2:991. 1995.

Local Name : Bana Ruar

Fls. & Frts. : November-May.

Locality : Bhitarkanika, Salendrasarei, Kalibhanjadhia, Ekakula.

Uses: Fuel wood, timber for construction.

Occurrence in India: Orissa, Sunderbans in West Bengal, Andamans

Aegiceras corniculatus (L.) Blanco, Fl. Filip. 79.1837; Saxena & Brahmam, Fl. Orissa 2:1001. 1995.

Local Name : Khalsi

Fls. & Frts. : November-July.

Locality : Khola, Bhitarkanika, Dangamal, Kalibhanjadiha.

Uses: Furniture making, used for asthma, diabetes, wood for fishing boat

Occurrence in India: Orissa, Sunderbans in West Bengal, Sea coast of India.

Avicennia alba Bl. Bijdr. 821. 1826; Saxena & Brahmam, Fl. Orissa 3:1398. 1995.

Local Name : Bani

Fls. & Frts. : January-June.

Locality : Khola, Dangamal, Gupti, Kalibhanjadia, Talachua port, Ekakula, Sunei Rupei, Salendra Sarai.

Uses : Fire wood, charcoal, fodder, honey forage and bark as medicine for skin and wounds.

Occurrence in India : Orissa, Sunderbans in West Bengal, Eastern Coast of India, Peninsula

Avicennia marina (Forssk.) Vierh. Akad. Wiss. Wien. Math-Natur-wiss. Kl. Denk. 71. 435. 1907; Saxena & Brahmam, Fl. Orissa 3:1399. 1995.

Local Name : Dhalabani

Table-1
Floristic analysis of
Bhitarkanika mangrove forest

Types of plant	No. of families	No. of genera	No. of species
Dicotyledons	58	112	144
Monocotyledons	8	19	24
Total	66	131	168

Fls. & Frts. : February-June.

Locality : Gupti, Sunei Rupei.

Uses : Medicinal, fodder, fuel wood, timber.

Occurrence in India : Orissa, Sunderbans in West Bengal, Bombay, Thana and Saurashtra.

Avicennia officinalis L. Sp. Pl. 110. 1753; Saxena & Brahman, Fl. Orissa 3:1400. 1995.

Local Name : Kalabani

Fls. & Frts. : March-July.

Locality : Bhitarkanika, Dangamal, Gupti, Kalibhanjadia, Ekakula, Talachua port, Sunei Rupei, Salendra sarai.

Uses : Fire wood, charcoal, fodder, honey. forage and bark as medicine for skin and wounds.

Occurrence in India: Orissa, Sunderbans in West Bengal

Brownlowia tersa (L.) Kosterm. Reinwardtia 4: 536.1959; Saxena & Brahman, Fl. Orissa 1: 192. 1994.

Local Name : Lata Sundari

Fls & Frts. : January-June.

Locality : Khola, Bhitarkanika, Dangamal, Kalibhanjadia, Sunei Rupei.

Uses : Fuel, fruits for relief of boils.

Occurrence in India : Sunderbans, Orissa

Bruguiera cylindrica (L.) Bl. Enum. Pl. Jav. 1:92. 1827. Saxena & Brahman, Fl. Orissa. 2:649. 1995.

Local Name : Kaliachuah

Fls. & Frts. : April - July

Locality : Ekakula, Gupti, Sunei Rupei, Salendra sarai.

Uses : Fire wood, timber, condiment from barks, tannin.

Occurrence in India : Orissa, Sunderbans in West Bengal, throughout Indian Coast.

Bruguiera parviflora (Roxb.) Wight and Arn. ex Griff. in Trans. Med. Phys. Soc. Cale. 8:10. 1836; Saxena & Brahman, Fl. Orissa 2:651. 1995.

Local Name : Dot

Fls. & Frts. : January - August

Locality : Kalibhanjadia.

Uses : Firewood, timber, wood for poles, fishing traps and fishing stakes

Occurrence in India : Orissa, Sunderbans in West Bengal, throughout Indian Coast

Bruguiera sexangula (Lour.) Poir. In Lam. Encycl. Meth. Bot. Suppl. 4:262. 1816; Saxena & Brahman, Fl. Orissa 2:651. 1995

Local Name : Bandari

Fls. & Frts. : January - August

Locality : Khola, Dangamal, Bhitarkanika, Salendra Sarai.

Uses : Charcoal, timber, firewood, tannin, tender leaves and hypocotyls are consumed as vegetables

Occurrence in India: Orissa, Sunderbans in West Bengal, Kerala, Andaman & Nicobar.

Bruguiera gymnorrhiza (L.) Savi. in Lam. Encycl. 4:696. 1798; Saxena & Brahman, Fl. Orissa 2:650. 1995.

Local Name : Bandari

Fls. & Frts. : March-April.

Locality : Gupti.

Uses : Hypocotyls eaten as vegetable, fodder, wood for fishing boat, firewood

Occurrence in India: Orissa, Sunderbans in West Bengal, throughout Indian Coast

Ceriops decandra (Griff.) Ding-Hou in Steenis, Fl. Males. 1. 5:471. 1958; Saxena & Brahman, Fl. Orissa 2:656. 1995.

Local Name : Gharana

Fls. & Frts. : January-June.

Locality : Kalibhanjadia, Ekakula, Gupti-Krisnapurapur, Sunei Rupei, Dangamal, Bhitarkanika.

Uses : Fire wood, fruit paste are used against ulcers, malaria; fuel wood, honey collection.

Occurrence in India : Orissa, Sunderbans in West Bengal, Andhra Pradesh.

Excoecaria agallocha L. Sp. Pl. (ed.2). 1451. 1763; Saxena & Brahman, Fl. Orissa 3:1642. 1995.

Local Name : Guan

Fls. & Frts. : March-June.

Locality : Dangmal, Gupti, Khola, Bhitarkanika, Ekakula, Sunei Rupei, Salendr Sarai

Uses : Fire wood, paper pulp, match wood, basket, honey, forage.

Occurrence in India: Orissa, Sunderbans in West Bengal, tidal forest of India

Heritiera fomes Buch.-Ham. Symes Ava Emb. 1.28. 1800; Haines. Bot. Bihar and Orissa 1:81. 1961.

Local Name : Kala Sundari

Fls. & Frts. : February-August.

Locality : Kalibhanjadia, Bhitarkanika, Dangamal.

Uses : Fuel, timber, furniture, house building, boat building, seeds for relief in diarrhoea and piles.

Occurrence in India: East and west coast of India, Orissa

Heritiera littoralis Dryand. in Aiton, Hort. Kew. (ed.1). 3:546. 1789; Mooney, Suppl. Bot. Bihar and Orissa 247. 1950.

Local Name : Dhala Sundari

Fls. & Frts. : October-February.

Locality : Khola, Bhitarkanika, Dangamal, Kalibhanjadia, Sunei Rupei.

Uses : Wood for boats, canoes and ships, furniture, diarrhea, firewood, timber

Occurrence in India: Andhra Pradesh, Tamil Nadu, Kerala, Karnataka, Gujarat, Maharashtra and Andamans, Orissa.

