Maejo Int. J. Sci. Technol. **2009**, *3*(02), 235-247

Maejo International Journal of Science and Technology

ISSN 1905-7873

Available online at www.mijst.mju.ac.th

Full Paper

Identification of microsatellite markers (SSR) linked to a new bacterial blight resistance gene *xa33*(t) in rice cultivar 'Ba7'

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Received: 24 October 2008 / Accepted: 9 April 2009 / Published: 4 May 2009

Abstract: This study attempts to identify a new source of bacterial blight (BB) resistance gene and microsatellite makers (SSR) linked to it. A total number of 139 F_2 progenies generated from a cross between the resistant donor 'Ba7'and 'Pin Kaset' were developed and used for this study. A Thai *Xoo* isolate, TXO16, collected from Phitsanulok province, was used to evaluate the resistance reaction in the F_2 population. The segregation ratio of resistance (R) and susceptibility (S) was statistically fitted to 1R:3S model indicating single recessive gene segregation. Twenty F_2 individuals consisting of 10 resistant and 10 susceptible plants were chosen for DNA analysis. Sixty-two polymorphic markers covering all rice chromosomes were used to identify the location and linked markers of the resistance gene. Four SSR markers, viz. RM30, RM7243, RM5509 and RM400, located on the long arm of rice chromosome 6, could clearly discriminate between resistant and susceptible phenotypes, and 161 $BC_2F_{2:3}$ individuals carrying BB resistance gene were developed through MAS using these SSR markers. This population was inoculated with TXO16 to validate and confirm the location of the gene and linked markers. The segregation ratio was statistically fitted to 1R:3S model confirming a recessive nature of the gene action in this germplasm. Phenotypic-genotypic association including five additional markers suggested that RM20590 was tightly linked to this resistance gene (R^2=59.12 %). The BB phenotype was controlled by a recessive gene with incomplete dominance of susceptible allele providing intermediate resistance to *Xoo* pathogen in heterozygotes. The location of the gene was in the vicinity of a dominant gene, *Xa7*, which was previously reported. However, the resistance gene identified here was different from *Xa7* because of the different nature of gene action. Consequently, this gene was tentatively designated as *xa33*(t). The resistance gene from rice cultivar 'Ba7' and the closely linked markers found in this study will be useful for rice breeders as a source to improve BB resistance through MAS in rice breeding programs.

Keywords: bacterial blight, rice, SSR marker, *Xanthomonas oryzae* pv. *oryzae*, *xa33*(t)

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Introduction

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most destructive diseases in rice-producing areas in Thailand and it is especially prevalent in irrigated and rainfed lowland rice growing areas. BB was first characterised in Fukuoka (Japan) in 1884 [1]. In Thailand, the damage of rice crop from BB was first reported in 1963 in Pathum Thani province [2]. BB disease can cause yield loss typically ranging from 20-30%, but in severely cases it can cause as high as 50% yield reduction [1]. Control measures for BB include cultural practices, chemical control, biological control, disease forecasting, and most importantly, host genetic resistance. Since the most effective chemical control is not yet available, the utilisation of resistant varieties carrying resistance genes has been considered to be the most effective way to control the disease [3]. Most researchers are interested in utilising BB-resistant varieties, and this goal is certainly achievable provided that an easy strategy to identify resistance genes is available.

At present, identification, cloning and functional analysis of a gene can be performed more rapidly. Up to date, more than 40 disease-resistant genes have been identified in dicot and monocot plants [4]. In the case of BB resistance, more than 30 BB resistance genes have been identified in cultivated rice and the wild relatives [3, 5-6]. Eleven of them are recessive resistance genes (*xa5, -xa5(t), xa8, xa13, xa15, xa19, xa20*, *xa24*, *xa28*, *xa31* and *xa32*) [3, 6-7], while six of them are cloned (*Xa1, xa5*, *xa13*, *Xa21 Xa26* and *Xa27*) [8-13]. Among all BB resistance genes, three of them were reported and mapped on rice chromosome 6. The first one, *Xa7*, is a dominant resistance gene originally identified in rice cultivars 'DV85' and 'DV87'. The second BB resistance gene, *Xa27*, identified in wild rice *O. minuta*, has also been mapped to the same region as *Xa7* [14-15], and the resistance gene has been cloned afterward [13, 16]. The last one, a recessive BB resistance gene *xa32*, identified in wild rice *O. barthii*, is located on the short arm of the same chromosome [6].

The majority of BB resistance genes were identified in rice *O. sativa* ssp. *indica* and wild rice *O. longistaminata*, *O. rufipogon*, *O. minuta* and *O. officinalis*, while some of them were identified in *O. sativa* ssp. *japonica* [16-17]. Most of these genes follow the classic gene-for-gene concept for the race-specific interaction between rice and *Xoo* [18]. Avirulent gene in bacteria exhibits the specificity for resistance gene in the rice plant. Some resistance genes are effective only in adult plants, while others are effective at all stages of growth. Some genes confer resistance to a broad spectrum of *Xoo* races, whereas others do so against only one or a few races. This observation could be influenced by particular geographical locations [3]. The developmental control of disease resistance has been observed in other plant-pathogen systems. Several rice resistance genes are expressed at the highest level only at the adult stage [19-20]. *Xa21*-mediated resistance increases progressively from susceptible juvenile stage to full resistance at the later adult stage, while *Xa7* shows broad resistance only in adult plants [21]. However, the effective gene at all growth stages appears to be *xa5* gene as it can confer resistance and exhibit a broad spectrum of resistance to *Xoo* isolates throughout Asia except India and Nepal [22].

The first step towards rice improvement via marker-based selection and map-based cloning of the resistance genes is the identification of molecular markers that are tightly linked to the genes of interest. Recent advances in molecular marker technology have made it easier to identify and introgress resistance genes to desired genetic backgrounds. Several major resistance genes against bacterial blight pathogen have been tagged by restriction fragment length polymorphism (RFLP) and randomly

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amplified polymorphic DNA sequence (RAPD) markers [23-25]. In addition, simple sequence repeat (SSR) markers have been extensively used to identify disease resistance genes in rice [26-27]. They provide several advantages over the other two types of markers when applied in a plant breeding program. Markedly, they are based on the polymerase chain reaction (PCR) technique, represent single loci, and can detect high levels of polymorphism.

In this study, we aim at using SSR markers to identify the BB resistance gene in rice cv. 'Ba7' and finding the markers tightly linked to this gene. These markers would be useful for the improvement of BB-resistance rice breeding program through marker-assisted selection (MAS).

Materials and Methods

Plant materials

The *indica* rice cultivar 'Ba7' was used as a BB resistance donor, in a cross with the recurrent parent, 'Pin Kaset' (PK) to develop an F_2 population. The population consisted of 139 progenies that were used as plant materials to identify the genomic location of a BB resistance gene. The backcross breeding and MAS strategies were used to develop the backcross population to validate the linked markers and confirm the location of resistance gene. The F_2 resistant plant was crossed with the recurrent parent to generate 98 BC_1F_1 individuals. DNA markers indentified in the F_2 population were used to select BC_1F_1 plants carrying the resistance gene, and 10 selected BC_1F_1 plants based on desired plant type were then crossed with PK to generate 122 BC_2F_1 individuals. DNA markers were used to identify the 52 BC_2F_1 plants carrying the resistance gene. A heterozygous plant was self-pollinated to produce 838 BC_2F_2. After that, four heterozygous BC_2F_2 plants were self-fertilised to produce 161 $BC_2F_{2:3}$ plants. All these plants were used individually to validate the effect of the resistance gene and the relationship between BB resistant phenotype and linked DNA markers (Figure 1).

Bioassay of BB resistance

A *Xoo* isolate, TXO16, collected from Wang Thong district, Phitsanulok province, Thailand, in 2003 was used in this study for the BB resistance evaluation. This isolate showed an incompatible reaction to 'Ba7' and a compatible reaction to PK. The isolate was grown in peptone sucrose agar medium (5 g peptone, 20 g sucrose and 15 g agar, adjusted to 1 litre with distilled water) for 72 hours at 28^{o}C. The bacterial cells were suspended in sterile water adjusted to 10^9 CFU/ml. TXO16 was assayed for a resistance reaction in F_2 and $BC_2F_{2:3}$ population (Figure 1). BB inoculation was done in the greenhouse using the leaf-clipping method [28]. Resistance reactions were recorded based on the mean of lesion length (LL) of an individual plant. One hundred and thirty-nine F_2 plants were inoculated 30 days after sowing, whereas 161 $BC_2F_{2:3}$ plants were inoculated 60 days after sowing. Three to four fully expanded leaves of each plant were inoculated. LL was measured at 12-14 days after inoculation. Reaction to BB was classified as resistant (R) when the LL was less than or equal to that of the donor parent (Ba7), and as susceptible (S) when it was longer.

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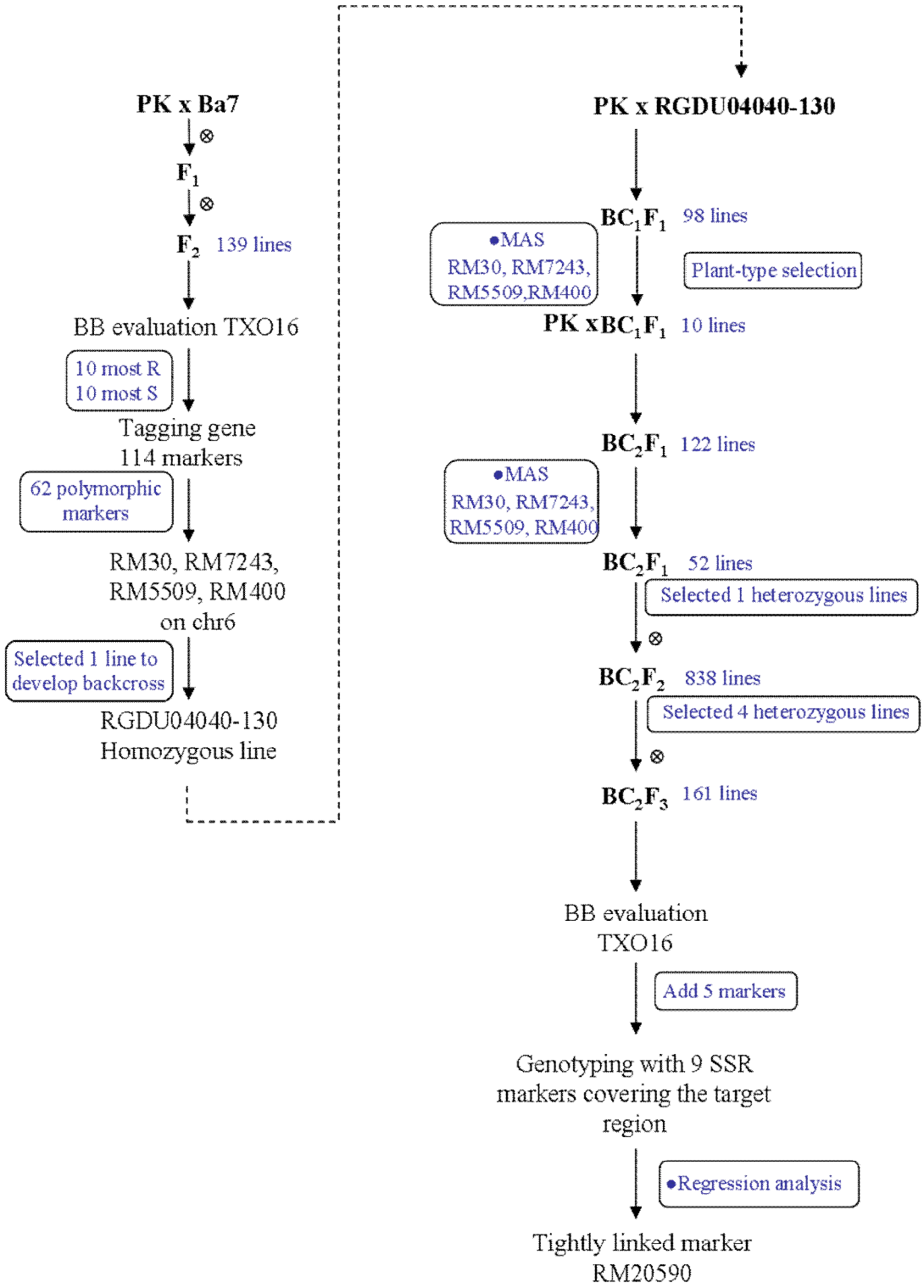

Figure 1. The development of rice lines for identifying SSR markers linked to the resistance gene in rice cultivar 'Ba7'

SSR markers and association analysis

F_2

One hundred and fourteen rice SSR markers covering the 12 linkage groups were analysed for polymorphism between 'Ba7' and PK. Ten most resistant and susceptible F_2 plants were selected based on their LL and genotypes as identified by 62 polymorphic SSR markers.

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$\underline{BC_2F_{2:3}}$

Four flanking SSR markers for BB resistance genes, viz. RM30, RM7243, RM5509 and RM400, were used for MAS and genotyping the $BC_2F_{2:3}$ progenies to validate the resistance gene and identify tightly linked markers. Five SSR markers tightly linked to BB resistance genes *xa33*(t), RM20523, RM20536, RM3430, RM20590 and RM340, were added into the $BC_2F_{2:3}$ genotype. These markers were obtained from the public database released by Gramene (http://www.gramene.org/). All of the genetic associations were analysed based on simple linear regression and ANOVA in STATGRAPHIC 2.1 program.

DNA extraction and PCR amplification

The DNA extraction of F_2 and $BC_2F_{2:3}$ population was conducted using the DNA trap® kit (DNA Technology Laboratory). The PCR amplification reactions for SSR markers were carried out with a total volume of 10 µl containing 20 ng of genomic DNA, 0.02 µM of each primer, 0.2 mM each of dNTPs, 2.5 mM $MgCl_2$, 0.2 unit *Taq* polymerase, and 1X PCR buffer. Amplification was performed for 35 cycles (30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C) followed by a final extension of 5 min at 72°C. Amplified products were separated by 4.5% denaturing acrylamide gel electrophoresis and were detected by the silver staining method.

BB resistance reaction patterns between Ba7 and IRBB7

Rice variety 'IRBB7' developed by IRRI was known to carry the dominant gene *Xa7*. It was used to compare BB resistance reaction pattern with 'Ba7'. The *Xa7* and *xa33*(t) were located in the same region on chromosome 6. Sixty-three *Xoo* isolates, collected from major rice growing areas in the north and north-east of Thailand, were used for BB evaluation. The resistance reaction was classified as resistant (R), moderately resistant (MR), moderately susceptible (MS) and highly susceptible (S) when the LL was 0-3 cm, 3.1-6.0 cm, 6.1-9.0 cm and more than 9.0 cm respectively [29].

Results

Phenotypic distributions

Continuous distributions of LL were observed in F_2 and $BC_2F_{2:3}$ population (Figures 2-3). Averages of LL ranged from 0.9 - 2.1 cm and 6.0 - 8.6 cm for 'Ba7' and PK respectively. When the cutoff was based on the mean and standard error of 'Ba7', the numbers of resistant and susceptible F_2 and $BC_2F_{2:3}$ were 37 and 102, and 38 and 124 respectively. These segregation ratios fit well with the expected 1R:3S at χ^2=0.19, p=0.65 and χ^2=0.21, p=0.65 respectively, thereby confirming that the major quantitative trait locus (QTL) for BB resistance in 'Ba7' was governed by a single recessive gene. In our study, inoculations of plants were conducted at two different growth stages, i. e. seedling (30-day-old plants) and tillering (60-day-old plants) stages. It should be noted that LL at the seedling stage was a little bit longer than one at the tillering stage.

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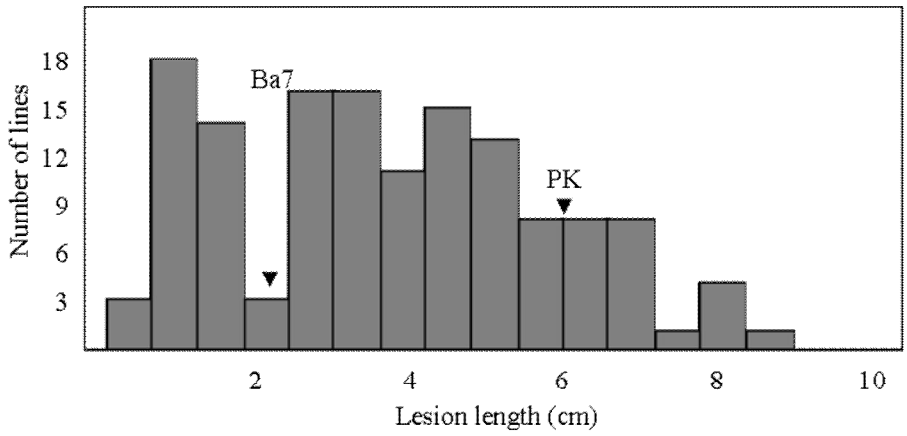

Figure 2. Distribution of LL after inoculation with Thai *Xoo* strain, TXO16, in a sample consisting of 139 individuals from a F_2 population derived from a cross between 'Ba7' and PK. The average LL of 'Ba7' and PK were 2.1 ± 1.4 cm and 6.0 ± 1.6 cm respectively.