Kandelia candel (L.) Druce, Rep. Bot. Exch. Club. Soc. Brit. Isles. 1913. 3:420. 1914; Saxena & Brahmam, Fl. Orissa 2:658. 1995.

Local Name : Sinduka

Fls. & Frts. : November-May.

Locality : Khol, Kaliabhanjadia, Gupti, Talachua port.

Uses : Fuel, tannin and radicles as vegetable.

Occurrence in India: Orissa, Sunderbans in West Bengal.

Lumnitzera racemosa Willd. New. Schr. Ges. Naturf. Fr. New Schr. 4:137. 1803; Saxena & Brahmam, Fl. Orissa 2:669. 1995.

Local Name : Thunda

Fls. & Frts. : November-May.

Locality : Bhitarkanika, Gupti, Kalibhanjadia, Dangamal, Ekakula, Salendra Sarai.

Uses : Fire wood, timber and tannin.

Occurrence in India : Orissa, Sunderbans in West Bengal, Peninsula

Phoenix paludosa Roxb. Fl. Ind. 3:789. 1832; Saxena & Brahmam, Fl. Orissa 4:2024. 1996.

Local Name : Hental

Fls. & Frts. : March-July.

Locality : Bhitarkanika, Dangmal, Gupti, Khola, Kalibhanjadia, Sunei Rupei, Salendra Sarai.

Uses : Edible fruit, fencing, popularly used as thatching material, rope.

Occurrence in India: Orissa, Sunderbans in West Bengal, Andaman

Rhizophora apiculata Bl. Enum. Pl. Jav.1:91. 1827; Saxena & Brahmam, Fl. Orissa 2: 659. 1995.

Local Name : Candelaria

Fls. & Frts. : April-June.

Locality : Bhitarkanika, Khola, Dangmal, Gupti, Sunei Rupei. Usually common in saline soil and dominant element of mangrove forests.

Uses : Timber, fire wood, tannin, adhesive, charcoal as substitute for petroleum coke.

Occurrence in India: Orissa, throughout Indian Coast

Rhizophora mucronata Poir. in Lamk. Encycl. Meth. 6:189. 1804; Saxena & Brahmam, Fl. Orissa 2:660. 1995.

Local Name : Rai

Fls. & Frts. : April-June.

Locality : Bhitarkanika, Gupti, Kalibhanjadia, Dangmal, Sunei Rupei.

Uses : Timber, fire wood, tannin, adhesive, charcoal as substitute for petroleum coke, decoction of bark is used for haematuna, diarrhoea, dysentery, leprosy, stop bleeding and neck inflammation.

Occurrence in India : Orissa, Sunderbans in West Bengal, throughout Indian Coast

Sonneratia alba J. Smith in Rees, Cycl. 33. 1819; Saxena & Brahmam, Fl. Orissa 2:701. 1995.

Local Name : Orua

Fls. & Frts. : June-September.

Locality : Khola.

Uses : Fruits edible, juice said to check haemorrhage

Occurrence in India: Orissa, Andaman and Nicobar

Sonneratia apetala Buch.-Ham. Syne. Emb. Ava. 3:313. 1800; Saxena & Brahmam, Fl. Orissa 2:703. 1995.

Local Name : Kerua

Fls. & Frts. : February-May.

Locality : Bhitarkanika, Kalibhanjadia, Dangmal, Ekakula, Gupti, Sunei Rupei.

Uses : Fire wood, paper pulp, match wood. Leaves and fruits are very good fodder.

Occurrence in India: Orissa, West Bengal, Peninsula

Sonneratia caseolaris (L.) Engl. Nachtr. 261. 1897; Saxena & Brahman, Fl. Orissa 2:703. 1995.

Local Name : Urua

Fls. & Frts. : June-August.

Locality : Khola, Ekakula.

Uses : Leaf decoction is used against diarrhea, fuelwood, vegetable

Occurrence in India : Orissa, Sunderbans in West Bengal, Deccan Peninsula

Xylocarpus granatum Koen. Naturf. 20:2. 1784; Saxena & Brahman, Fl. Orissa 1:285. 1994.

Local Name : Sisumar

Fls. & Frts. : January-May.

Locality : Khola, Bhitarkanika, Dangmal, Sunei Rupei, Gupti, Kalibhanjadia.

Uses : Timber, furniture, tannin, oils from seeds used for hairs, wood for pencils and scales.

Occurrence in India : Coastal regions of India, Orissa

Xylocarpus gangeticus (Prain) Parkinson, Ind. For. 40: 140. 1914; Mooneyl, Suppl. BBO. 249. 1950.

Local Name : Pitamari

Fls. & Frts. : March-July.

Locality : Bhitarkanika.

Uses : Bark is used as an astringent, for curing dysentery and diarrhea

Occurrence in India: Tidal forests of West Bengal, Orissa, Andamans.

Xylocarpus mekongensis (Prain) Pierre, Fl. Cochinch. t.359B. 1897; Banerjee & Rao, Fl. Mangrove of Orissa Coast 48:1990.

Local Name : Pitamari

Fls. & Frts. : February-June.

Locality : Khola, Bhitarkanika, Dangmal, Gupti, Ekakula .

Uses : Wood for furniture making, source of tannin.

Occurrence in India: Sunderbans in West Bengal, Orissa

MANGROVE ASSOCIATES

Acrostichum aureum L. Sp. Pl. 1069. 1753; Saxena & Brahman, Fl. Orissa 4:2586. 1996.

Local Name : Kharkhari.

Fls. & Frts. : April-June

Locality : Bhitarkanika, Dangmal, Khola.

Uses : Fodder, applied on wounds and boils, rheumatism, dried fronds are used for thatching.

Occurrence in India: Orissa, Sunderbans in West Bengal, throughout India.

Aglaia cucullata Roxb. Corr. Pl.3:54. t. 258. 1819; Saxena & Brahman, Fl. Orissa 1:268. 1994.

Local Name : Oanra

Fls. & Frts. : October-January

Locality : Bhitarkanika, Khola, Dangmal.

Uses : Wood used for construction

Occurrence in India: Sunderbans in West Bengal, Andamans, Orissa

Acanthus illicifolius L. Sp. Pl. 639. 1753; Saxena & Brahman, Fl. Orissa 3:1326. 1995.

Local Name : Harakancha

Fls. & Frts. : March-June.

Locality : Khola, Dangamala, Bhitarkanika, Kalibhanjadia, Gupti, Sunei Rupei, Salendra sarai.

Uses : Fuel, fruit pulp used as blood purifier, leaf for rheumatism.

Occurrence in India : Orissa, Sunderbans in West Bengal, Sea coast of India

Cerbera manghas L. Sp. Pl. 208. 1753; Saxena & Brahman, Fl. Orissa 2:1059. 1995.

Local Name : Paniamba

Fls. & Frts. : January-May.

Locality : Dangmal, Bhitarkanika, Khola, Gupti.

Uses : Bark is purgative, fruit is narcotic and poisonous.

Occurrence in India : Orissa, Sunderbans in West Bengal, Deccan Peninsula

Clerodendrum inerme (L.) Gaertn. Fruct. Sem. Pl. 1:271. 1788; Saxena & Brahman, Fl. Orissa 3:1406. 1995.