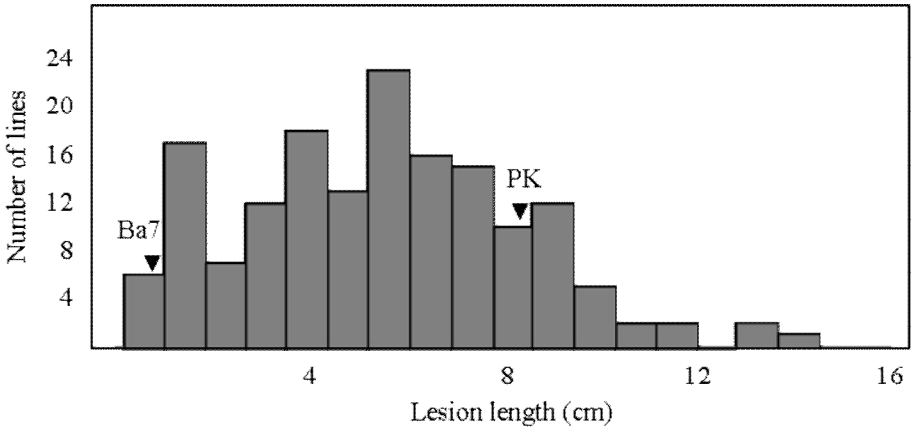

Figure 3. Distribution of LL after inoculation with Thai *Xoo* strain, TXO16, in a sample consisting of 161 individuals from the $BC_2F_{2:3}$ population derived from a cross between 'Ba7' and PK. The average LL of 'Ba7' and PK were 0.9 ± 0.8 cm and 8.6 ± 1.7 cm respectively.

Comparison of BB resistance in Ba7 and IRBB7

Race-specific BB resistance of 'Ba7' and 'IRBB7' were compared at the seedling stage (30 days after sowing) using 63 *Xoo* isolates collected from major rice growing areas in the north and north-east of Thailand (data not shown). 'Ba7' was resistant to 44 isolates, whereas 'IRBB7' was resistant to 41 isolates. Out of 63, 12 isolates showed different patterns or degrees of resistance between 'Ba7' and 'IRBB7' (Table 1). 'Ba7' was resistant to TXO56, while 'IRBB7' was susceptible to TXO56. In contrast, 'Ba7' was susceptible to TB0096 but 'IRBB7' was resistant to TB0096. These results indicated that 'Ba7' and 'IRBB7' were different in their specificity to BB isolates.

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Table 1. Twelve *Xoo* isolates showing different resistance patterns between 'Ba7' and 'IRBB7'

Xoo isolate	Collection area		Resistance pattern	
	Province	Region	Ba7	IRBB7
TB0096	Phitsanulok	North	S	R
TB0304	Chiang Rai	North	S	MS
TB9602	Chiang Mai	North	R	MR
TXO53	Phrae	North	S	MS
TXO55	Chiang Rai	North	MR	S
TXO56	Chiang Rai	North	R	S
TXO103	Ubon Ratchathani	North-east	MS	MR
TXO111	Ubon Ratchathani	North-east	MR	MS
TXO114	Khon Kaen	North-east	MR	R
TXO116	Khon Kaen	North-east	MS	S
TXO121	Udon Thani	North-east	R	MR
TXO122	Udon Thani	North-east	MR	MS

Tagging the major QTL with SSR markers

Out of 114 SSR markers tested for polymorphism, 62 markers revealed clear discrimination between 'Ba7' and PK. These markers were used to identify the genotype of twenty F_2 plants (10 resistant and 10 susceptible plants) and their parents. Four SSR markers, RM30, RM7243, RM5509 and RM400, produced distinguishable band patterns between resistant and susceptible plants as shown in Figure 4. All of them were located on the long arm of rice chromosome 6.

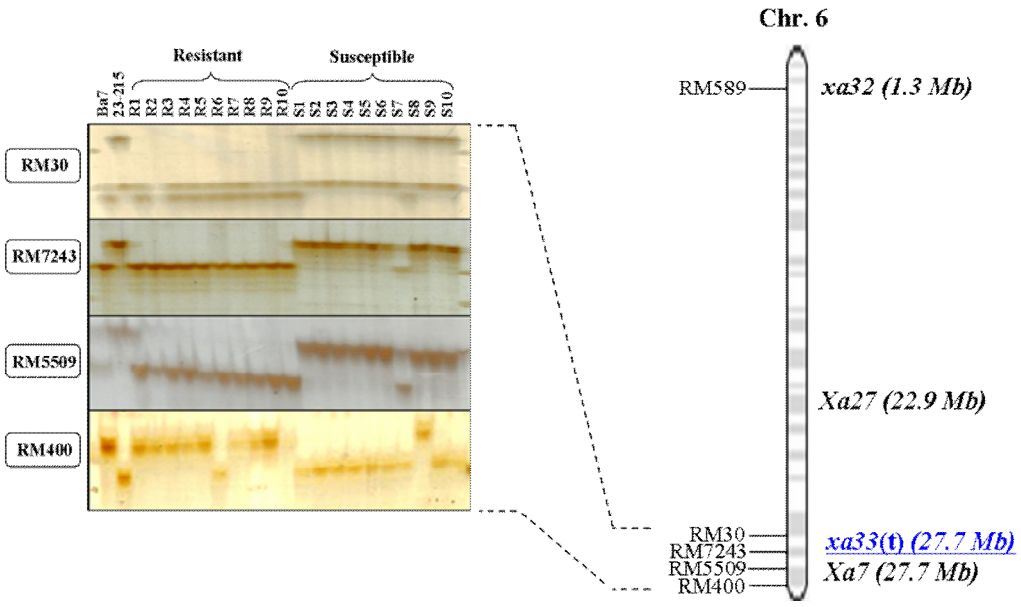

Figure 4. The SSR markers RM30, RM7243, RM5509 and RM400, located on the long arm of chromosome 6, showed distinguishable band patterns of resistant and susceptible F_2 plants and were identified as the possible linked markers to a BB resistance gene in 'Ba7'. These markers were located in the vicinity of reported *Xa*7.

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Identification of the major QTL by phenotype-genotype association

Four selected BC_2F_2 plants that showed heterozygozity via 4 SSR markers mentioned above were self-pollinated to generate 161 $BC_2F_{2:3}$ individuals. These individuals were evaluated for BB resistance reaction and their genotypes were classified by TXO16. RM20523, RM20536, RM3430, RM30, RM7243, RM5509, RM400, RM20590 and RM340 covering this QTL region. Regression analysis confirmed that LL was significantly associated with eight markers designated RM20523, RM20536, RM3430, RM30, RM7243, RM5509, RM20590 and RM400 (27.15-28.43 Mb). Multiple regression analysis indicated that RM20590 and RM5509 were closer to the targeted BB resistance gene than the others. The RM20590 explained 59.12 % of LL variation and appeared to be the closest linked marker in this experiment as shown in Table 2.

Table 2. Phenotype-genotype association analysis using ANOVA and regression analysis in the $BC_2F_{2:3}$ population from the cross between PK and Ba7. The mean of LL was significantly associated with eight SSR markers. (Ba7 = homozygous Ba7, H = heterozygous, and PK = homozygous Pin Kaset.)

Marker	Genome position (Mb)	R^2	Mean of LL (cm)			
			Ba7	H	PK	
RM20523	27.15	58.20**	1.6a	5.1b	8.3c	**
RM20536	27.16	48.88**	1.7a	5.0b	8.0c	**
RM30	27.25	58.20**	1.65a	5.09b	8.34c	**
RM3430	27.43	52.00**	1.94a	5.04b	8.24c	**
RM7243	27.56	57.16**	1.48a	5.11b	8.16c	**
RM5509	27.82	58.33**	1.53a	5.17b	8.31c	**
RM20590	28.01	59.12**	1.49a	5.13b	8.41c	**
RM400	28.43	52.23**	1.60a	5.28b	8.30c	**
RM340	28.59	ns	4.48a	5.27a	5.59a	ns

Notes:
** = significant at 0.01 level
ns = not significant
Means of LL followed by different letters in the same row were significant at $P < 0.01$ by Least Significant Difference (LSD).

Gene action of the major QTL

The LL of $BC_2F_{2:3}$ progenies after inoculation with *Xoo* isolate TXO16 showed a continuous distribution comprising of three phenotypic classes, as seen in Table 2 and Figure 5. Out of 161 $BC_2F_{2:3}$ individuals screened with the closely linked marker RM20590, the results indicated that 27 $BC_2F_{2:3}$ plants were resistant, 99 were moderately resistant, and 35 were susceptible, corresponding to homozygous 'Ba7' alleles (*xa33/xa33*), heterozygous alleles (*Xa33/xa33*), and homozygous PK (*Xa33/Xa33*) alleles respectively. The plants carrying heterozygous alleles (*Xa33/xa33*) exhibited an

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intermediate resistance in response to the *Xoo* pathogen, demonstrating that the inheritance of *xa33*(t) is a recessive gene with an incomplete dominance of susceptible allele in gene action.

Figure 5. Distribution of LL of $BC_2F_{2:3}$ population after inoculation with TXO16. The red, yellow and blue bars represented homozygous Ba7 plants, heterozygous plants and homozygous PK plants respectively. Based on RM20590 genotypes, the Ba7 homozygotes, the heterozygotes and the PK homozygotes showed resistant, moderately resistant and susceptible phenotypes respectively.

Discussion

The study of identification, tagging, cloning and functioning of a gene can be performed effectively and rapidly in recent years. The near isogenic lines analysis [30] and bulked segregant analysis (BSA) [31] are used to rapidly identify DNA markers linked to the resistance genes. The use of these methods requires the construction of extremely resistant and susceptible bulks, and the reliability of the experimental results depends on the accuracy of phenotype validation [32]. In our study, we modified the BSA method by analysing individual plants from extremely resistant and susceptible groups. The advantage of this modified method is the higher resolution that it provides with consequent high tendency to the location of genes in comparison to the conventional BSA. In addition, we can monitor the linkage tendency of the molecular markers for the resistance gene, while traditional BSA does not allow us to do so because individuals are bulked for analysis. Although, after tagging the gene, we did not analyse the association between phenotypes and genotypes, nevertheless we could use the flanking molecular markers from the tagged gene for MAS directly.

The resistance gene which we identified in rice 'Ba7' was located on the long arm of chromosome 6. In this region, two other dominant genes (*Xa7* and *Xa27*) have been reported [15, 33]. *Xa7* was originally identified in rice cultivar 'DV85' [33]. A tightly-linked marker, RG1091, was mapped to the position 107.5 cM on the Rice Genome Research Program (RGP) map [34]. Other studies with various molecular markers including AFLP, SSR and STS indicated that *Xa7* was located at 107.3 cM [35-36]. Later, Chen et al. [37] reported the high-resolution mapping and the genetic prediction of resistance gene *Xa7*. This gene was mapped to the 0.21 cM interval between the STMS (GDSSR02) and the SSR marker (RM20593). The SSR markers RM20589, RM20590 and RM20591 were reportedly located between these two flanking markers. In our experiment, RM20590 was

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identified as the closest *xa33*(t)-linked marker. Although, in this study, *Xa7* and *xa33*(t) shared common linked markers, they had shown different gene actions.

Based on our present study, *Xa7* and *xa33*(t) are not growth-stage dependent genes. They confer resistance to many Thai *Xoo* isolates at both seedling and booting stages. Sidhu et al. [33] reported that *Xa7* confers BB resistance only at the flowering stage. This is not the case with the Thai isolates. Seedling resistance is reportedly controlled by a number of known major genes conferring a high level of resistance throughout crop growth [38]. Adult plant resistance is characterised by a high level of resistance at the adult stage but the plants are very susceptible at their seedling stage [39]. Resistance at the seedling stage is more stable than resistance in adult plants [40]. Although *Xa7* and *xa33*(t) conferred BB resistance at seedling and tillering stages in our experiments, their resistance patterns against Thai BB isolates were different. The race specificity of the resistance genes indicated that *Xa7* and *xa33*(t) are not the same gene.

Amongst BB resistance genes identified on chromosome 6 including *Xa7*, *Xa27* and *xa32*(t), *Xa27* was reportedly located between RFLP markers RG424-RG162 (70.4-104.6 cM, Cornell map) on the long arm of chromosome 6, which is about 22.1 cM away from *Xa7*. It was originally found in wild rice *O. minuta* Acc 101141 [14] and introgressed into cultivated varieties. The gene *xa32*(t) was identified in wild rice *O. barthii* [6] and it was mapped on the terminal region of chromosome 6 at a distance of 9.3 cM from RM588 (16.1 Mb). Thus, *xa33*(t) in 'Ba7' was certainly different from *Xa27* and *xa32*(t) genes.

The gene *xa33*(t) conferred recessive gene action with incomplete dominance of susceptible allele because its heterozygous plants exhibited moderate susceptibility to *Xoo* strain. There are few reports on the genetics of incomplete susceptibility of BB found in rice. The *Xa27* gene conferred semi-dominant resistance to *Xoo* isolates PXO99 and T7174 in CO39 genetic background but provided complete resistance at the tillering stage in IRBB27 background while the seedling stage was susceptible. The inheritance of *Xa27* as a semi-dominant resistance gene was also observed in the genetic backgrounds of five parental lines of the Chinese hybrid rice when the plants were heterozygous at the resistance locus [15]. In the same way, *Xa21* and *Xa7* showed incomplete dominance in the heterozygous background of rice hybrid Minghui 63 by infection with GX325 and KS-1-21. The homozygous alleles were more resistant than the heterozygous ones [41]. Moreover, the *Xa4* resistance gene conferred from rice cultivar Teqing acted as a dominant resistance gene against *Xoo* strains CR4 and CXO8. On the contrary, it acted as a recessive factor against *Xoo* strain CR6 [42]. These incidents demonstrated that the *Xoo* strain which is used to evaluate the population, genetic background and developmental stage plays important roles in determining gene action.

In this study, we have identified the new BB resistance gene designated as *xa33*(t) in rice cv. 'Ba7'. The closely linked markers found will be useful for improvement of BB resistance through MAS in rice breeding programs.

Acknowledgements

This study was supported by grants from Research and Development of Rainfed Lowland Rice Varieties using Biotechnology, a collaborative project between BRRD and BIOTEC. The experiment in

this paper was conducted at Rice Gene Discovery Unit, Kasetsart University, Khampaeng Saen campus, Nakhon Pathom, Thailand.

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Maejo International

Journal of Science and Technology

ISSN 1905-7873

Available online at www.mijst.mju.ac.th

Full Paper

Composting of tobacco plant waste by manual turning and forced aeration system

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Received: 14 July 2008 / Accepted: 4 May 2009 / Published: 12 May 2009

Abstract: The efficiency of tobacco plant waste composting, by the manual turning and the forced aeration system, was compared. Tobacco plant waste, cow manure, urea fertiliser, and a compost inoculum mixture at a 100:10:0.2:0.01 ratio respectively, with 60% (w/v) moisture content, were set up in piling forms. The piles of the manual turning system were provided with turning aeration by hand at intervals of 7 days during the composting process. For the forced aeration system, each pile was aerated by a 3-HP air pump with a flow rate of 19 litres min^{-1} for 15 minutes every morning and evening. The completely randomised design of turned and force-aerated piles was performed in triplicate. The composting activity of both systems during the composting period was measured by several parameters: temperature, pH, moisture content, C/N ratio, growth of microorganisms, cellulase activity, and nicotine degradation in the set-up piles. Both systems had similar temperature, pH, and moisture content conditions in the piles during the composting process. However, the forced aeration system was more advantageous for the growth of mesophilic and thermophilic microorganisms, for cellulase activity from cellulase-producing microorganisms, and for nicotine degradation, when compared to the manual turning system. In conclusion, the forced aeration system was more efficient than the manual turning system in composting and is a viable alternative method for the composting process.

Keywords: composting, tobacco waste, manual turning, force-aerated system

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Introduction

It is estimated that roughly 300,275 tons of nicotine waste are produced globally every year [1]. In Thailand, approximately 500-650 tons of tobacco plant waste is generated annually at various stages during post-harvest processing and manufacturing of tobacco products. Tobacco plant waste has no immediate use, and cigarette companies are required to pay for its disposal. The majority of tobacco plant waste is burned, although this has a high cost of operation because of air pollution problems. On the other hand, several soil microorganisms, such as *Arthrobacter nicotianae*, *A. nicotinovorans*, *A. globiformis*, *Enterobacter cloacae*, *Psedomonas putida*, *Cellulomonas* sp., and *Alcaligenes paradoxus* are able to degrade toxic nicotine [2-7]. Therefore composting, which utilises several types of microorganisms, may be used as an alternative method for organic waste treatment, similar to that of sewage sludge and animal manure. Composting toxic tobacco plant waste from the agricultural industry would minimise waste and be useful for agricultural purposes. Currently, composting is used in the agro-industrial process to obtain products that can be applied to soil in order to increase the organic matter content as well as to enhance the soil structure and cation exchange capacity [8]. Utilisation of organic waste composts is particularly important for unfruitful soils that have low organic matter content. Likewise, many Asian agricultural regions have defective soils since farmers have used inorganic fertilisers for many years without regard to their long-term effect on soil structure and thus greatly need this type of treatment.