Local Name : Phuljholi

Fls. & Frts. : April-December.

Locality : Dangmal. Bhitarkanika, Ekakula, Gupti, Sunei Rupei

Uses : Fuel and fibre from stem are used against stomach pains and also as antiseptic.

Occurrence in India : Orissa, Sunderbans in West Bengal, Sikkim and Assam, Peninsula

Cynometra iripa Kostel., Allg. Med. Pharm. Fl.4:1341. 1835; Saxena & Brahman, Fl. Orissa 1:394. 1994.

Local Name : Singada

Fls. & Frts. : June-December.

Locality : Khola, Bhitarkanika, Dangamal, Kalibhanjadia, Sunei Rupei.

Uses : Fire wood, seed produce oil.

Occurrence in India: Peninsular India, West Bengal, Orissa

Dalbergia spinosa Roxb. Fl. Ind. 3:223. 1832; Haines, Bot. Bihar and Orissa 2:310. 1961; Saxena & Brahmam, Fl. Orissa 1:494. 1994.

Local Name : Gohirakanta

Fls. & Frts. : April-October.

Locality : Dangmal, Gupti, Sunei Rupei, Bhitarkanika.

Uses : Fire wood, fodder.

Occurrence in India: Orissa, Sunderbans in West Bengal, Orissa, Andhra Pradesh, Tamil Nadu.

Derris scandens (Roxb.) Benth. J. Linn. Soc. Bot. 4(Suppl.) 1:103. 1860;; Saxena & Brahmam, Fl. Orissa 1:496. 1994.

Local Name : Kentia

Fls. & Frts. : July-December.

Locality : Dangamal.

Uses : Fibre.

Occurrence in India : Orissa, Sunderbans in West Bengal, throughout India

Derris trifoliata Lour. Fl. Cochinch. 433. 1790; Saxena & Brahmam, Fl. Orissa 1:497. 1994.

Local Name : Katei lata

Fls. & Frts. : February-June.

Locality : Khola.

Uses : Stimulant, anti-spasmodic

Occurrence in India: Orissa, Assam, Bengal, Maharashtra, Peninsular India, Andaman and Nicobar

Dodonaea viscosa N. Jacq. Encl. Syst. Pl. Carib. 10.1760; Saxena & Brahmam, Fl. Orissa 1:346. 1994.

Local Name : Mahada

Fls. & Frts. : January-November.

Locality : Ekakula.

Uses : Wood is extremely tough and durable

Occurrence in India: Orissa

Myriostachya wightiana (Nees ex Steud.) Hook. f. Fl. Brit. Ind. 7:327. 1896; Saxena & Brahmam, Fl. Orissa 4:2417. 1996.

Local Name : Nalia

Fls. & Frts. : June-December

Locality : Bhitarkanika, Gupti, Sunei Rupei, Kalibhanjadia, Dangamal, Ekakula.

Uses : Good fodder for deer and pigs.

Occurrence in India: Orissa, Bengal

Pentatropis capensis (L.f.) Bullock, Kew. Bull. 9:284. 1955; Saxena & Brahmam, Fl. Orissa 2:1100. 1995.

Local Name : Raigidi

Fls. & Frts. : March-December.

Locality : Gupti, Sunei Rupei.

Uses : Decoction of the bark is given to cure abdominal disorder.

Occurrence in India: Orissa, North eastern and Peninsular India

Porteresia coarctata (Roxb.) Tateoka, Bull. Nat. Sci. Musc. Tokya 8:406. 1965; Saxena & Brahmam, Fl. Orissa 4:2461. 1996.

Local Name : Dhaniedhana

Fls. & Frts. : August-January.

Locality : Bhitarkanika, Kalibhanjadia, Dangmal.

Uses : Pioneer soil binder and fodder.

Occurrence in India : Orissa, Sunderbans in West Bengal, Tamil Nadu.

Salacia chinensis L. Mant. Pl. 2:293. 1767; Saxena & Brahmam, Fl. Orissa 1: 304. 1994.

Local Name : Not known

Fls. & Frts. : January-May.

Locality : Dangamala, Kalibhanjadia, Sunei Rupei, Salendra Sararai

Uses : Ripe fruits are eaten, roots are used as antidiabetic drug for normalizing menstruation and invigorating blood circulation.

Occurrence in India : Sunderbans in West Bengal, Eastern and Western Peninsula, Assam, Bihar and Andaman and Nicobar, Orissa

Salicornia brachiata Roxb. Fl. Ind. 1:82. 1820; Saxena & Brahmam, Fl. Orissa 3:1525. 1995.

Local Name : Ninia

Fls. & Frts. : October-February.

Locality : Bhitarkanika, Salendra sarai.

Uses : Leaves and young shoots are eaten.

Occurrence in India : Orissa, Sunderbans in West Bengal, Sea coast of India

Sarclobus carinatus Wall. As. Res. 12:570. t. f. 1816; Saxena & Brahmam, Fl. Orissa 2:1102. 1995.

Local Name : Raigidi

Fls. & Frts. : April-September.

Locality : Khola.

Uses : Fruits edible, leaves and roots are said to be medicinal.

Occurrence in India : Orissa, Sunderban, Andamans

Sarcolobus globosus Wall. As. Res. 12:568, t. f. 1816; Saxena & Brahman, Fl. Orissa 2:1103. 1995.

Local Name : Raigaddi

Fls. & Frts. : September-February.

Locality : Khola.

Uses : Treatment of rheumatic pain.

Occurrence in India : Orissa, Sunderbans, West Bengal, Nicobar Island.

Sesuvium portulacastrum (L.) L. Syst. Nat. (ed.10). 1058. 1759; Saxena & Brahman, Fl. Orissa 2:772. 1995.

Local Name : Goda bani

Fls. & Frts. : January-May.

Locality : Kalibhanjadia, Gupta.

Uses : Indicator of saline soils, extraction of salts, fodder and food during famine.

Occurrence in India : Orissa, Sunderbans in West Bengal, Sea Coast of India

Suaeda maritima (L.) Dumort. Fl. Belg. 22. 1827; Saxena & Brahman, Fl. Orissa 3:1526. 1995.

Local Name : Giryia

Fls. & Frts. : February-May.

Locality : Kalibhanjadia, Bhitarkanika, Ekakula, Salendra Sarai.

Uses : Improve soil conditions for establishment of mangrove species, hepatitis, eaten as leafy vegetable.

Occurrence in India: Orissa, Sunderbans in West Bengal, Sea coast of India

BACK MANGROVES

Barringtonia acutangula (L.) Gaertn. Fruct. 2:97. t. 101. 1791; Haines, Bot. Bihar and Orissa 2:384. 1961; Saxena & Brahman, Fl. Orissa 2:688. 1995.

Local Name : Hinjala

Fls. & Frts. : April-September.

Locality : Bhitarkanika.

Uses : Fruit is bitter, anthelmintic, astringent. Leaf juice is given in diarrhea

Occurrence in India: Orissa, throughout India

Caesalpinia bonduc (L.) Roxb. Fl. Ind. 2:362. 1832; Saxena & Brahman, Fl. Orissa 1:378. 1994.

Local Name : Gilo

Fls. & Frts. : August-February.

Locality : Dangamala, Ekakula.

Uses : Fuel, fodder, fruit for relief of pain and rheumatism.

Occurrence in India : Sunderbans, Orissa

Caesalpinia crista L. Sp. Pl. 380. 1753; Saxena & Brahman, Fl. Orissa 1:379. 1994.

Local Name : Nentei

Fls. & Frts. : January-May.

Locality : Dangmal, Khola, Gupti, Sunei Rupei.

Uses : Fuel, fodder, fruit for relief of pain and rheumatism.

Occurrence in India : Sunderbans in West Bengal, Orissa

Crateva magna (Lour.) DC. Prodr. 1: 243. 1824; Saxena & Brahman, Fl. Orissa 1:67. 1994.

Local name : Baruna

Fls. & Frts. : March-May.

Locality : Bhitarkanika, Dangamala, Sunei Rupei.

Uses : Leaves or skin of bark is applied in cervical adenitis, abscess and edematous wounds. The same paste is salutary in rheumatic joint for relief of pain. The pulp of leaves is applied on abdomen in spleen enlargement, with great benefit.

Occurrence in India : Deccan Peninsula, Gangetic Plain, Bihar, Assam, Orissa

Cuscuta reflexa Roxb. Pl. Corom. t. 104. 1799; Saxena & Brahman, Fl. Orissa 2:1203. 1995.

Local Name : Nirmuli

Fls. & Frts. : January-May.

Locality : Bhitarkanika, Salendra sarai.

Uses : Used to cure intestinal worms, edema, skin diseases, diarrhoea, headache, fever malignancy and paralysis.

Occurrence in India : Orissa, throughout India

Dendrophthoe falcata (L.f.) Etting in Denschr. Acad. Wissen. Math.-Naturw. Cl. 32:52. 53. 58. t. 13. f.14. 1872; Saxena & Brahman, Fl. Orissa 3:1579. 1995.

Local Name : Malanga

Fls. & Frts. : November-February.

Locality : Dangmal.

Uses: Used as astringent, aphrodisiac, narcotic and diuretic and is useful in treating pulmonary tuberculosis, asthma, menstrual disorders, swelling wounds, ulcers, used by women as an anti-fertility agent and possess anticancer activity.

Located in India : Orissa, throughout India

Pongamia pinnata (L.) Pierre, Fl. Forest. Cochin. t. 385. 1899; Saxena & Brahmam, Fl. Orissa 1:570. 1994.

Local Name : Karanja

Fls. & Frts. : July-February.

Locality : Bhitarkanika, Khola, Kalibhanjadia, Dangamal, Ekakula, Gupti, Sunei Rupei.

Uses : Medicinal (piles, ulcers); seed-oil used for skin diseases; fodder.

Occurrence in India : Orissa, throughout India

Diospyros ferrea (Willd.) Bakh. Gard. Bull. Sing. 7:162. 1933; Saxena & Brahmam, Fl. Orissa 2:1020. 1995.

Local Name : Gua koli

Fls. & Frts. : March-June.

Locality : Bhitarkanika.

Uses : The wood is said to be used for cabinet making. The bark can be used to make a cordage. Fruit improves blood circulation.

Occurrence in India : Orissa, Peninsular India

Dolichondrone spathacea (L.f.) K. Schum. Fl. Kais. Wilh. Land. 1 23. 1889; Saxena & Brahmam, Fl. Orissa 2:1297. 1995.

Local Name : Gosinga

Fls. & Frts. : April-January.

Locality : Bhitarkanika.

Uses : Wood is light and thus was preferred for making saddles. It is also used in floats for fishing nets and for wooden shoes.

Occurrence in India : Orissa, Coastal India

Hibiscus tiliaceus L. Sp. Pl. 1753; Haines, Bot. Bihar and Orissa 1:71. 1961; Saxena & Brahmam, Fl. Orissa 1: 148. 1994.

Local name : Bania

Fls. & Frts. : January-June.

Locality : Bhitarkanika, Dangmal, Khola, Gupti, Sunei Rupei, Salendra Sarai.

Uses : Fuel, timber, leaf decoction is used against dysentery and as a laxative.

Occurrence in India : Along the eastern and western coasts, Sunderban and Andaman Islands, Orissa.

Hoya parasitica (Roxb.) Wall. in Wt. Contrib. Bot. Ind. 37.1834; Saxena & Brahmam, Fl. Orissa 2:1095. 1995.

Local Name : Not known

Fls. & Frts. : January-June.

Locality : Bhitarkanika.

Uses : Not known

Occurrence in India : Orissa, Assam, Sunderban, Andamans

Lansea coromandelica (Houtt.) Merr. J. Arn. Arb. 19. 353. 1939; Saxena & Brahmam, Fl. Orissa 1:360. 1994.

Local Name : Mahi

Fls. & Frts. : February-May.

Locality : Dangamal.

Uses : Tender branches and gum, in coma caused by overdose of narcotics, dyspepsia, general debility, gout and dysentery, sore eyes, leprosy, sprains and bruises.

Occurrence in India : Orissa

Manilkara hexandra (Roxb.) Dubard, Ann. Inst. Bot. Geol. Colon. Marseille (Ser.3) 23:9. f. 2. 1915; Saxena & Brahmam, Fl. Orissa 2:1011. 1995.

Local Name : Khirini

Fls. & Frts. : November-June.

Locality : Dangmal.

Uses : Ripe fruits are tasty and edible.

Occurrence in India : Orissa, Deccan Peninsula, Gujarat

Pandanus fascicularis Lam. Encycl. 1:372. 1785; Saxena & Brahmam, Fl. Orissa 4:2030. 1996.

Local Name : Ketakikia

Fls. & Frts. : August-December.

Locality : Bhitarkanika, Khola, Dangamal.

Uses : Flowers for distillation of perfumes; leaves for making mats, baskets; prop-roots as rope and cordage

Occurrence in India : Orissa, throughout the Coast of India.

Pandanus foetidus Roxb. Fl. Ind. 3:742. 1832; Saxena & Brahmam, Fl. Orissa 4:2030. 1996.

Local Name : Luni kiya

Fls. & Frts. : November-June.

Locality : Bhitarkanika.

Uses : Medicinal; for leprosy, small pox, syphilis, scabies and heart and brain diseases.

Occurrence in India : Orissa, Bihar, Assam

Thespesia populnea (L.) Sol. ex Corr. Ann. Mus. Natl. Hist. Nat. 9:290, t. 8. f. 1.1807; Saxena & Brahmam, Fl. Orissa 1: 168. 1994.

Local Name : Habeli

Fls. & Frts. : August-January.

Locality : Dangmal, Kalibhanjadia, Ekakula, Gupti, Sunei Rupei.

Uses : Fodder, medicinal (dermatitis, scabies, stomach ailments).

Occurrence in India : Coastal regions of India, Orissa

Tinospora cordifolia (Willd.) Hook. f. & Thoms. Fl. Ind. 184. 1855; Haines, Bot. Bihar and Orissa 1:18.1961; Saxena & Brahmam, Fl. Orissa 1: 39. 1994.

Local name : Guduchi

Fls. & Frts. : May-October.

Locality : Bhitarkanika.