In order for composting to be accepted, development of the operating strategy, followed by the success of the composting process, is necessary. Therefore, proper evaluation of composting systems is required if an acceptable product is to be generated, and the efficiency of the system must be maximised [9-10]. Manual turning is often labour intensive, creates air pollution (e.g. dust), and requires additional space for the pile. Therefore, other operating strategies that can reduce manpower and space are worth exploring. In addition, the composting time can be shortened by other composting strategies. In one report, force-aerated composting, which maintains temperatures in the upper thermophilic range and provides an effective inactivation of pathogens, was a more efficient composting method [11]. Force-aerated windrow composting uses a ventilation unit (centrifugal blower) to force air into the perforated pipe system, located underneath the compost pile, to induce air convection movement into the material and to deliver oxygen to the microorganisms [11,12-14]. Bulking agents, such as wood chips, straw, peat, or sawdust, are often mixed with the compost material to give the required open structure and to ensure adequate aeration [12,15-16]. This composting system is also a non-turning method and therefore saves space compared to the conventional composting method.

The present study compares the composting efficiency of conventional composting systems to force-aerated composting systems. In this study, chemical and biological parameters were determined to assess the maturity of the compost from tobacco plant waste. Meanwhile, the reduction of nicotine was determined for both composting systems.

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Materials and Methods

Raw materials

The tobacco plant waste was collected from the storehouse of the Chiang Mai Tobacco Office, Thailand Tobacco Monopoly. The tobacco waste was air dried for 4 weeks prior to use.

Cow manure was obtained from the cow farm, located at the Faculty of Agriculture at Chiang Mai University, Chiang Mai, Thailand. Sub-samples of cow manure were air dried at 80°C for 24 h and ground and pressed through a 0.2 mm sieve. The samples were then stored prior to usage.

Compost inoculum (CMU) was produced in the Department of Biology at Chiang Mai University. The CMU consists of 4 strains of bacteria in the genus *Bacillus*, 1 strain of actinomycetes in the genus *Streptomyces*, and 2 strains of fungi in the genus *Aspergillus ,* and one unknown strain.

Experimental establishment

The tobacco plant waste, cow manure, urea fertiliser, and compost inoculum, in a 100:10:0.2:0.01 ratio respectively, were homogeneously mixed, and the moisture content was adjusted to 60% (w/v) with tap water before piling. The completely randomised design (CRD) with two treatments, i.e. manual turning and forced aeration, were performed in triplicate. Each pile was triangular in shape, and approximately 2.5×3.5 m at the base and 1.5 m in height [17]. For the turned pile, aeration was performed manually by turning the piles every 7 days. During the composting process, the ambient temperature and temperature at a depth of 60 cm was monitored every 2 days in the first week and every 10 days thereafter. For the force-aerated pile, 10-cm diameter polyvinyl chloride (PVC) pipes perforated in lengths of 2.5 m, were laid at the base of the piles. Wood chips were used to cover the perforated sections of the pipes to prevent blockage of the holes. Aeration for the piles was supplied by a 3-HP air pump, with an average flow rate of 19 litres min^{-1} and a maximum output of 24 litres min^{-1}. The air pump was operated 15 minutes in the morning and 15 minutes in the evening during the entire period of composting. Every two days in the first week and then every 10 days thereafter, the temperature of each pile was measured in the following locations: at the top, 130 cm from the base of the pile; in the middle, 75 cm from the base of the pile; at the bottom of the pile, 30 cm from the base of the pile; and at the surface of the pile, 5 cm from the surface of the pile.

Analytical sample

Composite samples were taken at five symmetrical locations in each pile at the starting time and then every 10 days until the end of the composting process (50 days).

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Chemical analysis

1) Moisture content

The moisture content in the composting piles was determined by the weight loss of 10 g samples, which were dried for 48 h at 105°C in an incubator, or until the weight of the compost mixture was constant. The percentage of moisture content was then calculated.

2) pH value

To obtain the pH value in the compost, 10 g of solid sample was extracted with deionised water, at a sample-to-water ratio of 1:5 (v/v). After an equilibration time of 30 min, with occasional stirring, the pH was measured with a pH metre.

3) C/N ratio

The total organic carbon content of the 10 g of compost sample was determined by oxidation with potassium dichromate in an acid medium. The excess of the dichromate was measured using Mohr's salt, according to the method previously described [18].

The total nitrogen content of the 10 g of compost sample was determined using the regular-Kjeldahl method [19]. Kjeldahl nitrogen was quantified by mineralisation within a strong acid medium, containing 98% sulfuric acid, followed by steam distillation and titrimetric determination of NH^+_4/NH_3. Organic nitrogen was obtained by subtraction of NH^+_4/NH_3 nitrogen from Kjeldahl nitrogen. The C/N ratio was then calculated by dividing the total organic carbon content by the total nitrogen content.

4) Cellulase activity

The compost sample (5 g) was mixed with 25 ml distilled water for 30 min, using an ultrasonic bath, and was then centrifuged for 15 min at 6000 RPM. The cellulase activity was assayed by measuring the reduced sugars, as represented by glucose, using the dinitrosalicylic acid method [20]. The reaction mixture contained 0.5 ml of the above supernatant and 0.5 ml of 1% carboxymethylcellulose (Sigma Co.) in a 0.05 M potassium phosphate buffer at pH 7.0. After incubation at 50°C for 30 min, the reaction was terminated by adding 3 ml of dinitrosalicylic acid. The colour of the reaction mixture was developed in a boiling water bath in 15 min, and the absorbance was read at 540 nm. Sugar values were read from a glucose calibration curve. One unit of enzyme activity was defined as the amount of enzyme which released 1 μg of reducing sugar per min.

5) Microbiological analysis

The growth of both mesophilic and thermophilic bacteria and fungi were determined. In the preliminary experiments, various media for bacteria and fungi were tested, and the media in which the largest number of isolates appeared was adopted as the plate count media. The media utilised in this work was Trypticase soy agar (BBL Microbiology Systems) media for bacteria (trypticase peptone, 17 g; phytone peptone, 3 g; NaCl, 5 g; K_2HPO_4, 2.5 g; glucose, 2.5 g; agar, 20 g; distilled water, 1 litre [pH 7.3]), and Rose Bengal agar media for fungi (peptone, 5 g; dextrose, 10 g; KH_2PO_4, 1 g; $MgSO_4$, 0.5 g; agar, 15 g; Rose Bengal 35 mg; distilled water, 1 litre [pH 5.2]). In order to determine the total counts of microorganisms, 10 g of sample was subjected to 10-fold serial dilutions using a sterile

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phosphate buffer solution at pH 7. From each dilution, a 0.1 ml aliquot was spread onto plates containing media, as described above, in duplicate. The incubation temperatures were 30°C for mesophile growth and 50°C for thermophile growth. The incubation time was 24 h for mesophilic bacteria, 48 h for thermophilic bacteria, and 72 h for mesophilic and thermophilic fungi. The cultures were prepared from the plates, and those that corresponded to the same dilution (from duplicates) and showed between 30-300 colonies were selected, and their microbial numbers, as CFU g^{-1}, were calculated.

6) Nicotine analysis

In order to extract nicotine, 5 g of compost sample was added to a distillation flask with 50 ml of alkali-salt solution and then distilled with a current of steam. The distillate was diluted, and the spectrophotometric method was used to measure the percentage of nicotine residue using the AOAC official method 960.07 [21].

Results and Discussion

Temperature profile

The initial temperature of the turned pile was 29°C and rapidly rose to a peak of 62°C after a 4-day composting period. The high temperature continued until 20 days of composting, after which the temperature dropped to 36°C by the 30th day of composting. Thereafter the temperature varied within a narrow range (36-32°C) (Figure l). The temperature change of the force-aerated pile and turned pile were similar. The pattern of these temperatures, i.e. first an increase to a high temperature, persistence of a high temperature, and then a decrease to a low temperature, is a typical temperature profile of the composting process, especially for cow manure and wheat straw composts [22], citrus waste compost [23], spent pig manure and sawdust litter composts [24], and filter cake and bagasse composts [25]. The temperature levels in the compost piles tended to increase and reach 50-60°C due to the energy released from the biochemical reactions of the microorganisms in the compost piles, while the temperature levels in the compost piles tended to decrease after the thermophilic phase due to a loss of the substrate and a decrease in microbial activity [26]. The temperature in the compost piles can be used as an indicator of the compost maturity [19,27]. It was previously reported that the compost material can be considered mature when an ambient temperature of 28°C is reached, and that the compost in a force-aerated pile, after 30 days of the composting process, is closer to maturity than the one in a turned pile [19,27].

pH Change

During the composting process, the pH of the turned piles and the force-aerated piles were similar (Figure 2). At first, the pH of both piles increased from an initial pH of 9.54 and 9.10 respectively, to a pH of 11.78 and 10.97 after 10 days. This increase in pH during the composting process could be due to the production of ammonium as a result of the ammonification process [24]. Subsequently, the pH of both piles decreased from 11.78 and 10.97, to 7.78 and 7.80 respectively.

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This may be due to a high organic carbon content in these piles that was subsequently degraded to organic acids by the acid-forming bacteria that exist in the compost piles [28]. In addition, the pH decrease may also be caused by the mineralisation of organic acids and the large quantity of carbon dioxide released during the composting process [9,19,22,24,25,28]. Our results show that the pH of both composts decreased to a final, mature pH of approximately 8.0, based on the compost regulations of pH 5.0-8.0 for the US, and pH 5.5-8.0 for the Council of European Communities (CEC) [47].

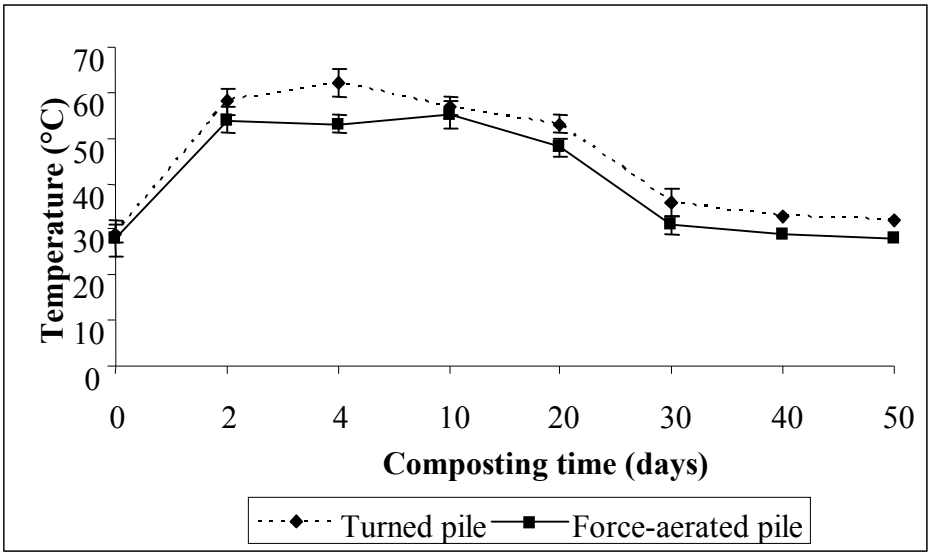

Figure 1. Temperature profiles during composting of tobacco plant waste

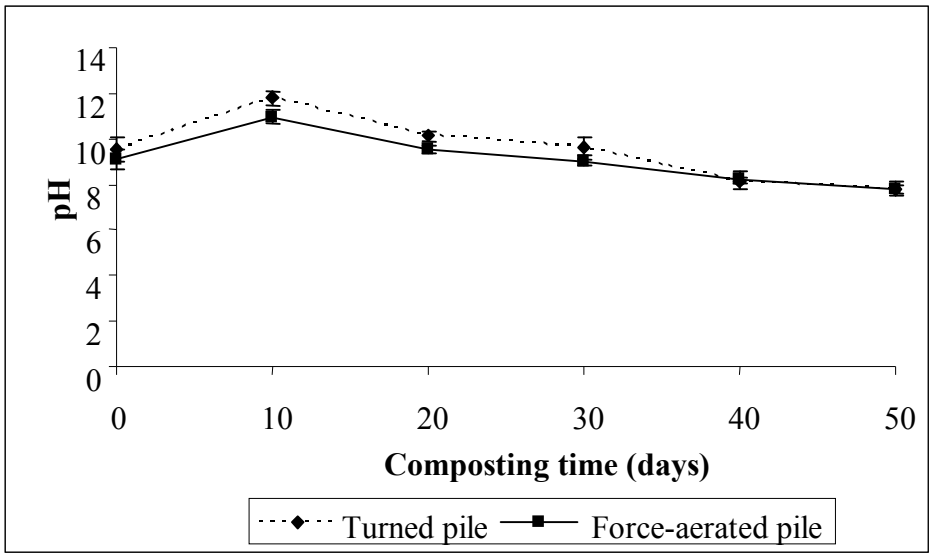

Figure 2. Change of pH during composting of tobacco plant waste

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Moisture content

At the initial stage of composting, the moisture content of the turned and force-aerated piles were approximately 60-63% and then dropped gradually throughout the composting time (Figure 3). The moisture content of both piles slowly decreased to a final concentration of approximately 42-46% at the end. An optimum level of moisture content was previously reported to have a strong effect on the oxygen consumption rate of aerobic heterotrophic microorganisms and was efficient between 50-60% for composting spent litter, and therefore, during the entire composting process. All of the compost piles were adjusted to have a moisture content of 60% [9]. Water evaporation occurred as an effect from the heat generated from the microbial reactions during composting, which resulted in a decrease in moisture content in the compost pile [30]. When an ambient temperature was reached, the addition of water was stopped, although the composting process continued. The force-aerated pile, after a 30 day composting period, was thought to have achieved an acceptable level of quality for mature compost, with a less than 50% moisture content [31].

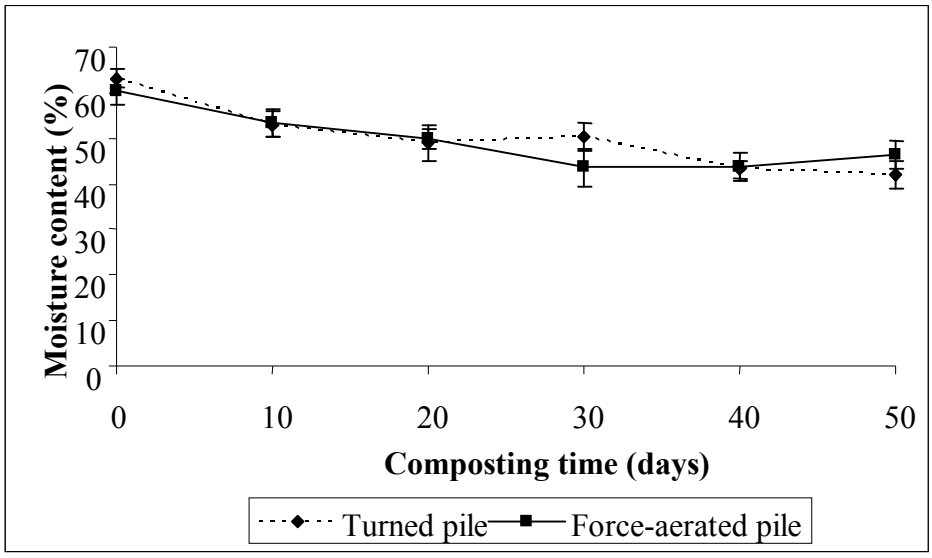


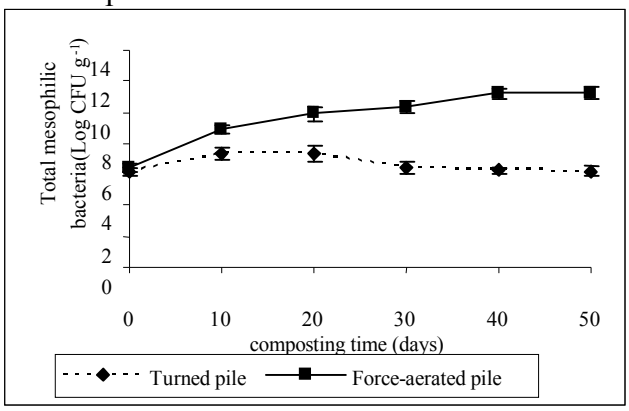
Figure 3. Change of moisture content during composting of tobacco plant waste

Growth of microorganisms

The growth of both the mesophilic and thermophilic bacteria and fungi, at various stages of the composting process, was determined (Figures 4-5). The growth of the mesophilic bacteria in a force-aerated pile regularly increased with time and reached a maximal growth of approximately 10^{11} CFU g^{-1} after 40 days of composting, while the growth of the mesophilic bacteria in the turned pile was retarded and was only 10^{6}-10^{7} CFU g^{-1} (Figure 4a). Thermophilic bacteria in the force-aerated pile first grew rapidly, reaching CFU of approximately 10^{7}-10^{9} CFU g^{-1} within 20 days of decomposition and then slowly declined to 10^{8} CFU g^{-1} at the end of composting time. The thermophilic bacteria in the turned pile was reduced to 10^{6}-10^{7} CFU g^{-1} (Figure 4b). The growth of the mesophilic and

thermophilic fungi in the force-aerated pile increased with time and reached a maximum titre of 10^9-10^{10} CFU g^{-1} after 30 and 40 days of composting time respectively (Figures 5a-5b). Their titres declined thereafter. The growth rates of the mesophilic and thermophilic fungi in the turned pile were mostly parallel to those in the force-aerated pile but resulted in overall lower cell counts. These results indicate that composting through force-aerated pile allowed the mesophilic and thermophilic bacteria and fungi to grow better in comparison with the turned pile composting. During the decomposition, the temperature of the material was raised, which favours the growth of bacteria and fungi during that particular stage of composting. The proliferation of the mesophilic and thermophilic microorganisms during composting is related to the mesophilic and thermophilic stages of the composting system [32-35]. Microbial succession plays a key role in the composting process and in the appearance of certain microorganisms that reflect the quality of the maturing compost [34,36].

a. Mesophilic bacteria



b. Thermophilic bacteria

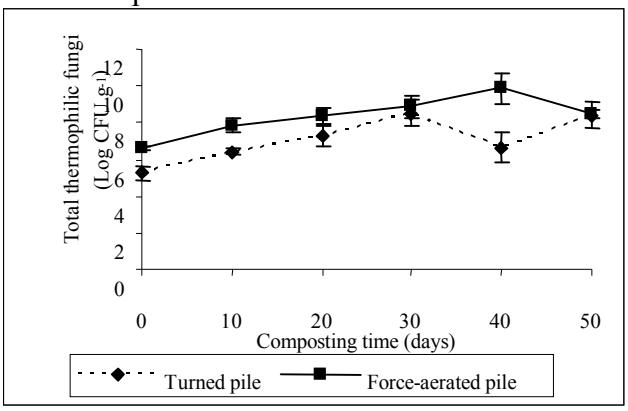
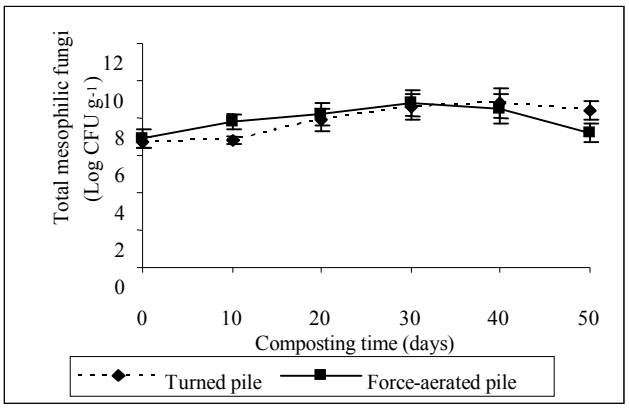


Figure 4. Change of mesophilic (a) and thermophilic (b) bacterial growth during the composting of tobacco plant waste

a. Mesophilic fungi



b. Thermophilic fungi

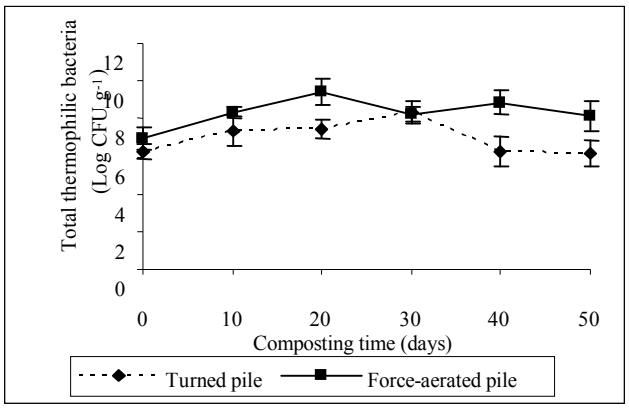


Figure 5. Change of mesophilic (a) and thermophilic (b) fungi growth during the composting of tobacco plant waste

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Change in C/N ratio

Both the turned and force-aerated piles decreased in C/N ratio with the composting time and reached maturation with a C/N ratio of approximately 13 after roughly 50 days of composting (Table 1). The initial C/N ratio was the main factor that affected the time required for compost maturity [27]. Based on the nutritional requirements of the microbes that are active in composting, the C/N ratio of the organic matter should be on the order of 20-25 parts carbon to 1 part nitrogen; a departure from this ratio leads to slow composting. On the other hand, the 20/1 ratio is critical in terms of crop production. If the C/N ratio is higher than 20/1, there is a strong possibility of nitrogen shortage for the crop plants [37-38]. Composts with a C/N ratio of 20, but not higher, are required for proper maturation [39]. Several previous studies have concluded that a C/N ratio of 20-25 indicates the maturity of the final compost product [40-41]. However, although the C/N ratio is a factor used for indicating compost maturation, it cannot be used in this study as an absolute indicator of compost maturation since the initial C/N ratio was below 20. For instance, the C/N ratio of compost at maturity was 13-27 for the co-composting of chestnut burr and leaves with solid poultry manure [42] and 11-17 for the composting of bagasse with sewage sludge [43].

Table 1. Change of C/N ratio during composting of tobacco plant waste

	C/N ratio	
Treatment	day 0	day 50
Turned pile	17.76	12.27
Force-aerated pile	19.04	13.75

Cellulase activity

The cellulase activity of both the turned and force-aerated piles is presented in Figure 6. The results show that the force-aerated pile had a rapid increase in cellulase activity during the first 30 days of the composting period followed by a prompt decline in cellulase activity, which continued until the end of the composting period. In the turned pile, the cellulase activity was similar to that of the force-aerated pile, but with an overall lower activity level. These results are consistent with a previous report that also stated that the cellulase activity increased to a maximum level at 30 days in all treatments, followed by a decline in cellulase activity until 60 and 90 days [44]. In general, cellulose decomposition limits the rapid production of compost more than any other substrate [45]. Cellulase activity, involved in the degradation of cellulose, depends on the type of cellulolytic microorganisms that develop in organic waste [45]. For the most part, fungi are involved in the decomposition of cellulose, hemicellulose, and lignin that are present in the organic matter. The force-aerated system can provide aerobic conditions for microorganisms, and this is favourable for cellulase-producing fungi in

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order to decompose the cellulose of the organic matter and allow for higher availability of nitrogen [46].

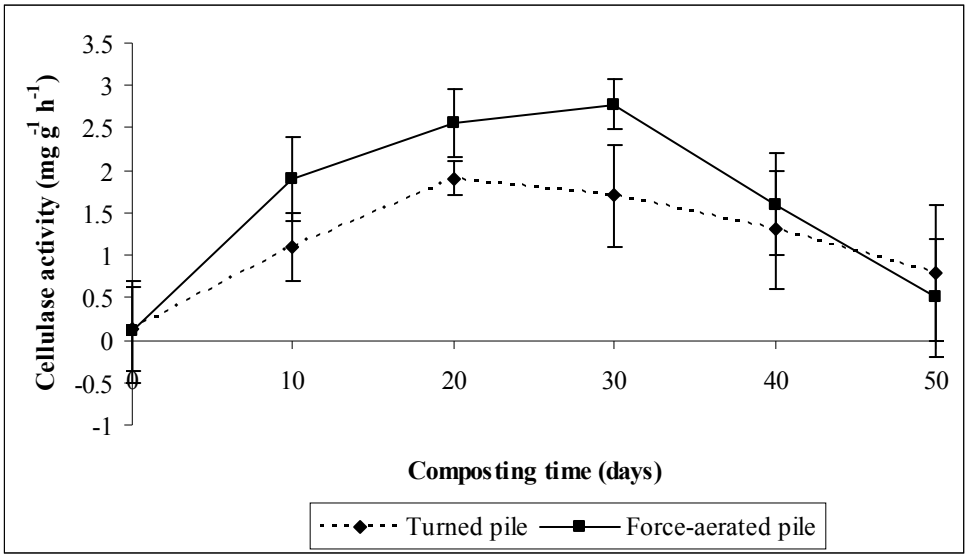

Figure 6. Change of cellulase activity during composting of tobacco plant waste

Nicotine

As shown in Table 2, the nicotine content dropped at the end of the composting process from 1.75 to 1.51% in the turned pile and from 1.75 to 1.18% in the force-aerated pile. The compost obtained by the forced aeration system had significantly ($p<0.05$) less nicotine residue (5700 mg kg^{-1} loss) than that by the manual turning system (2400 mg kg^{-1} loss). A similar result was obtained previously, and it was reported that at the end of composting the tobacco waste, the nicotine content dropped from 2000 to 450 mg kg^{-1} [47]. These results are supported by an additional report that stated that composting with tobacco waste could accelerate the breakdown of nicotine and result in the production of a less toxic and more useful organic improvement [48]. The forced aeration system, with its higher aeration, may encourage microbial growth, which may decompose the nicotine residue more quickly [1-7].

Table 2. Percentage and loss of nicotine residue in turned piles and force-aerated piles

	Nicotine (%)		Loss of nicotine (mg kg^{-1})
Treatment	day 0	day 50	After 50 days
Turned pile	1.75 b	1.51 ab	2400
Force-aerated pile	1.75 b	1.18 a	5700

Note: Different letters in the table indicate significant differences at $p<0.05$.

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Conclusions

This investigation has revealed that the composting activities of both the turning and forced aeration systems are similar in temperature, pH, and moisture content. However, the forced aeration system had higher titres of both the mesophilic and thermophilic bacteria and fungi and a higher cellulase activity from cellulose-degrading organisms compared to the turning system. This indicates that the forced aeration system may supply more aerobic conditions for both microorganism growth and cellulase activity, which are vital to the composting process. As a result of this study, we have concluded that the forced aeration system of composting enhances the growth of the related microorganisms, cellulase activity, and degradation of nicotine content in tobacco waste compared to composting by the manual turning system. Therefore, the forced aeration system is an alternative method for the composting process; however, the proper duration and interval times of the forced aeration system should be established in order to improve the composting process. Nevertheless, compost from both turned and force-aerated piles apparently reached maturity faster (within 30-40 days, as indicated by a decrease to ambient temperatures) than previous studies.

Acknowledgements

The authors wish to thank the Graduate School of Chiang Mai University, Thailand and the Department of Environmental Quality Promotion, Ministry of Natural Resources and Environment, Thailand for financial support. We also thank Mrs. Dermot Rodgers for English grammar checking.

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Maejo International

Journal of Science and Technology

ISSN 1905-7873

Available online at www.mijst.mju.ac.th

Full Paper

A valued Indian medicinal plant – *Begonia malabarica* Lam. : Successful plant regeneration through various explants and field performance

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Received: 10 November 2008 / Accepted: 17 May 2009 / Published: 21 May 2009

Abstract: A cost-effective and efficient protocol has been described in the present work for large-scale and rapid in vitro propagation of a valuable medicinal herb *Begonia malabarica* Lam. (Begoniaceae) by shoot auxillary-bud proliferation and organogenesis on MS medium supplemented with 6-benzylaminopurine (BA; 0.0-8.8 mg/l) and indole-3-acetic acid (IAA; 0.0-2.88 mg/l) at different concentrations, either alone or in combinations. Initiation of callus formation from the base of the leaf lamina was observed on MS supplemented with BA, IAA and adenine sulphate. Root induction on shoots was achieved on full strength MS with IAA/ indole-3-butyric acid (IBA) at different concentrations. MS medium with 4.4 mg/l BA and 1.4 mg/l IAA elicited the maximum number of shoots (10 multiple shoots) from nodal explants. Leaf-based callus differentiated into more than 28 shoots on MS with 150 mg/l adenine sulphate. The regenerated shoots were rooted on MS with 1.2 mg/l IBA within ten days. Almost 95% of the rooted shoots survived hardening when transferred to the field. The regenerated plants did not show any morphological change and variation in levels of secondary metabolites when compared with the mother stock. Thus, a reproduction of *B. malabarica* was established through nodal and leaf explants. This protocol can be exploited for conservation and commercial propagation of this medical plant in the Indian subcontinent and might be useful for genetic improvement programs.

Keywords: *Begonia malabarica*, regeneration, explants, field performance

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Introduction

Plants are an important source of medicines and play a key role in world health [1,2]. In almost all regions and cultures of the world, from ancient times till today, plants have been used as medicines [3]. Today's medicinal plants are important to the global economy as approximately 80% of traditional medicinal preparations involve the use of plants or plant extracts [4]. The increasing demand for herbal medicines in recent years due to their fewer side effects in comparison to synthetic drugs and antibiotics has highlighted the need for conservation and propagation of medicinal plants. An efficient and most suited alternative solution to the problems faced by the phytopharmaceutical industry is development of in vitro systems for the production of medicinal plants and their extracts. In vitro propagation technology has sound and extensive potential for commercial rapid multiplication of medicinal plants and horticultural crops because it is a quick method, allows round-the-year propagation/production of identical plants, and produces plants free from diseases [5]. In vitro propagation has been successfully employed for the conservation of medicinal crop genetic resources, particularly with those crops which are vegetatively propagated [6,7]. Propagation through seed is unreliable due to poor seed quality, erratic germination and seedling mortality as under natural field conditions [8].

Begonia malabarica Lam. (Begoniaceae) is an important medicinal plant whose main secondary metabolites are luteolin, quercetin and β-sitosterol [9]. The leaves are used for the treatment of respiratory infections, diarrhoea, blood cancer and skin diseases [10]. Very few reports on cultivation, breeding and improvement programmes and in vitro studies of *B. malabarica* are available despite its commercial importance. This paper deals with the standardisation of a technique for its micropropagation through multiple shoot formation. The protocol provides early bud-break with a high frequency of shoot multiplication from axillary bud and leaf explants with comparatively reduced requirement for plant growth hormones, as well as successful acclimatisation of plants in the soil. The performance of regenerated plantlets was also evaluated in the field.

Materials and Methods

The methods of plant tissue culture used were the standard methods as described by Gamborg and Phillips [11]. The nodal explants and leaves of one-year-old plants of *B. malabarica* were collected from the foot-hills of Anamalai around Aliyar Dam, Coimbatore district, Tamilnadu, India. They were washed first under running water (30 min) and treated with 0.2% (v/v) aqueous surfactant (Teepol, BDH, India) for 10 min. followed by repeated rinsing with distilled water. Subsequently, they were treated (20 min) with 0.1% (w/v) carbendenzim (BASF, India). Further sterilisation was done under aseptic condition in a laminar air-flow hood. The plants were surface-sterilised with 50% (v/v) ethanol (1 min) and then by 0.07% (w/v) $HgCl_2$ (3 min). Finally, the plants were washed thoroughly (3-5 times) with sterilised distilled water. The plant nodes were cut into an appropriate size (0.6 cm) and young-leaf laminas with midrib (0.5 cm) were cut and cultured on Murashige and Skoog (MS) medium.

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Throughout the experiment a full strength MS medium of 3% (w/v) sucrose and gelled with 0.8% (w/v) agar (Qualigens, India) was used. The pH of all media were adjusted to 5.8 prior to autoclaving (15 min). The cultures were incubated for 16 hr in a culture room at 25±1°C. Photoperiod (50μE/m^2/s) was provided by cool-white fluorescent tubes.

The basal medium was supplemented with 6-benzylaminopurine (BA; 0.0-8.8 mg/l) and indole-3-acetic acid (IAA; 0.0-2.88 mg/l), either alone or in combinations. Initiation of callus formation from the base of leaf lamina was observed on MS medium supplemented with BA, IAA and adenine sulphate (Ads). Root induction on shoots was achieved on full strength MS with IAA/ indole-3-butyric acid (IBA) at different concentrations. Well-developed rooted shoots were removed from the culture vessels, washed gently under running tap water and planted in plastic cups containing a potting mixture of sand, soil and farmyard manure in the ratio of 1:1:1. The plantlets were kept in net house for acclimatisation (2-3 weeks) before their subsequent transfer to the field. All cultures were visually examined periodically. Humidity was maintained by sprinkling water regularly throughout the day [12]. The plants were gradually exposed to the normal conditions and transferred to the medicinal garden of Kongunadu Arts and Science College (Autonomous), Coimbatore, Tamilnadu, India.

The experiments were set up in a completely randomised design. There were ten cultures per treatment and each experiment was repeated thrice; sub-culturing was carried out at an interval of 4 weeks. Effects of different treatments were quantified on the basis of the percentage of cultures showing responses in regeneration of shoots, mean number of shoots/node, mean-number of roots/shoot and root length, which were statistically analysed. Qualitative analysis of secondary metabolites was carried out by thin layer chromatography. The shade-dried roots and leaves of in vitro raised plants and mother plants were crushed into powder form and were subjected to phytochemical analysis [13].