Uses : It is antiperiodic, antipyretic, alterative, diuretic, anti-inflammatory

Occurrence in India : Tropical India, Orissa

Tylophora fasciculata Buch-Ham. ex Wight & Arn. in Wight Contrib. 50. 1834; Saxena and Brahmam, Fl. Orissa 2:1107, 1995.

Local Name : Dhala Anatamula

Fls. & Frts. : June-November.

Locality : Bhitarkanika

Use : Poisonous, used as a rat poison. Roots and leaves emetic and purgative.

Occurrence in India : Orissa, Deccan Peninsula

BEACH FLORA

Canavalia maritima (Aubl.) Thouars, J. Bot. (Desvaux) 1:80. 1813; Saxena & Brahmam, Fl. Orissa 1:465. 1994.

Local Name : Luna simba

Fls. & Frts. : March.

Locality : Ekakula.

Uses : Young pods and seeds are used as vegetables.

Occurrence in India : Coast of western peninsula, Orissa

Ipomoea pes-caprae (L.) R. Br. in Turkey, Narr. Exp. Congo, App. 477. 1818; Saxena & Brahmam, Fl. Orissa 2:1180. 1995.

Local Name : Kansari nata

Fls. & Frts. : Throughout the year.

Locality : Ekakula.

Uses : It is a sand binder, leaves and roots are useful for gonorrhea, rheumatism skin infection and stomachache.

Occurrence in India : Orissa, Sea shore of India.

The remaining 107 non-mangrove species of the ecosystem are not included here. However, the list

of apparently rare and endangered species occurring in Bhitarkanika Mangrove forest is given in Table-2.

Table-2
List of rare and endangered species

Name of the plants	Family
<i>Mucuna gigantea</i> (Willd.) DC.	Fabaceae
<i>Xylocarpus granatum</i> Koen.	Meliaceae
<i>Aglaia cucullata</i> Roxb.	Meliaceae
<i>Merope angulata</i> (Kurz) Swingle	Rutaceae
<i>Heritiera fomes</i> Buch.-Ham.	Sterculiaceae
<i>Cerbera manghas</i> L.	Apocynaceae

The enumeration of species is based on a recent survey, collection and identification of the mangroves of Bhitarkanika ecosystem during the survey period from March, 2008 to April, 2010. Interestingly, *Cerbera manghas*, *Xylocarpus granatum*, *Xylocarpus moluccensis*, *Heritiera littoralis*, *Sapium indicum*, *Amoora cucullata*, *Intsia bijuga*, *Salicornia brachiata*, etc. are presently seen only in Bhitarkanika National Park and exhibit very poor growth. The species like *Merope angulata*, *Sarcobolus carinatus*, *Sonneratia caseolaris* and *Acanthus volubilis*, which were abundant in the past, are now represented by a few viable populations. The area of occurrence of species like *Cerbera manghas* and *Xylocarpus granatum* has shown considerable shrinkage in the recent times. The mangrove forests of Bhitarkanika National Park are under severe biotic and natural pressure. This is largely due to reclamation of the mangrove forests for paddy cultivation, prawn culture, forest clearing for human settlement and over exploitation of economically important mangrove taxa to meet the ever-increasing human needs. In addition, loss to mangrove ecosystem is caused by severe floods, cyclones and other natural disasters in the coastal belt. Thus, it is high time to conserve the existing mangrove forests and take up reclamation and revegetation programmes to save these plant resources and maintain ecological balance. Though late, still there is time to set priority for conservation and protection of the existing mangrove ecosystem, which have profound ecological and economic importance for the State of Odisha.

Acknowledgement

The authors are thankful to the Professor and Head of P.G. Dept. of Botany, Utkal University, Bhubaneswar for providing necessary facility to carry out this work. The financial assistance from MoEF, Govt. of India through the DFO, Mangrove Forest Division, Rajnagar, Department of Forest, Govt. of Orissa is gratefully acknowledged.

References

- Alongi, D.M. (1996). The dynamics of benthic nutrient pools and fluxes in tropical mangrove Forest. *J Mar. Res.* **54**: 123-148.
- Banerjee, L.K. and Das, G.C. (1972). New distributional records from Orissa coast. *Bull. Bot. Surv. India* **14**(1-4): 184-186.
- Banerjee, L.K. (1984). Vegetation of the Bhitarkanika sanctuary in Cuttack District, Orissa. *J. Econ. Tax. Bot.* **5**(5): 1065-1079.
- Banerjee, L.K. (1986). Comparative study of mangroves of the Sunderbans and that of the Mahanadi Delta in Eastern India. In: Bhosale, L.J. (ed.) *Proc. Nat. Symp. Biological Utilization & Conservation of Mangroves*. Shivaji University, Kolhapur, India, pp. 527.
- Biswal, A.K. and Choudhury, B.P. (1993). Bhitarkanika wildlife sanctuary: exploration and management. *Neo-Botanica* **1**(182): 17-22.
- Choudhury, B.P. (1984). A glimpse into the vegetation of Bhitarkanika wildlife sanctuary in the State of Orissa. *Indian Bot. Repr.* **3**(2): 121-124.
- Choudhury, B.P. (1986). Nomenclature and distribution of some mangrove elements in the Bhitarkanika wildlife sanctuary. *Newsletter Nature Wild-life Cons. Soc.* **4**(1&2): 58-60.
- Choudhury, B.P. (1990). Bhitarkanika: mangrove swamps. *J. Env. Sci.* **3**(1): 1-16.
- Choudhury, B.P., Biswal, A.K. and Subudhi, H.N. (1991). Mangroves of Orissa and aspects of their conservation. *Rheedia* **1**(1&2): 62-67.
- Choudhury, B.P. (1994). A comparative study of mangrove vegetation in Bhitarkanika and Paradeep area in the state of Orissa. In: Subba Rao, M.V. (ed.) *Forest, Wildlife and Environment*, Andhra University, Vishakhapatnam, India, pp. 33-41.
- Choudhury, B.P. (2001). Biodiversity of Orissa mangroves "Management and Conservational strategies for threatened taxa". *Nat. Bot. Soc.* **55**: 63-72.
- Haines, H.H. (1921-1925). *The Botany of Bihar and Orissa*, 6 parts. London.
- Mooney, H.F. (1950). *Supplement of the Botany of Bihar and Orissa* Catholic Press, Ranchi.
- Mishra, R.K., Sahu, J.R. and Upadhyay, V.P. (2005). Species diversity in Bhitarkanika mangrove ecosystem in Orissa, India. *Lyonia*, **8**(1): 73-87.
- Rao, T.A. and Banerjee, L.K. (1967). Some plant records for Orissa coast. *J. Bombay Nat. Hist. Soc.* **64**: 583-584.
- Rao, T.A., Banerjee L.K. and Mukherjee, A.K. (1970). Some interesting plant records from the Orissa coast. *J. Bombay Nat. Hist. Soc.* **66**: 659-660.
- Rao, T.A. and Sastry, R.K. (1974). An ecological approach towards classification of coastal vegetation of India II. Estuarine border vegetation. *Ind. For.* **100**: 438-452.
- Rao, T.A. and Banerjee, L.K. (1982). Tidal mangroves of Mahanadi delta, Utkal coast, India. In: *Proc. Sem. Res. Dev. & Env. in Eastern Ghats*, Waltair, pp. 219-226.
- Reddy, C.S., Pattanik C., Dhal, N.K. and Biswal, A.K. (2006). Vegetation and floristic diversity of Bhitarkanika National Park, Orissa, India. *Ind. For.* **132**(6): 664-680.
- Samal, R.C. and Patnaik, J.M. (1989). Management strategies for Bhitarkanika mangrove forest. In: *INDO-US Workshop on Wetlands. Mangroves and Biosphere Reserve: Government of India, Ministry of Environment and Forests*, New Delhi, India. pp. 96-99.