Results and Discussion

Bud break on the nodal segments was achieved on MS with 4.4 mg/l BA and 1.4 mg/l IAA (Figure 1a). When MS supplemented with different concentrations of BA and IAA was used, multiple shoots emerged from the nodal explants within two weeks of incubation (Figure 1b). In vitro response varied greatly in various plants depending upon the age of explants [14] as well as on the culture maintenance conditions [15]. Among different concentrations of the growth hormones tested, 4.4 mg/l BA and 1.4 mg/l IAA elicited the maximum number of shoots (10 multiple shoots) from nodal explants (Table 1). Direct shoot regeneration from nodal explants has been reported earlier [16,17] on MS medium with 27.2 mg/l Ads + 2.46 mg/l IBA. Similarly, Verma et al. [18] reported rapid propagation of *Plumbago zeylanica* with a maximum of four multiple shoots per nodal segment with 8.87 mg/l BA and 0.49 mg/l IBA. The present study exemplifies a positive modification on shoot induction efficacy on MS medium with low concentrations of auxin and cytokinin. The elongation of shoots (3-4 cm) was observed on the same proliferation medium within two weeks of incubation

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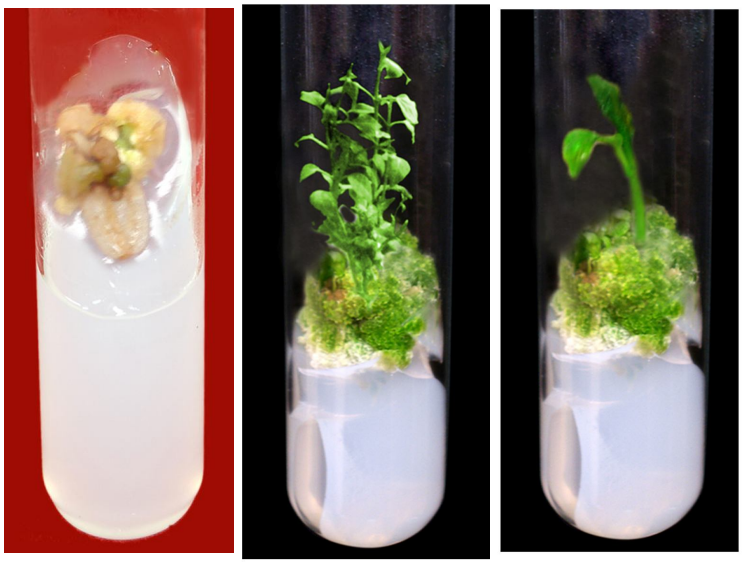
(Figure 1c). Similarly, Chaplot et al. [19] reported rapid propagation of *P. zeylanica* with a maximum of 12 multiple shoots per nodal segment with 4.4 mg/l BA and 1.4 mg/l IAA.



a **b** **c**

Figure 1. Micropropagation through axillary bud proliferation and leaf callus of *B. malabarica*
a. Bud break from nodal explant
b. Multiple shoot formation on MS medium containing BA (4.4 mg/l) and IAA (1.4 mg/l)
c. Shoot formation from callus on MS medium with BA (4.4 mg/l), IAA (1.4 mg/l) and Ads (150mg/l)

Table 1. Influence of different combinations of BA and IAA in MS medium on shoot formation through nodal explants of *B. malabarica*

Concentration (mg/l)		Response (%)	Number of shoots/node* (Mean ± SD)
BA	IAA		
0.0	0.0	0.0	0.0
2.0	0.5	12	1.5 ± 0.2
4.4	0.5	48	4.2 ± 0.4
4.4	1.4	83	10.2 ± 1.4
4.4	2.8	62	5.6 ± 0.4
6.4	1.4	54	4.6 ± 0.6
8.8	1.4	52	4.4 ± 0.3

* Values are from four replications, ten cultures per replicate, and scored after three weeks.

Callus initiation was observed from young leaves on MS medium supplemented with BA (0.0 - 8.8 mg/l), IAA (0.0-2.8 mg/l) and Ads (150 mg/l). Callus formation from the explant leaf of *B. malabarica* is in agreement with results obtained by Chaplot et al. [19] for *P. zeylanica*, although the hormonal combinations were different. The best callus (nodular) formation was observed on MS

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medium containing 4.4 mg/l BA, 1.4 mg/l IAA and 150 mg/l Ads. The higher number of shoots from the callus was observed on MS with 4.4 mg/l BA, 1.4 mg/l IAA and 150 mg/l Ads (Table 2). An average of 28 shoots was recorded in callus cultures through organogenesis. Present results are consistant with earlier reports on *P. zeylanica* [19] and Ashwagandha (*Withania somnifera*) [20] in that cytokinin and auxin influence shoot bud regeneration.

Table 2. Influence of different combinations of growth regulators in MS with 150 mg/l Ads on shoot bud regeneration from leaf callus of *B. malabarica*

Concentration (μM)		Response	Number of shoots/node*
BA	IAA	(%)	(Mean ± SD)
0.0	0.0	-	-
2.0	1.4	10	1.04 ± 0.2
2.0	2.8	26	2.08 ± 0.4
4.4	1.4	92	28.04 ± 1.4
4.4	2.8	84	22.06 ± 1.2
6.4	1.4	72	15.04 ± 1.2
6.4	2.8	70	12.8 ± 1.1
8.8	1.4	64	6.34 ± 1.2

* Values are from four replicates, ten cultures per replicate, and scored after three weeks.

Well-developed shoots (3-4 cm with three nodes) generated through axillary-bud proliferation and leaf callus were excised and cultured on MS medium with different concentrations of auxins for root induction, which was found to be more prominent in the medium containing IAA (0.56 mg/l) or IBA (1.2 mg/l) (Figure 2; Table 3). Earlier workers [17,19,21] had reported smaller numbers of roots on half-strength MS medium containing 0.57 mg/l IAA, 4.92 mg/l IBA and 2 mg/l IBA respectively. While profuse rooting was observed on full strength MS supplemented with IAA or IBA (Table 3), the best result (16 roots) was obtained on MS with IBA (1.2 mg/l) within 10 days. The potency of IBA in root induction has been reported in Petunia [22] and in *P. zeylanica* [18]. In our case, maximum frequency (96%), number of roots/shoot (around 16) and mean root length (13.62 cm) were achieved within 10 days when shoots were cultured on MS with IBA.

The ultimate success of the in vitro propagation lies in the successful establishment of plants in the soil. In this study, an 80% transplantation success of in vitro hardened plantlets in the field was observed in comparison to the 60-90% survival of plantlets recorded in the experiments of previous workers [17,21]. The high survival rate of in vitro *B. malabarica* plants in the present study indicates that this procedure may be easily adopted for large scale multiplication and cultivation. The in vitro propagated plantlets resemble the general growth and morphological characteristics of the donor plants (Figure 3). The in vitro raised plants and the seed-grown plants were then uprooted from the field for root harvesting. A significantly higher number of roots (16.0 ± 0.8) per plant for the former were observed compared to that for the seed-generated stock (4.2 ± 1.2). There was a threefold increase in root biomass on fresh weight basis of the in vitro raised plants (148.3 ± 1.8 g) in

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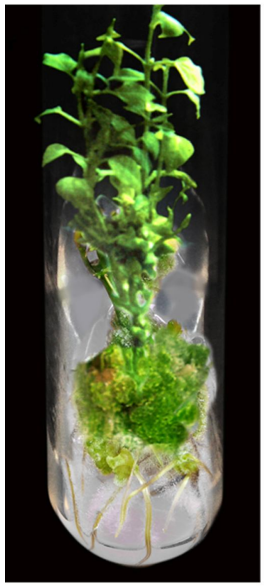

Figure 2. Induction of roots on regenerated shoots on MS medium containing IBA

Table 3. Effect of different auxins in MS medium on root induction from regenerated shoots

Concentration (μM)		Number of roots/shoot* (Mean ± SD)	Root length (cm)
IAA	0.0	-	-
	0.56	13.62 ± 1.2	11.2 ± 0.42
	1.42	6.84 ± 1.4	5.12 ± 0.28
IBA	0.0	-	-
	0.48	4.2 ± 0.42	5.04 ± 0.02
	1.2	16.04 ± 1.2	13.62 ± 0.04
	2.48	8.36 ± 1.4	6.14 ± 0.02

* Values are from four replicates, ten cultures per replicate, and scored after two weeks.

comparison with that of the seed-generated plants (42.4 g ± 1.2 g). Similar observations have been reported by Roja and Heble [23] for in vitro generated plants (*Rauwolfia serpentina*) with thick root stumps (fresh weight 60.56 g) compared to the long slender roots (fresh weight 11.92 g per plant) of the conventionally grown counterparts. Present results are also in agreement with the recent report by Chaplot et al. [19] who obtained a higher number of roots (18.0 ± 0.6) and fresh weight (5.1 ± 1.4 g) in in vitro raised *P. zeylanica* as compared to what were observed in rooted cuttings. Further qualitative analysis of secondary metabolites in roots and leaves of plantlets showed the presence of flavonoids, alkaloids, phenols, saponins and tannins in both the in vitro generated and seed-grown plants without showing any levels of variation.

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Figure 3. Hardened *B. malabarica* plant in potting mixture of sand: soil : FYM (1:1:1) ready for transplantation in the field.

Conclusions

A reproduction protocol for *B. malabarica* has been established through nodal and leaf explants. This protocol can be exploited for conservation and commercial propagation of this medicinal plant in the Indian subcontinent and may be useful for genetic improvement programs.

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Maejo International

Journal of Science and Technology

ISSN 1905-7873

Available online at www.mijst.mju.ac.th

Short Communication

Comparative study of proteolytic activity of protease-producing bacteria isolated from *thua nao*

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Received: 26 August 2008 / Accepted: 20 May 2009 / Published: 21 May 2009

Abstract: *Thua nao* is a Thai traditional fermented soya bean product with distinct flavour and aroma. This study was carried out to screen the potential protease-producing bacteria during the fermentation process. Of 171 strains, there were 169 bacterial isolates (98.8%) exhibiting proteolytic activity. Two potential isolates namely TN51 and TN69 showing highest activity as indicated by the widest clear zones (2.73 and 2.65 cm, respectively) were selected for further study. Using five different protein-based media, we found that the medium composition had an influence on the enzyme activity.

Keywords: *thua nao*, fermented soya bean, protease-producing bacteria, *Bacillus*

Introduction

There are various types of fermented soya bean from different parts of the world such as Indian *kinema*, Nigerian *dawadawa* (or *daddawa*), Japanese *natto*, and Thai *thua nao*. Typically, these soya

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bean foods are produced in similar manner: soya beans are washed, soaked overnight and cooked by boiling or steaming. The boiled or steamed soya beans are then fermented naturally at ambient temperature for 2-3 days. Among these fermented soya bean foods, Japanese *natto* is one of the best characterised products and, at present, has been produced commercially using the pure starter culture of *Bacillus subtilis natto* strain [1].

Thua nao is an alkali fermented soya bean widely consumed in the northern part of Thailand. Its use is versatile ranging from making ready-to-eat products after steaming or roasting to producing essential condiments in a variety of traditional dishes. Several studies have shown that, like other fermented soya beans, *Bacillus* species are responsible for the fermentation process of *thua nao* and they are abundantly isolated from the products [2-4]. In this present study, we aim to investigate the proteolytic activity of *Bacillus* species isolated from *thua nao* products. Further experiment was also undertaken to determine whether different protein-based media could have an effect on their protease activity.

Materials and Methods

Bacterial cultures

The bacterial cultures used in this study were isolated by Chukeatirote et al. [4]. They were initially characterised by morphological, physiological, and biochemical properties. These included Gram-staining, presence of spore, oxygen requirement, catalase test, ability to grow in 5 and 7 % NaCl, VP (Voges-Proskauer) test, and citrate and starch utilisation [5,6]. Additionally, *B. natto* ASA isolated from Asachiban *natto*, *B. natto* BEST195 [7], *B. subtilis* TISTR008 and *B. licheniformis* TISTR1109 obtained from Thailand Institute of Scientific and Technological Research (TISTR) were also used as reference strains in proteolytic activity study.

Determination of proteolytic activity

Initially, the bacterial strains isolated from *thua nao* were screened for their proteolytic activity. For this, a single colony of these bacteria was inoculated into a test tube containing 3 ml of nutrient broth containing 1 % peptone, 0.5 % beef extract, and 0.5 % NaCl. The culture was then grown at 37 ^{o}C for 24 h. Fifty microlitres of the overnight culture was subsequently transferred to a new test tube containing 3 ml of the nutrient broth and the culture was further incubated at 37 ^{o}C for 24 h. After incubation, the bacterial cells were harvested at 14,000 rpm for 15 min at 4 ^{o}C. The supernatant was collected for further use as the source of the protease enzymes and thus referred to the "crude extract". For preliminary assay, 5 µl of the crude extract were used to spot on a skim milk agar plate (containing 0.5 % peptone, 0.3 % beef extract, 0.5 % skim milk, and 1.5 % agar). The inoculated plate was then incubated at 37 ^{o}C for 24 h. The presence of a clear zone was recorded and used to indicate the bacterial ability to produce proteases.

In addition, further investigation was carried out using five different protein-based media: i) skim milk agar (SK: 1 % skim milk, 2 % agar); ii) skim milk agar supplemented with 0.02 % sodium azide (SK + SA); iii) soya protein agar (SPA: 1 % soya protein, 2 % agar); iv) nutrient agar

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supplemented with 1 % soya protein (NA + SP); and v) nutrient agar supplemented with 1 % gelatin (NA + G). For this, the selected bacteria were initially cultured in these media, centrifuged, and the supernatant was then used to determine the proteolytic activity as mentioned above. In general, the proteolytic activity was carried out at least in triplicate. The value of the relative index of enzyme activity (i.e. protease activity in this case) was also introduced in order to express appropriate results using the ratio between the diameter of the clear zone and that of the bacterial colony.

Results and Discussion

In this study, 171 bacterial strains previously isolated [4] were screened for their protease production. Using skim milk agar, the proteolytic activity could be determined by observing the presence of the clear zone. Preliminary results showed that, of 171 strains tested, 169 isolates representing approximately 99 % could produce protease enzymes. However, it was also evident that these bacteria had different proteolytic activity. To group these protease-producing bacteria, the size of the diameter of the clear zone was used as an indicator as illustrated in Table 1. Only two isolates did not show proteolytic activity on skim milk agar. The majority of the testing bacteria (119 isolates representing ~70 %) produced clear zones between 1.51 and 2.00 cm. The two bacterial isolates, namely TN51 and TN69, which exhibited highest proteolytic activity as indicated by the widest clear zones of 2.73 and 2.65 cm respectively, were selected for further experiment.

Table 1. Distribution and proportion of protease-producing bacteria isolated from *thua nao* based on the size of the clear zone diameter

Width of clear zone (cm)	Number of isolates	Proportion (%)
0 – 1.00	2	1.17
1.01 – 1.50	30	17.54
1.51 – 2.00	119	69.59
2.01 – 2.50	16	9.36
> 2.50	4	2.34

Based on cell morphology, 158 isolates (92.34 %) were rods and only 13 isolates were cocci. In addition, it was found that the endospore-forming bacteria were a major group representing ~76 % (120 isolates) of the rod-shaped bacterial group. According to Chukeatirote et al. [4], the endospore-forming bacteria were present throughout the 72-h fermentation period, whereas those non-sporeforming bacilli were found only at the beginning of the fermentation (less than 24 h). The bacteria TN51 and TN69, which had the strongest proteolytic activity were Gram-positive endospore-forming, rod-shaped bacteria (Figure 1) and thus were referred to as *Bacillus* spp. strains TN51 and TN69 respectively. Their detailed characteristics are given in Table 2.

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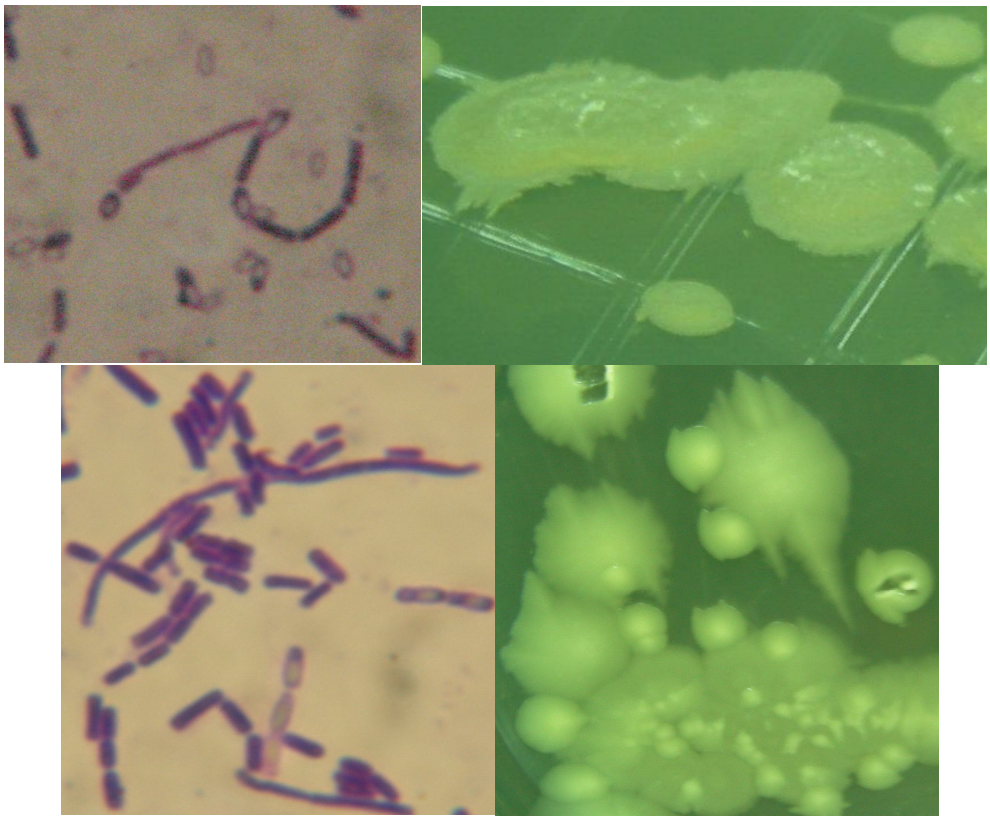

Figure 1. Cell and colony morphology of the bacteria TN51 (top) and TN69 (bottom)

Table 2. Morphological, physiological and biochemical characteristics of the bacteria TN51 and TN69

Characteristic	Bacteria TN51	Bacteria TN69
Gram-staining	Positive	Positive
Presence of spore	+	+
Shape	Rod	Rod
Catalase test	+	+
Voges Proskauer test	+	+
Starch hydrolysis	+	-
Citrate utilisation	-	-
Nitrate reduction	+	+
Oxygen requirement	Facultative anaerobe	Facultative anaerobe
Growth in 5% NaCl	+	+
Growth in 7% NaCl	-	-

Note: + = positive; - = negative

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Subsequently, we used five different protein-based media to evaluate whether this might affect the proteolytic activity. For this, the bacterial cells were cultured in these media, centrifuged and their supernatants collected and used as crude enzymes for evaluation of activity. As shown in Table 3, our results clearly suggest that the protein substrate can significantly affect the production of proteases. Sodium azide, known to prevent microbial growth, is shown to reduce (or even inhibit) the protease production, possibly as a result of inhibition of bacterial growth. Skim milk agar and NA supplemented with soya protein or gelatin are shown to be the appropriate media for induction of proteases enzyme of the tested bacteria. However, it should be noted that the bacterial cell growth could still be observed despite the fact that the crude extracts were used (Figure 2). Besides, it was clear that the growth of the bacterial colony had a major effect on the size of the clear zone; a large bacterial colony tended to induce a wide clear zone. The proteolytic activity could then be misinterpreted unless the size of the bacterial colony was taken into account. Consequently, to provide a better interpretation, the relative index of enzyme activity was calculated as the ratio of diameter (mm) of the clear zone to that of the bacterial colony and was used for this comparative analysis. According to Table 4, *Bacillus* spp. strains TN51 and TN69 exhibit highest activity of proteases when cultured in skim milk agar (SK) and nutrient supplemented with gelatin (NA + G), in which their relative indices of enzyme activity, respectively, are 2.0 and 1.8 for SK, and 1.9 and 1.6 for NA + G. The difference in the ability to utilise different protein sources is likely due to a difference in substrate specificity of the enzymes produced.

Table 3. Influence of different protein-based media on the production of extracellular proteases. (Figures represent mean width of clear zone diameter (mm) ± SD from three replicates.)

Bacterial isolate	SK	SK + SA	SPA	NA + SP	NA + G
Bacillus subtilis	16.0 ± 0	6.0 ± 0	0	20.0 ± 0	24.0 ± 0
B. licheniformis	3.8 ± 0.58	0	0	0	ND
B. natto ASA	17.0 ± 0	7.0 ± 0	11.67 ± 0.58	20.0 ± 0	27.0 ± 1.22
B. natto BEST195	17.0 ± 0	10.0 ± 0	10.67 ± 0.58	18.33 ± 1.53	24.0 ± 0.71
Bacillus TN51	16.67 ± 0.58	7.67 ± 0.58	10.33 ± 0.58	18.33 ± 0.58	24.4 ± 0.89
Bacillus TN69	22.67 ± 0.58	0	17.67 ± 0.58	19.0 ± 1.00	19.0 ± 1.00

Note: SK = skim milk agar; SK + SA = skim milk agar supplemented with 0.02% sodium azide; SPA = soya protein agar; NA + SP = nutrient agar supplemented with soya protein; NA + G = nutrient agar supplemented with 1% gelatin; ND = not determined.

The degradation of proteins has been previously described as the most important biochemical change occurring during the soya bean fermentation [8-10]. In addition, it has been suggested that such proteolysis is strongly relevant to dominating *Bacillus* species, especially for the *dawadawa* case [8,11]. Previous investigations including this present study have also shown that the predominant microbes present in *dawadawa* and *thua nao* are capable of producing proteases [3,12].

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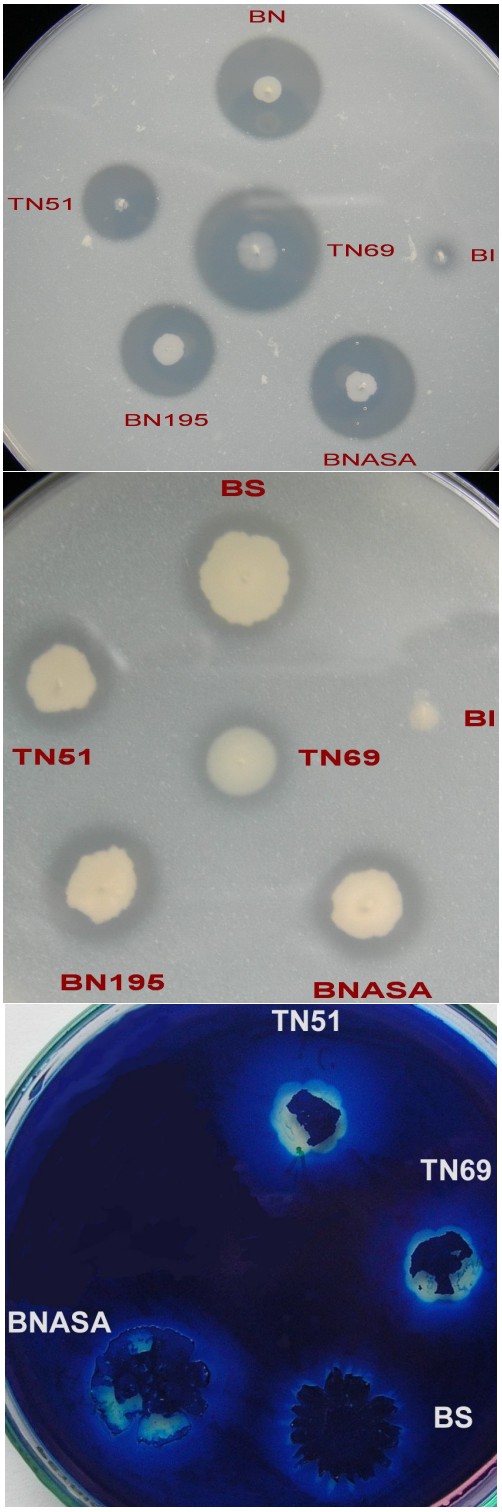

Figure 2. Proteloytic activity of *Bacillus* species when cultured in skim milk agar (top), NA supplemented with soya protein (middle), and NA with gelatin (bottom). BS = *Bacillus subtilis*; Bl = *B. licheniformis*; BNASA = *B. subtilis* (natto) ASA; BN195 = *B. subtilis* (natto) BEST195; TN51 and TN69 = *Bacillus* spp. strains TN51 and TN69

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Table 4. Relative index of protease activity of *Bacillus* species when cultured in skim milk agar (SK) and NA supplemented with 1% gelatin (NA + G)

Bacterial isolates	SK	NA + G
Bacillus subtilis	1.1 ± 0.1	1.6 ± 0.14
B. natto ASA	1.1 ± 0.04	1.4 ± 0.46
Bacillus TN51	2.0 ± 0.09	1.9 ± 0.08
Bacillus TN69	1.8 ± 0.09	1.6 ± 0.06

Note: SK = skim milk agar; NA + G = nutrient agar supplemented with 1% gelatin

Conclusions

The present study has shown that nearly all bacterial species isolated from *thua nao* are capable of producing protease enzymes. Furthermore, the production of extracellular proteases can be induced using appropriate media. The *Bacillus* species TN51 and TN69 previously isolated are of great importance due to their strong proteolytic activity. Both strains are now being studied for their potential as pure starter cultures in the improvement of *thua nao* fermentation process.

Acknowledgement

The authors would like to thank Dr. Mitsuhiro Itaya of the Institute for Advanced Biosciences, Keio University, Japan for providing the *Bacillus subtilis* (natto) strain BEST195.

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Maejo International Journal of Science and Technology

ISSN 1905-7873

Available online at www.mijst.mju.ac.th

Short Communication

Surface investigation of chitosan film with fatty acid monolayers

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Received: 18 December 2008 / Accepted: 12 May 2009 / Published: 22 May 2009

Abstract: The surface pressure- molecular area (π-A) isotherm curves of two fatty acids of different chain lengths, i.e. stearic (C_{18}) and arachidic (C_{20}) acids, were obtained by using Langmuir-Blodgett (LB) technique. Results showed clear isotherm plots with limiting mean molecular area around 21 $Å^2$ for both acids. However, the monolayer was found to collapse at higher than 33 mN m^{-1} and 21 mN m^{-1} for stearic acid and arachidic acid respectively. The effect of Langmuir-Blodgett monolayers of the acids was investigated by atomic force microscopy (AFM). Chitosan film, before and after dipping in water, was also studied by means of AFM so that it could be used for comparison. It was found that the surface of chitosan was more homogeneous and smoother after dipping in water. In addition, more homogeneous surfaces were achieved after transferring a layer of the fatty acid onto the substrate.

Keywords: chitosan, fatty acids, Langmuir-Blodgett, atomic force microscopy

Introduction

Chitosan is an organic polysaccharide usually obtained by deacetylation of the most abundant naturally occurring chitin of crab and shrimp shells [1,2]. This fibril biopolymer is composed predominantly of unbranched chains of β -(1 $\rightarrow$ 4)-2-amino-2-deoxy-D-glucopyranose as shown in Figure 1. It is a non-toxic, biocompatible, and biodegradable polymer [3]. It has been widely used in diverse fields, ranging from waste management to food processing, medicine, and biotechnology [4-9].

Chitosan easily forms a film. In general, its films are clear and flexible, with good mechanical properties. In addition, they do not possess any pores [10]. Since chitosan degrades before melting, it is necessary to dissolve it in an appropriate solvent before casting into films. It is preferable to dissolve chitosan in acetic acid due to its non-toxicity and ease of removal.

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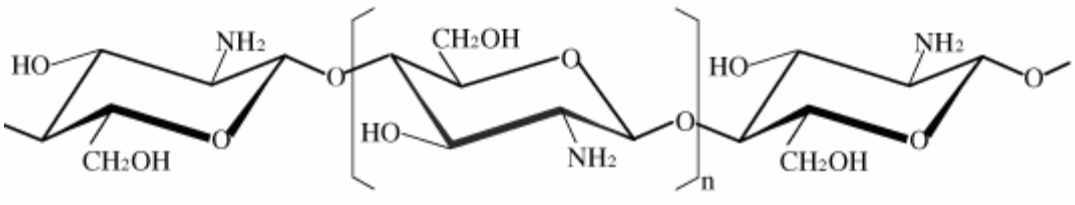

Figure 1. Chemical structure of chitosan

Generally, the film properties of chitosan depend on its morphology, which is effected by molecular weight, degree of N-acetylation, solvent evaporation, and free amine regenerating mechanism. In addition, the solvent used has an influence on the properties of chitosan films [11]. Chitosan coatings have been studied by several researchers to improve the quality and extend the storage life of food products [12-16].

The objective of this work is to characterise some of the surface properties of chitosan film after transferring a monolayer of stearic acid or arachidic acid onto its surface with a view to further explore the properties of the resulting thin film and its applications such as the production of contact lenses. These two fatty acids are known to form good-quality monolayers [17-19]. For this purpose we used Langmuir-Blodgett (LB) technique and atomic force microscopy (AFM).

Materials and methods

Materials

Shrimp-source chitosan was a gift from the chitin-chitosan laboratory of Universiti Kebangsaan Malaysia (UKM). Its degree of deacetylation (DD) was determined to be higher than 93 % by UV method according to the procedure reported by Muzzarelli and Rochetti [20]. Acetic acid (99.5%), stearic acid (99%) and arachidic acid (99%) were purchased from Sigma Chemicals. Chloroform (99.9%) was used as a solvent and deionised water (resistivity =18 MΩ cm) was used as a subphase.

Preparation of chitosan thin film

A certain amount of chitosan was dissolved in 1% acetic acid solution and cast into films at 75^{o}C on microscopic slides (size 25.4 ×76.2 mm, 1-1.2 mm thick), and left for 48 hours. This film was used as substrate for the LB monolayer study.

Methods

The molecular weight of chitosan was found to be about 7.9 $\times 10^5$ g mol^{-1} as determined by gel permeation chromatography (GPC) equipped with a Waters 1515 HPLC pump and Waters 2414 refractive index detector. The column used was PL aquagel-OH 30 (8 μm, 300 × 7.5 mm) and the solvent used was 1% acetic acid.

Surface pressure-area isotherms were recorded in a clean room using a KSV 3000 balance with symmetrical compression of the monolayers. A Whilhelmy plate was used as the surface pressure sensor. Stearic acid and arachidic acid were each dissolved in chloroform (1.0 mg mL^{-1}), and then an appropriate amount of each spreading solution (100 μL) was carefully injected onto the air-water

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interface. After the chloroform was allowed to evaporate for about 10 min, the monolayer film was compressed at a speed of 10 cm^2 min^{-1}. After that, the monolayer was transferred onto the chitosan substrate with a dipping speed of 5 cm^2 min^{-1} at 9.5 mN m^{-1}. Only one layer at the condensed phase was required in this work. All samples as well as the recording of isotherms were prepared or made in the clean room at room temperature.

Atomic force microscopic observation was performed on a digital AFM nanoscope, Dimension 3000 (Digital instruments, Santa Barbara, CA). AFM studies were carried out using the tapping mode under air atmosphere at ambient temperature. The scan size, set point and scan rate are shown in the images. The tapping set-point was adjusted to minimise probe-sample interactions.

Results and Discussion

Langmuir-Blodgett film

Figure 2 depicts the surface pressure-molecular area (π-A) isotherm curve of stearic acid monolayer. The isotherm shows considerable curvature indicating expanded monolayer behaviour. The surface pressure increases gradually, reaches a plateau, and then increases again at lower molecular area before collapsing. In the region of high surface pressure (above 20 mN m^{-1}), the isotherm seems to have a constant slope. The monolayer was found to collapse at higher than 33 mN m^{-1}. The limiting molecular area observed, i.e. the area occupied per molecule when the monolayer is closely packed, was 21 $Å^2$. This value was estimated from the π–A curve by extrapolating the condensed region to zero pressure. The liquid condensed region was around 7-10 mNm^{-1} and 19-22 $Å^2$. Longer liquid condensed region may suggest that a more stable condensed film is formed.

The arachidic acid isotherm is shown in Figure 3. In the region of high surface pressure, the isotherm has a nearly constant slope and this part of the isotherm has been extrapolated to zero surface pressure to evaluate the mean molecular area. The limiting mean molecular area is about 21 $Å^2$ while the pressure collapse is higher than 21 mNm^{-1}. The liquid condensed region is about 6-10 mNm^{-1} and 22-25 Å.

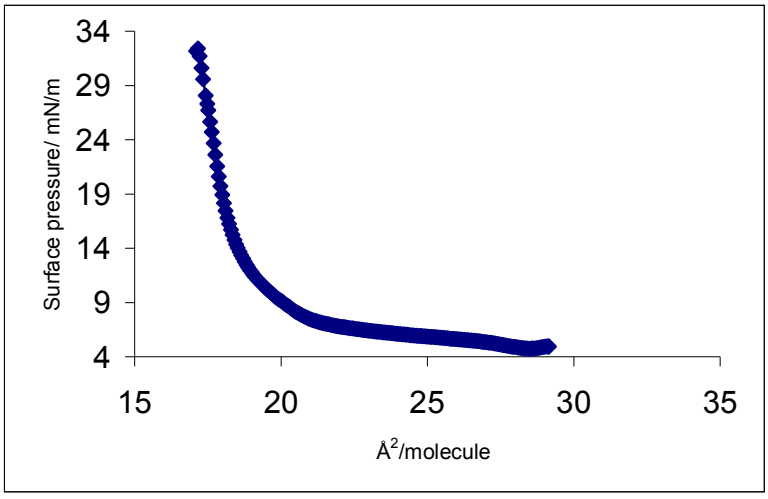

Figure 2. Surface pressure-area isotherm for monolayers of stearic acid

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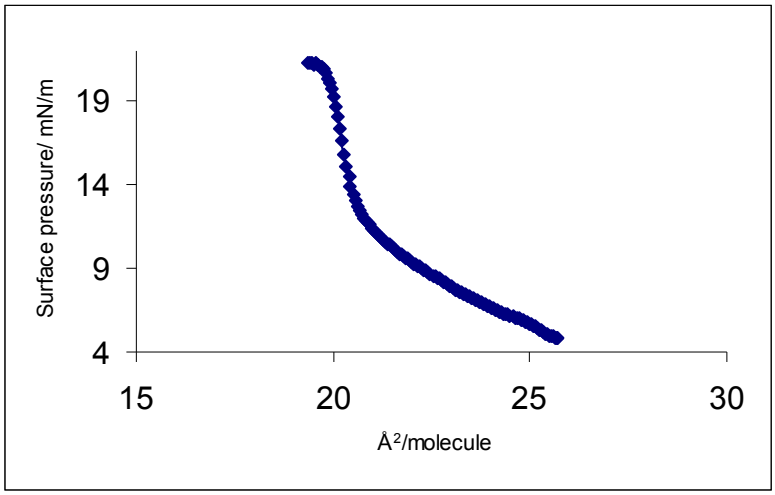

Figure 3. Surface pressure-area isotherm for monolayers of arachidic acid

Atomic force microscopy (AFM)

AFM was used to investigate the smoothness of the surfaces as well as detect possible orientational structures in the LB films. Tapping-mode AFM images can yield information about the surface features of the films.