Folklore claims of a few interesting ferns from Deogarh district, Odisha, India

S.C. Sahu¹, N.K. Dhal^{1*} and R.C. Mohanty²

¹Natural Products Department, Institute of Minerals and Materials Technology, Bhubaneswar-751013

²P.G. Department of Botany, Utkal University, Bhubaneswar-751004

(Received : 04 October, 2010; Revision received : 23 December, 2010)

A survey of medicinal plants used by the tribal people in Deogarh district, Odisha, India is reported. Ethnobotanical study of pteridophytes are very rare in India as a whole and Odisha in particular. Here is an attempt to study the medicinal uses of ferns for different ailments like skin disease, stomach problem, rheumatism, common cold, snake bite and insomnia etc. The present paper enumerates 21 fern species with their correct botanical name followed by family, vernacular names, locality, field number, occurrence, parts used, doses and mode of administration in respect to different diseases.

Key words: Deogarh district, ferns, folklore claims

Plants and plant-based medicaments have been employed since time immemorial for prolonging human life by combating various ailments. Traditional ethnobotanical studies have received much attention due to their wide local acceptability and clues for discovery of new or less-known medicinal plants (Tripathi, 2000). The collection of information about natural flora, classification, management and use of plants by the people holds importance among the ethnobotanists. This work concentrates on medicinal value of some selected ferns, commonly used by the tribal populace of Deogarh district.

The pteridophytic flora of India is comprised of 800-1000 taxa (Dixit, 1984) against the estimated number of about 9800 species of pteridophytes in the world flora (Mabberley, 1997). Dixit (1996) presented a taxonomic account of 141 species of pteridophytes that belong to 66 genera of 41 families in the 'Flora of Orissa' and arranged these taxa as per the classification of Pichi-Sermolli (1977). Panigrahi (1993 and 1998) studied the taxonomy and nomenclature of 136 species of pteridophytes that belong to 53 genera under 27 families and enumerated them according to modern system of classification based on Kramer and Green (1990) and Brummit (1992).

Deogarh district is located between 21° 31'53" N latitude and 84° 43'2" E longitude with a total area

of 6702 sq. km towards the north-west part of the state of Odisha. The study involves intensive explorations and critical study of specimens for 2 years. Regular field trips were made in such a way so as to cover all the areas of the district at regular intervals in different seasons from 2007 to 2008. The ferns with folklore claims were collected, processed, critically studied and identified consulting the regional floras (Haines, 1921-25; Mooney, 1950; Gamble, 1915-36; Saxena and Brahmam, 1994-96) and preserved in the herbarium of RRL-B at IMMT, Bhubaneswar.

The details have been provided in enumeration with correct botanical name followed by family, vernacular names, locality and field number, occurrence, parts used, doses and mode of administration in respect to different diseases.

The data on medicinal plants, which were collected from inhabitants in and around different forest pockets of Deogarh district, were pooled and analysed. The investigation revealed the medicinal ferns of 21 species and 16 genera belonging to 16 families, which are commonly used in various ailments by various tribes (G-Gonda, M-Munda, K-Kondha, S-Saura etc.) of the area surveyed. The enumeration and utilization of these plants are mentioned below.

* Corresponding author, Email : nkdhal@immt.res.in

Adiantum incisum Forssk. (Adiantaceae), 'Dodhali' (K)
Rhizome paste is externally applied against snake bite. (Katasaraghati, 11590)

Adiantum philippense L. (Adiantaceae), 'Dodhali' (G)
Tender leaf paste mixed with honey (1g: 1 tsp) is taken once a day for one week against severe cough. (Koradkot, 10063)

Alsophila gigantea Wall. ex Hook. (Cyatheaceae), 'Sankukuri' (G)

Leaf paste is applied on the affected part externally for early maturation of boils. (Khuludi, 12041)

Angiopteris evecta (Forst.) Hoffm. (Angiopteridaceae), 'Maranga' (S)

Tender leaf paste mixed with the equal amount of stem juice of 'Hadabhanga' (*Cissus quadrangula*) is applied twice a day for 15 days against Filaria. (Pallahara, 7168)

Blechnum orientale L. (Blechnaceae), 'Vomban' (M)
Rhizome paste (3g) is applied over the boil for its early maturation. (Koradkot, 10069)

Ceratopteris thalictroides (L.) Brongn. (Parkeriaceae), 'Kurla' (M)

Leaf paste is applied on the affected part against eczema. (Melichua, 11677)

Cheilanthes farinosa (Forssk.) Kaulf. (Cheilantheaceae), 'Chhota mahajal' (M)

Rhizome juice mixed with 'toddy' obtained from Khajuri (*Phoenix sylvestris*) juice (1 glass: 10 ml) is given as general tonic. (Dantaribahal, 10173)

Cheilanthes tenuifolia (Burm.f.) Sw. (Cheilantheaceae), Nanha dodhari (M)

Dried powder of whole plant (1 tsp) mixed in 1 glass of light boiled water is taken once a day for 15 days against indigestion and stomach problem. (Purumunda, 11617)