Figure 4 shows a tapping-mode AFM photograph of chitosan film. The image shows some holes and mountains and it seems to be inhomogeneous. However, the film becomes homogeneous and smoother and the bumps disappear after being dipped in deionised water (Figure 5), which is probably due to a layer of water that forms on the surface. In addition, the surface seems to be robust and not damaged at the tip. However, after transferring a layer of stearic acid or arachidic acid onto the chitosan film at the surface pressure of 9.5 mNm^{-1}, the images show smoother surfaces despite the presence of some bumps as shown in Figure 6. These observations may indicate the existence of interaction between chitosan and the two fatty acids, which is most likely due to the formation of intermolecular hydrogen bonding between the amino and hydroxyl groups in the chitosan and the hydroxyl groups in the fatty acids.

When the monolayers are transferred onto a solid substrate, molecules are fixed and their distribution is not changed. Figure 7 shows that the distribution of the fatty acids is quite uniform and the substrate (chitosan) surface seems to be covered by the film.

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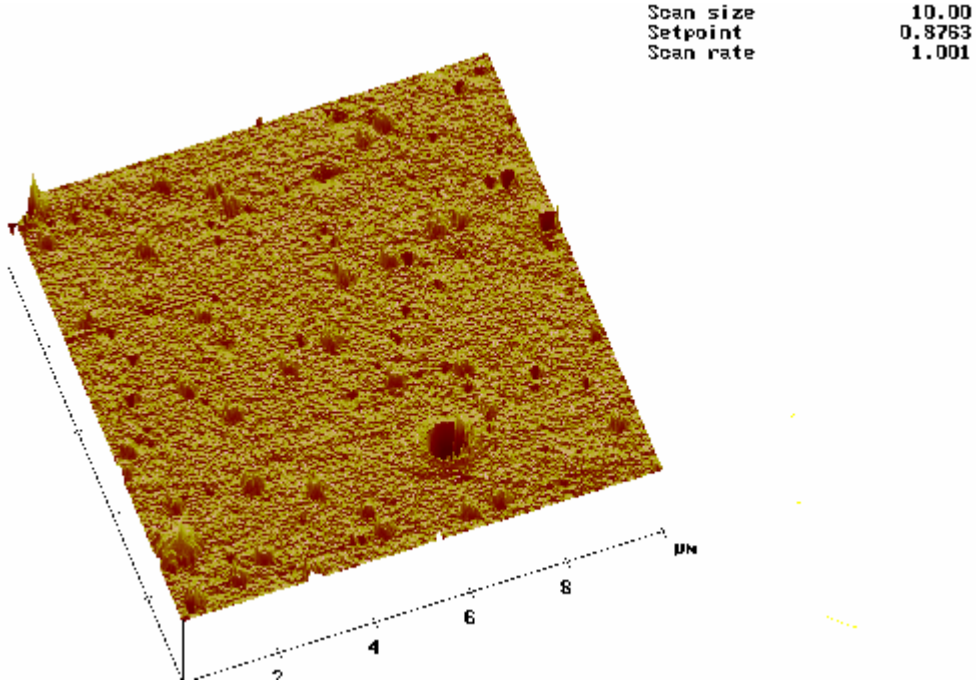

Figure 4. AFM image (10 x 10 μm) of chitosan film

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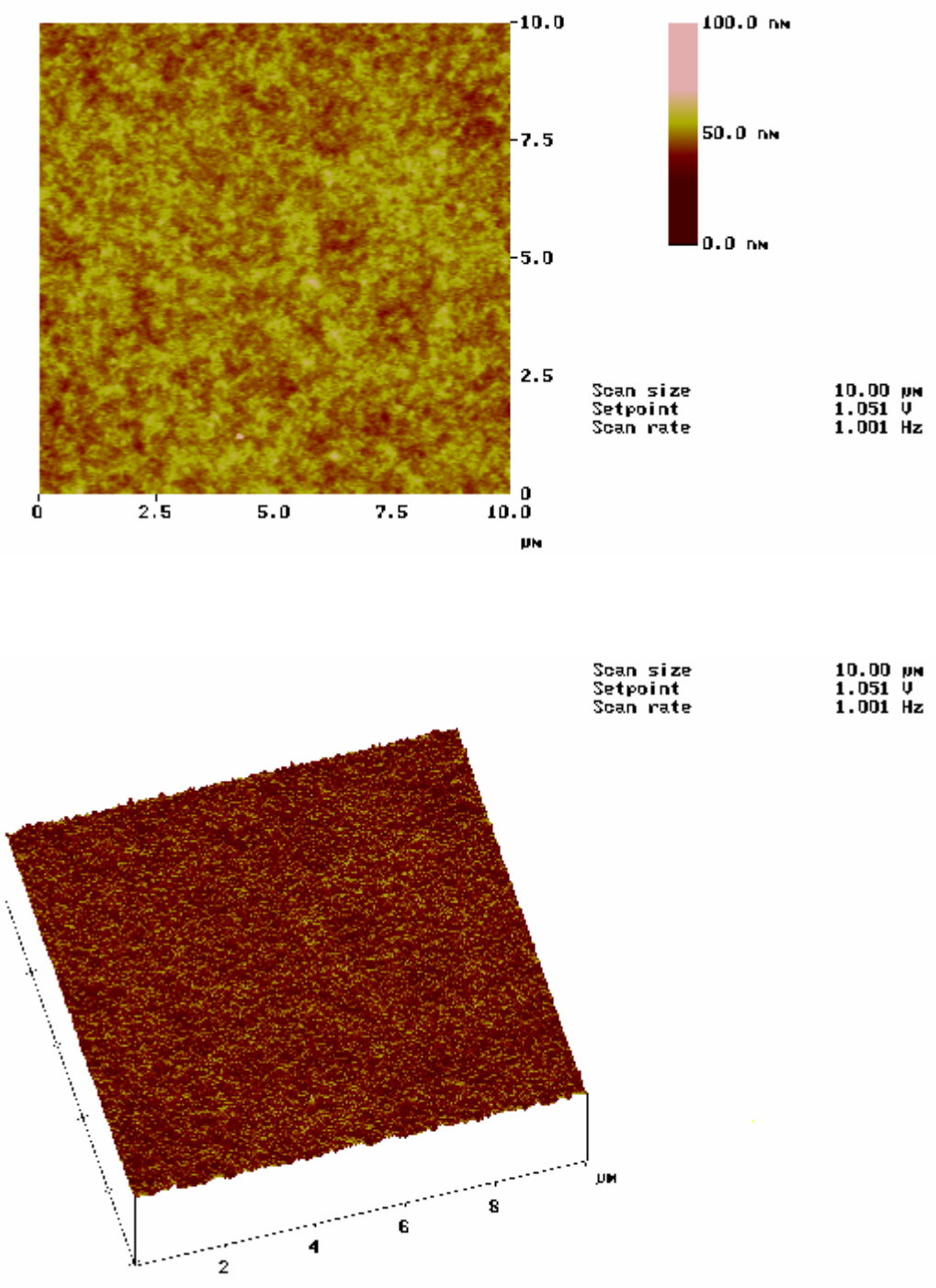

Figure 5. Topographic AFM images (10 x 10 µm) of chitosan film after being dipped in deionised water

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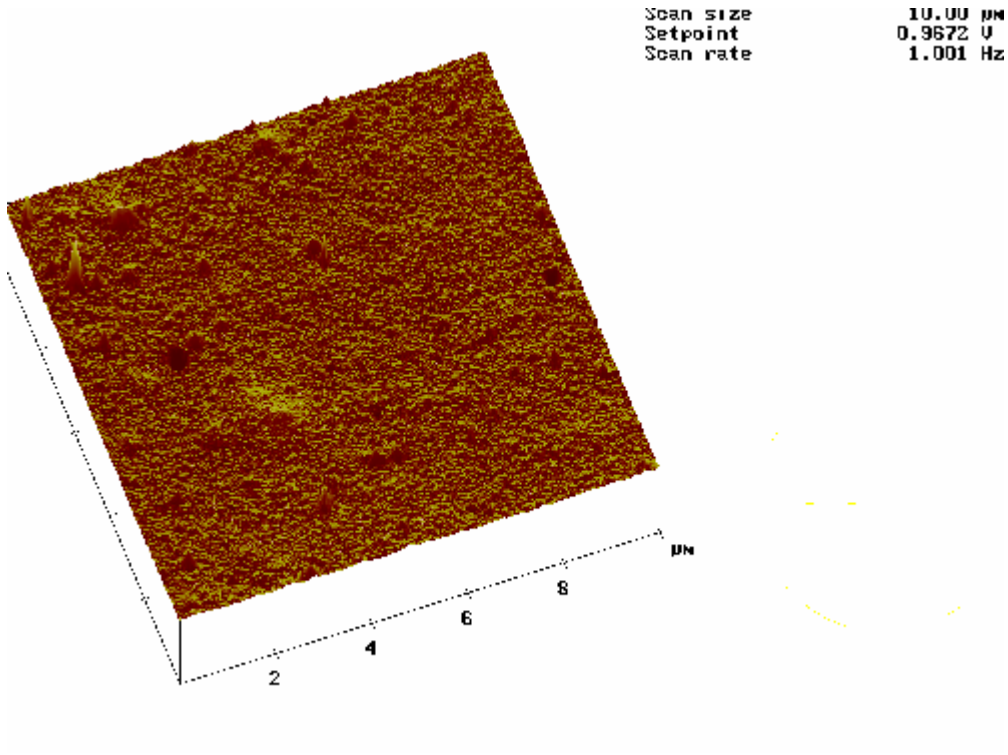
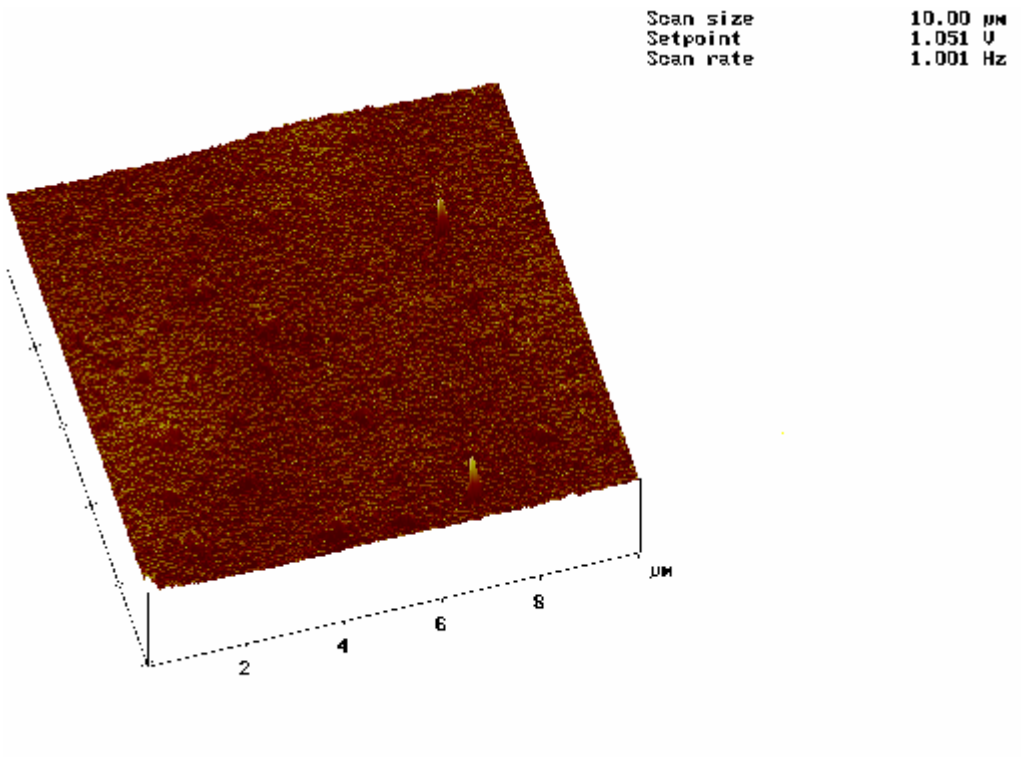


(A)



(B)

Figure 6. AFM photographs (10 x 10 µm) of a transferred monolayer of stearic acid at 9.5 mN m^{-1}(A) and arachidic acid at 9.5 mN m^{-1}(B) onto chitosan film

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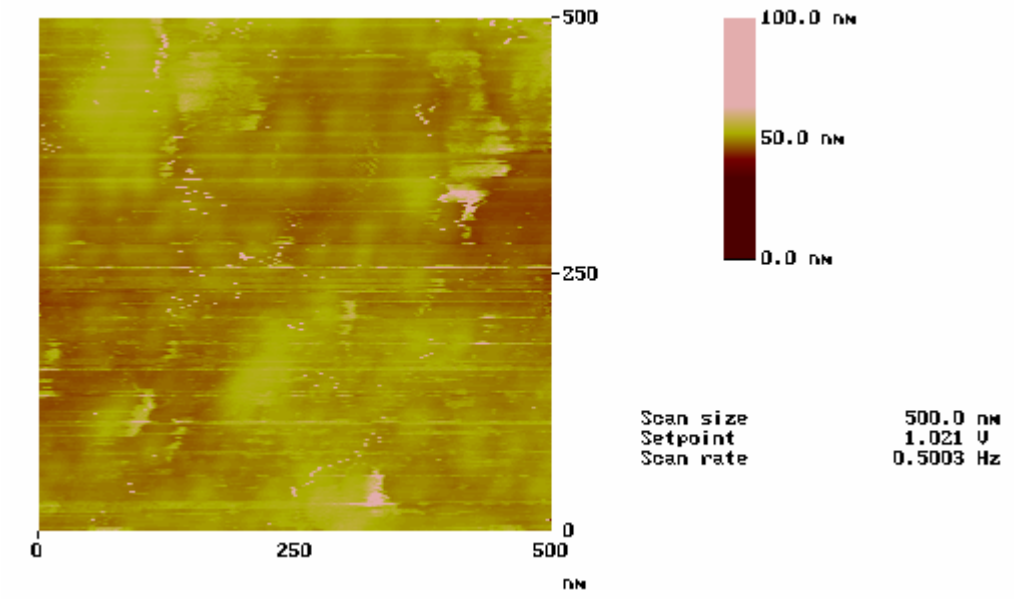
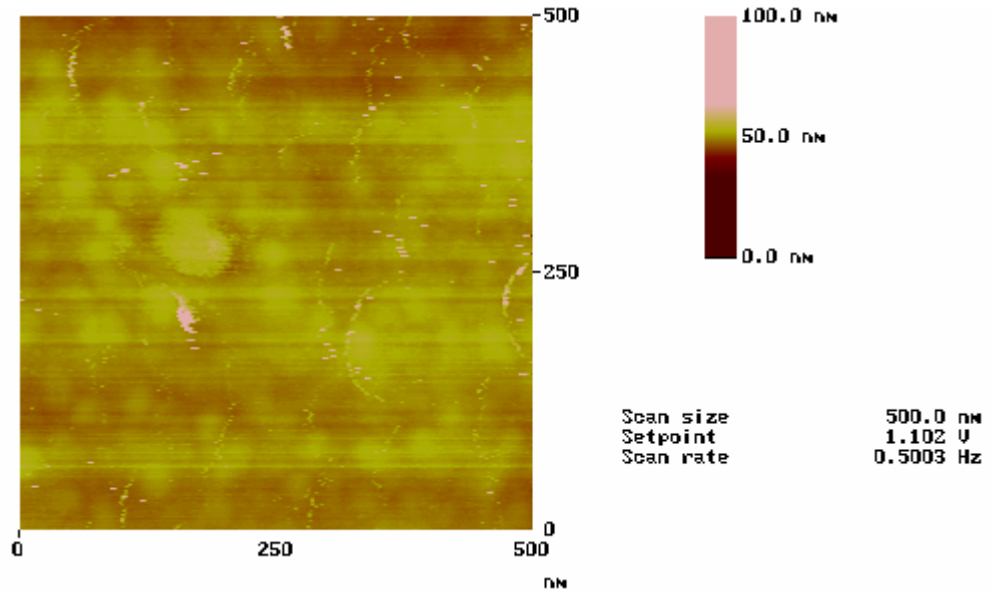


(A)



(B)

Figure 7. AFM photograph (0.5 x 0.5 μm) of LB film of a transferred monolayer of stearic acid at 9.5 mNm^{-1}(A) and arachidic acid at 9.5 mNm^{-1}(B) onto chitosan film

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Conclusions

This study has shown that the LB measurements of stearic acid and arachidic acid give distinct isotherm plots. AFM photographs show holes and mountains on the surface of chitosan film. However, improvement in the surface morphology of chitosan film can be achieved after transferring a monolayer of fatty acids onto the film, i.e. smoother surfaces are obtained after coating chitosan film with a fatty acid monolayer, which makes this film suitable for certain applications.