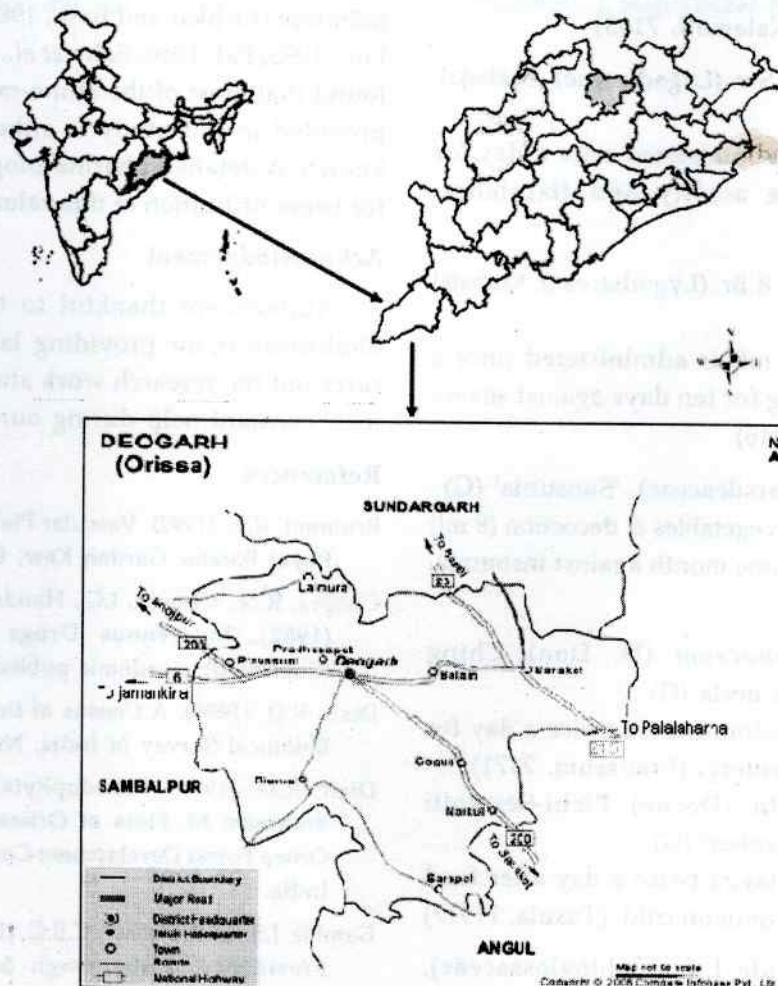


Fig. 1 : Map of the study area

Christella dentata (Forssk.) Brownsey & Jermy (Thelypteridaceae), 'Hadazoda' (M)

Rhizome paste is applied in case of spondylitis. (Kudapara, 10040)

Christella parasitica (L.) Lev. (Thelypteridaceae), 'Hadazoda' (G)

Rhizome paste (2 g) mixed with 4 g of young leaf paste of *Costus speciosus* is administered for fifteen days against rheumatism. (Dantaribahal, 10068)

Diplazium esculentum (Retz.) Sw. (Athyriaceae), 'Ranuran' (K)

Young leaves decoction (3 ml) mixed 4 ml tuber juice of 'Satabari' (*Asparagus racemosus*) is administered in empty stomach for ten days against general weakness. (Khuludi, 12042)

Equisetum diffusum D.Don (Equisetaceae), 'Langi' (M)
Powdered root (2 g) mixed with honey (10 ml) is administered twice a day for one week after food in case of severe cough. (Kalamati, 7163)

Lygodium flexuosum (L.) Sw. (Lygodiaceae), 'Mahajal' (M)

Rhizome paste (5 g) is administered once a day for 15 days against acute acidity and flatulence. (Koradkot, 10070)

Lygodium microphyllum R.Br. (Lygodiaceae), Mahajal (M)

Fresh leaf decoction (5 ml) is administered once a day in the early morning for ten days against severe dry cough. (Tusula, 11916)

Marsilea minuta L. (Marsileaceae), 'Sunsunia' (G)
Leaves are used as leafy vegetables & decoction (8 ml) is taken twice a day for one month against insomnia. (Tusula, 10912)

Microsorium membranaceum (D. Don) Ching (Polypodiaceae), Partha mula (G)

Rhizome paste (7 g) is administered twice a day for three days against dysentery. (Pravasuni, 7171)

Nephrolepis delicatula (Decne) Pichi-Sermolli (Nephrolepidaceae), 'Kanher' (G)

Rhizome paste (2 g) is taken twice a day after food for seven days against common cold. (Tusula, 11917)

Ophioglossum nudicaule L.f. (Ophioglossaceae), 'Mukuna' (S)

Rhizome paste is externally applied in affected part to check of joint pains. (Khulundipani, 7165)

Pteris biaurita L. (Pteridaceae), 'Phulka' (G)

A paste of matured leaves is applied externally against swollen shoulder. (Pravasuni, 9942)

Selaginella indica (Milde) Trayon (Selaginellaceae), 'Sanjibani' (K)

Whole plant paste mixed with the oil of 'Kusum' (*Schleichera oleosa*) is externally applied in case of wounds caused by scorpion bite. (Pallahara, 7161)

Selaginella repanda (Desv.ex Poir.) Spring (Selaginellaceae), 'Mrutusanjibani' (M)

Whole plant paste is externally applied to arrest bleeding in case of fresh cut. (Khandamunda, 11703)

In summary, this paper presents information on uses of 21 fern species of 16 families for different diseases among tribes of Deogarh district of Odisha. These information were checked with available literature (Kirtikar and Basu, 1980; Chopra *et al.*, 1982; Jain, 1995; Pal, 1980; Sahu *et al.*, 2009 & 2010). It was found that most of the ethno-medicinal information provided in this study is either interesting or less known. A detailed pharmacological study is needed for better utilization of this valuable natural resource.

Acknowledgement

Authors are thankful to the Director, IMMT, Bhubaneswar for providing laboratory facilities to carry out the research work and tribal informers for their constant help during our explorations.

References

- Brummit, R.K. (1992). Vascular Plant Families and Genera. Royal Botanic Garden Kew, U.K.
- Chopra, R.N., Chopra, I.C., Handa, K.L. and Kapur, L.D. (1982). Indigenous Drugs of India; 2nd Edn. (Reprinted), Academic publishers, New Delhi, India.
- Dixit, R.D. (1984). A Census of the Indian Pteridophytes. Botanical Survey of India, New Delhi, India.
- Dixit, R.D. (1996). Pteridophyta. In: Saxena H.O. and Brahmam M. Flora of Orissa Vol. 4. pp. 2536-2654, Orissa Forest Development Corporation, Bhubaneswar, India.
- Gamble, J.S. and Fischer, C.E.C. (1915-36). Flora of Madras Presidency. Bishensingh & Mahendrapal Singh, Dehradun, India.

- Haines, H.H. (1921-25). The Botany of Bihar and Orissa, 6 parts: Bishensingh & Mahendrapal Singh, Dehradun, India.
- Jain, S.K. (1995). A Manual of Ethnobotany; Scientific Publ. Jodhpur, India.
- Kirtikar, K.R. and Basu, B.D. (1980). Indian Medicinal Plants. Bishensingh & Mahendrapal Singh, Dehradun, India.
- Krammer, K.U. and Green, P.S. (1990). The Families and Genera of Vascular Plants, Pteridophytes and Gymnosperms, Vol. 1, Springer Verlag, Berlin, Germany.
- Mabberley, D.J. (1997). The Plant Book (2nd Edn.), Cambridge University Press, U.K.
- Mooney, H.F. (1950). Supplement to the Botany of Bihar and Orissa. Ranchi, India.
- Pal, D.C. (1980). Observations of folklore about plants used in veterinary medicine in Bengal, Orissa and Bihar. Bull. Bot. Surv. Ind. 22(1-4): 96-99.
- Panigrahi, G. (1993). Conspectus of the pteridophytic flora of Orissa, Part-I: The fern allies. In: Gupta, B.K. (ed.) Higher Plants of Indian Sub-Continent. Vedan Books, India, Vol. IV, pp. 103-117, Ind. J. Forest. Add. Ser. No. VII.
- Pichi-Sermolli, R.E.G. (1977). Tentamen pteridophytorum in genera taxonomicum ordinem redigendi. Webbia. 31: 313-512.
- Sahu, S.C., Dhal, N.K. and Mohanty, R.C. (2009). Ethnobotanical study of Deogarh district (Orissa) with respect to plants used for treating skin diseases. Ethnobotany, 21 (1&2): 46-50.
- Sahu, S.C., Dhal, N.K. and Mohanty, R.C. (2010). Potential medicinal plants used by the tribals of Deogarh district, Orissa, India. Ethnomedicine, 4(1): 53-61.
- Saxena, H.O. and Brahman, M. (1994-1996). Flora of Orissa Vol. I-IV. Orissa Forest Development Corporation Ltd., Bhubaneswar, India.
- Tripathi, Y.C. (2000). Ethnomedicinal treasure of tribal Rajasthan. J. Non-Timber For. Prod. 7(1-2): 77-84.

INSTRUCTIONS TO CONTRIBUTORS

Plant Science Research publishes original review articles and results of scientific research in Botany as full papers or short communications in English. It is open only to the members of the Society. Publication is facilitated if authors ensure that the manuscript and illustrations meet the requirements outlined below before they are submitted.

1. Two copies of the manuscript along with the illustrations should be sent to:
Prof. P.K. Chand
Managing Editor
Plant Science Research
P.G. Department of Botany, Utkal University, Bhubaneswar - 751 004 (India)
2. All parts of the manuscript should be typed double spaced with the left margin at least 40 mm wide. Each page of the article should be numbered.
3. The first page should carry only the title of the article, name(s) of author(s) and institution(s) where work was done. An additional sheet should carry the running title (not to exceed 50 letters and spaces) and the address for correspondence.
4. Papers should have an abstract in English not exceeding 200 words in the beginning and the usual pattern i.e. Introduction, Materials and methods, Results, Discussion and References, should be followed for the text. Short communication should have an abstract in the first paragraph but without any subheadings like Introduction, etc., and should not exceed 4 foolscap typed pages in double space.
5. Footnotes are not allowed, except under tables.
6. Symbols, units and nomenclature should conform to international usage. For all the numerical data metric system should be followed.
7. Text references should be made using the authors name followed by a year within parenthesis.
Example : Murashige and Skoog (1962).
8. The references should be listed alphabetically at the end of the paper and have the form:
 - i) *Journal*: Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.
 - ii) *Edited volumes*: Bell, J.M.B. and Mudd, C.H. (1976). Sulphur dioxide resistance in plant: A case study of *Lolium perenne*. In : Mansfield, T.A. (ed.) *Effects of Air Pollution on Plant*. Cambridge University Press, Cambridge, U.K., pp. 87-103.
 - iii) *Books*: Halliwell, B. and Gutteridge, J.M.C. (2007). *Free Radicals in Biology and Medicine*. 4th Edn. Oxford University Press, New York. U.S.A.Unpublished materials and personal communications are accepted only as references in the text, not in the bibliography.
9. Tables should be typed on separate sheets furnished with short explanatory heading and be referred to in text. They must be planned to fit in a printed page within a length of either 8 or 16 cm.
10. Illustrations should be drawn on separate sheets in Indian ink and photograph be printed black and white on glossy papers with strong contrast. They should be planned to appear with a maximum final width of either 8 cm or 16 cm. Letters, numbers and symbols must appear clearly, but should not be oversized. In diagrams, the top and right side should be boxed in by lines or the same type as the abscissa and ordinate. Each figure should be identified with author's name, figure number and indication of orientation. Combination of photographs and pen drawings in one plate should be avoided.
11. Authors receive one proof in which they should correct only printing errors. Major additions or change at the proof stage will be charged extra.
12. Printing cost at the rate of Rs.200/- (two hundred only) per page and actual cost of blocks are charged to the authors.
13. Twenty five reprints without cover will be supplied free of cost to the authors.
14. Acceptance of any paper is subject to the recommendations of the reviewers, who are specialists in their fields. The Editorial Board reserves the right to reject any paper found unsuitable for the journal.

PLANT SCIENCE RESEARCH

Vol. 32, 2010

TABLE OF CONTENTS

1. Physiological, genetic and molecular basis of submergence tolerance in rice: A review B.P. Das, M.K. Kar, P. Dash and A.T. Roy	1
2. Micropropagation of pomegranate through <i>in vitro</i> culture of meristems: A review S.K. Naik and P.K. Chand	9
3. Low cost <i>in vitro</i> propagation: A review A.K. Behera, S. Gochhait and U.B. Mohapatra	18
4. Effects of chelating agents and metal ions on photochemical activity, biochemical alterations and attenuation of nickel toxicity in wheat N. Patnaik, B. Satpathy and H.K. Patra	32
5. Effect of salinity on biochemical changes and OJIP fluorescence parameters of <i>Rhizophora mucronata</i> L. S. Acharya, M. Kar and P.K. Mohapatra	40
6. Nutrient-alginate encapsulated nodal explants of butterfly pea (<i>Clitoria ternatea</i> L.) as synseeds for germplasm conservation and exchange S.S. Swain, A. Pal, L. Sahu, C. Pradhan, D.P. Barik and P.K. Chand	48
7. <i>In vitro</i> propagation of <i>Acalypha indica</i> L.: A potential medicinal plant G. Rout, P.R. Biswal, L.P. Mishra and S.P. Rath	56
8. <i>In vitro</i> response of cotyledon and hypocotyl explants of flax <i>Linum usitatissimum</i> L. S. Gochhait, S. Dwibedy, A.K. Behera, D. Behera and U.B. Mohapatra	62
9. Metabolic performance of microbes in rice field under conventional and organic farming systems D. Chhotaray, P.K. Mohapatra and C.S.K. Mishra	68
10. Metal ions affect the growth and amylase activity of thermotolerant <i>Bacillus</i> sp. isolated from 'Atri' hot spring of Odisha (India) E. Maric, A.K. Dalai and B. Behera	75
11. Synthesis of biotechnologically relevant amylase from wheat bran using native isolates of <i>Aspergillus tamarii</i> B.K. Sethi, P.K. Bhuyan, S. Rout, P.K. Nanda and S.L. Sahoo	81
12. Production of value-added phenolic compounds from wheat bran using isolates of <i>Staphylococcus aureus</i> P.K. Sarangi and H.P. Sahoo	87
13. Identification of endophytic fungi associated with rice seeds by molecular techniques U. Dhua, B. Bhattacharjee, S.R. Dhua, A. Chhotaray, I. Sahoo and S. Nayak	92
14. Orange peel based fermented plant beverage against antibiotic resistant human pathogens P. Pradhan, R.C. Mohanty and A.K. Mishra	101
15. Influence of seasonal variation and HCH pre-treatment on survival and longevity of HCH degrading aerobic bacteria in soil S.K. Sahu, J. Chakrabarty, S. Panda, L. Choudhury, S. Biswas, N.K. Mohapatra and N. Sethunathan	107
16. Effect of soils of varied pH on the growth and N ₂ fixation of the water fern <i>Azolla</i> K.B. Satapathy	114
17. Changes in biochemical constituents in betel leaf (<i>Piper betle</i> L.) during storage using banana leaves N.P. Das, J.R. Rout, S.L. Sahoo and P.K. Mishra	119
18. Current vegetation status of mangroves and their associates of Bhitarkanika National Park, India S.K. Pattnaik, P.C. Panda and H.K. Patra	127
19. Folklore claims of a few interesting ferns from Deogarh district, Odisha, India S.C. Sahu, N.K. Dhal and R.C. Mohanty	138