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Maejo International Journal of Science and Technology

ISSN 1905-7873

Available online at www.mijst.mju.ac.th

Full Paper

A method to calculate the voltage-current characteristics of 4H SiC Schottky barrier diode

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Received: 29 December 2008 / Accepted: 14 June 2009 / Published: 18 June 2009

Abstract: The voltage-current characteristics of the Schottky barrier diode defined by the diode equation can be obtained by using iteration method and a C++ program. The diode equation is split into two functions and the current density for a specified forward voltage is evaluated at a point where the equality of these two functions is seen to hold. A set of values of current and voltage are generated using the C++ program. The device parameters, i.e. area, barrier height and doping level, were obtained from published work. These are found to tally well with experimental results. The analysis has been made using 4H silicon carbide diodes with contacts of nickel, titanium and gold.

Keywords: Schottky barrier diode, 4H silicon carbide (4H-SiC), barrier height, specific on-resistance

Introduction

The voltage-current relationships of the Schottky barrier diodes have been obtained by Bethe [1], Schottky [2] and Crowell and Sze [3]. These have been experimentally verified by Chang and Sze [4] with devices having pre-determined parameters and dimensions. However, it is sometimes useful to obtain a solution for the current density J_F in terms of forward drop directly from the diode equation for specific values of forward voltage V_F. Since the diode equation cannot be solved directly, in this paper a C++ program has been used which can give by iteration the equality of two functions into which the

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diode equation is split. The set of values of J_F and V_F obtained using the C++ program are found to tally well with experimental results by Saxena and Steckl [5] and Sochacki [6] for nickel metal contact, by Itoh et al. [7] for titanium metal contact, and by Itoh et al.[8] for gold metal contact.

Theory

The basic device structure using a partial metal overlap over the Schottky contact by Itoh et al. [7] is shown in Figure 1. The device is made using an n-type 4H SiC substrate on top of which an n-type epilayer is grown. The thickness of the epilayer is 'h' and boron implant from the top of the epilayer is made with a gap in the centre over which a metal contact with finite diameter 'd' is made. The diameter of the Schottky contact is 'a'.

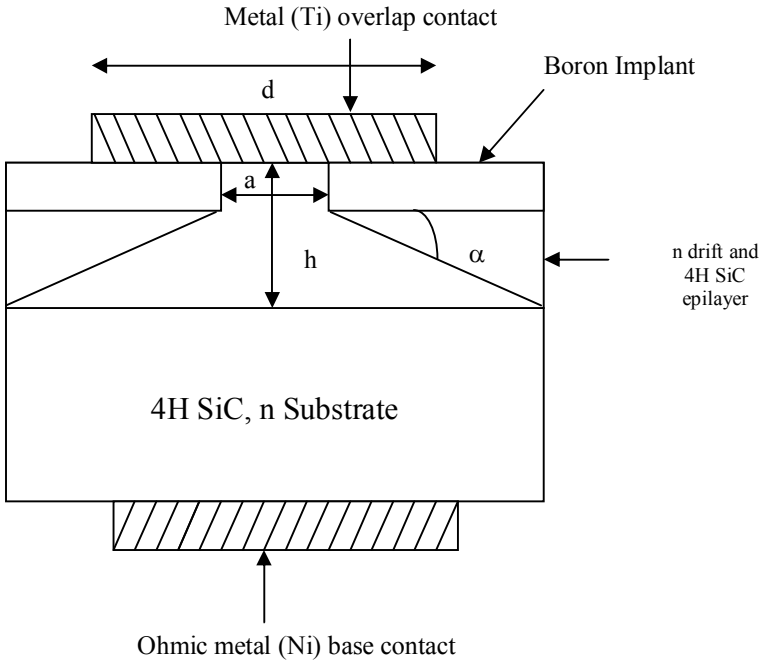

Figure 1. The basic 4H silicon carbide device structure [7]

The voltage-current (V-I) equation of the Schottky diode using thermionic emission theory is given by Saxena et al. [5]. This can be quoted as:

$$J_F = J_S\left[\exp\left(\frac{qV_D}{\eta KT}\right)-1\right], \qquad (1)$$

where

$$J_S = A^*T^2\exp\left(-\frac{q\phi_B}{\eta KT}\right), \qquad (2)$$

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and V_D is the voltage drop across the diode, i.e. at the Schottky contact, K is the Boltzman's constant (CV/K), q is the electronic charge in coulomb, A^* is the effective Richardson's constant in $\left[\frac{A}{cm^2}K^2\right]$, and ϕ_B is the Schottky barrier height in volt.

The Schottky barrier diode is shown in Figure 2. It has the series resistance, namely the specific on-resistance R_{on-sp}, which is due to the resistance of the drift region R_D, the substrate R_{sub} and the contacts. R_{on-sp} may be approximated at low and medium current levels to the resistance of the drift layer of thickness 'h'.

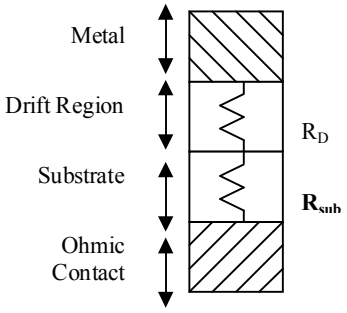

Figure 2. Equivalent circuit model of 4H-SiC Schottky barrier diode of Figure 1

The basic voltage-current equation for such a diode has been derived by Baliga [9] and Bhatnagar et al. [10]. The forward drop VF of the diode can be expressed as :

$$V_F = V_D + J_F R_{on-sp} \qquad (3)$$

Combining equation (3) with (1) and (2) above,

$$V_F = \frac{\eta KT}{q} \ln\left(\frac{J_F}{A^* T^2}\right) + \phi_B + J_F R_{on-sp} \quad (4)$$

The specific on-resistance, R_{on-sp}, can be calculated using the trapezoidal current flow model as mentioned by Baliga [9]. This is shown to be the same as in Figure 1. The ohmic contact at the base is grounded and the top metal contact is given a negative bias. The current flow in the device starts from the Schottky contact at the top and spreads out to form a trapezoid and α is the angle made by the inclined side of the trapezoid and the horizontal level . The specific on-resistance of the device can be expressed as [9]:

$$R_{on-sp} = \rho_D \frac{L_G}{\tan\alpha} \ln\left[1 + \frac{2h}{a}\tan\alpha\right], \qquad (5)$$

where $\alpha = 26°$.

The equation (4) can be rewritten as

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$$-\frac{\eta KT}{q}\ln\left(\frac{J_F}{A^*T^2}\right) = -V_F + \phi_B + J_F R_{on-sp} \quad (6)$$

The above equation can be divided into two functions. Let the left hand side be represented by function F_1 and that on the right hand side denoted by function F_2. The equation above can then be written as $F_1 = F_2$.

A C++ program was then developed and run for a fixed value of V_F with known values of other parameters for a given range of values of J_F. The value of J_F at which ($F_1 - F_2$) tends to zero or a minimum value is the required value of J_F for a fixed value of V_F. This process was repeated for other values of V_F and a set of values of 'J_F versus V_F' was obtained. This was performed in the case of nickel, titanium and gold metal contacts for 4H SiC. The results obtained were compared with those obtained experimentally [5-8] for the 4H SiC diodes. The comparisons are shown in Figures 3-5.

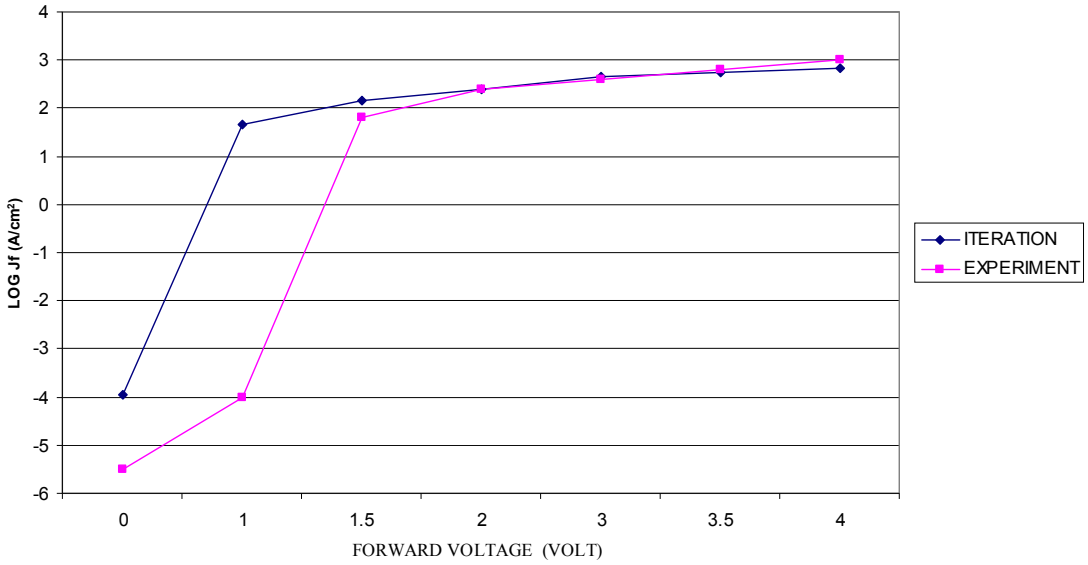

Figure 3. V_F-log J_F plot of 4H SiC with nickel metal contact

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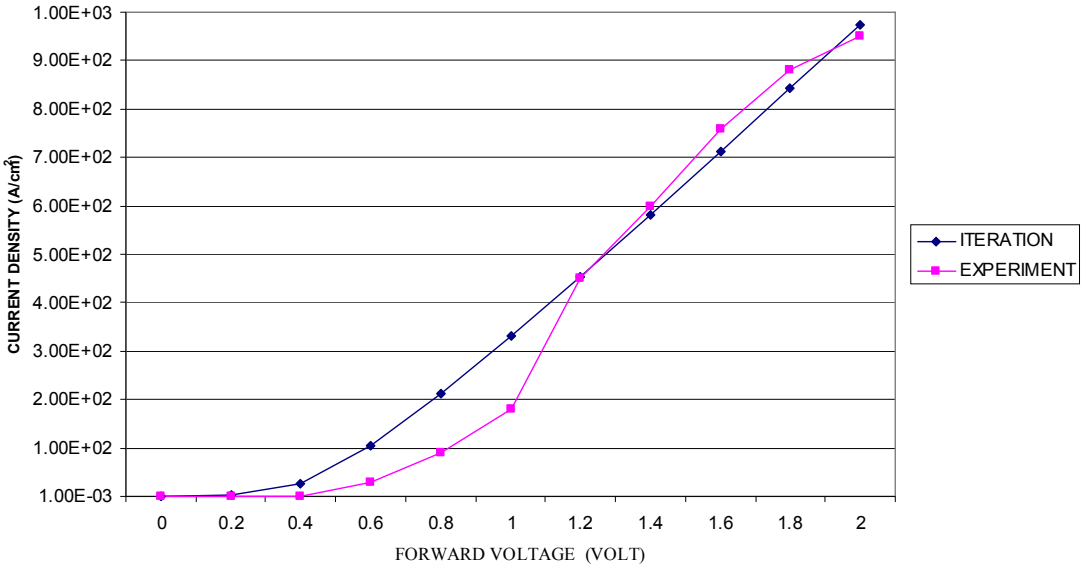

Figure 4. V_F-J_F plot of 4H SiC with titanium metal contact

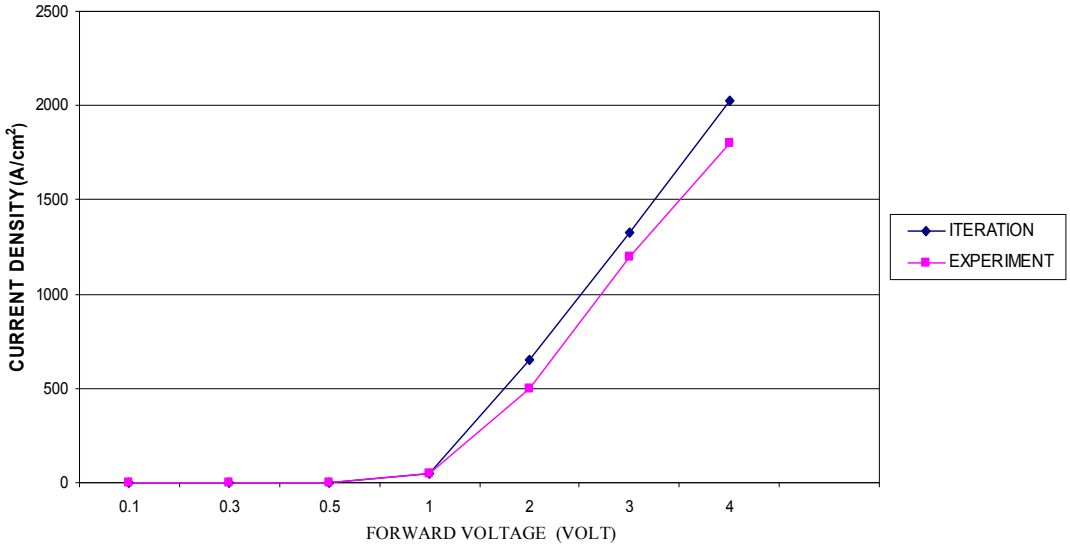

Figure 5. V_F-J_F plot of 4H SiC with gold metal contact

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The C++ program used is given below:

```
#include<iostream.h>
#include<conio.h>
#include<math.h>
void main()
{
clrscr();
long double q,rsp,n,k,t,bh,fv;
float jf=.001;
long double f1,f2;
int a=146;
q=1.602e-19 ;
t=300;
k=1.38e-23;
n=1.7;
rsp=34.94;
bh=1.55;
cout<<"enter the value of Ideality factor:";
cin>>n;
cout<<"enter the value of specific on resistance:";
cin>>Rsp;
cout<<"enter the value of barrier height:";
cin>>fv;
cout<<"enter the value of forward voltage:";
cin>>fv;
cout<<"difference is="<<bh-fv;
for(int i=1;jf<=.07;i++,jf=jf+.001)
{  f1=((n*k*t)/q)*(2.303*log((jf)/a*t*t)));

f2=(bh-fv)+jf*rsp;
cout<<"For value of Jf="<<jf<<endl;
        {
        cout<<(f1-f2)<<endl;
                if(i%10==0)
                        {
                                i=0;
                                getch();
                        }
                }
        }
}
```

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Discussion

The V_F-J_F plots of the 4H SiC diodes obtained by the C++ program tally well in the case of metal contacts of nickel, titanium and gold for some finite range of the forward drop V_F. In the case of nickel (Figure 3) the comparison between the result from the C++ program and the experiment obtained by Saxena et al. [5] shows a deviation in the low voltage region at V_F below about 1.5 V, but the curves tally with each other at higher values of V_F. Similarly, deviation exists for the titanium contact up to 1.2 V; at higher voltages the graphs match. This is shown in Figure 4, in which the result obtained using C++ programming is compared to that obtained by Itoh et al. [7]. The best match of the results can be seen in Figure 5 for gold contact metal, in which marginal deviation of the iterated curve exists at voltages exceeding 1.2 V and both curves merge with each other at V_F values below 1 V. The calculated result shown in Figure 5 is plotted alongside that experimentally obtained by Itoh et al. [8].

The discrepancy in the characteristic curves for nickel contact shown in Figure 3 is primarily because of the low value of Richardson's coefficient used in the experiment [5], the effective Richardson's coefficient A^* being found to be much lower than 146 A/cm^2/K^2. This results in a higher value of V_F for a specified value of J_F.

The experimental result for titanium contact shown in Figure 4 [7] have a large deviation from the result obtained by the C++ program in the low voltage region with V_F less than 1.2 V. However, the barrier height for titanium contact metal has been found to depend on the polarity of the crystalline phase, i.e. ϕ_B = 1.17 V for the C face and 1.1 V for the SiC face. Such variation in ϕ_B may be responsible for the voltage drop at the contact at high and low current levels. The present work has not taken into account such variation in ϕ_B.

The result of the C++ program for the gold metal contact shown in Figure 5 seems to tally well with experimental result of Itoh et al. [8] for all ranges of voltage. In this case, the effective Richardson's coefficient A^* of 146 A/cm^2/K^2 was taken for both theory and experimentation.

Conclusions

A method was devised to draw the voltage-current characteristics of the 4H-SiC Schottky barrier diode. The method would be useful in generating such characteristics where experimental facilities do not exist, which may help in analysing the differences that may arise between theoretical and experimental results so that a better theoretical model can be developed.

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Full Paper

A decision support tool for basin irrigation in northern Nigeria

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Received: 19 March 2009 / Accepted: 22 June 2009 / Published:1 July 2009

Abstract : Inadequate rainfall, water resources scarcity and attendant food security-related problems have made irrigation technology a necessity. This work presents the development of a decision support system for solving surface irrigation design problems in northern Nigeria. The arid northern states affected by desert encroachment constitute a good candidate and their climatological data was obtained from the Nigerian Metrological Agency. The interactive system was defined in terms of inputs and outputs. The inputs were properties of soil, surface irrigation method and climate. The outputs were mainly the quantity of water application, scheduling pattern, possible design configuration, advance time, cut-off time, application rate, and water use efficiency. The FAO Penman-Monteith equation was used to estimate evapotranspiration values of major crops grown in Nigeria. Mathematical models outlined by Walker and Skogerboe were adapted, and heuristics applied in determining the best configuration that achieves optimum water application efficiency. We encoded the knowledge base using Matlab® software. The application was successfully used for the modification of a farm irrigation scheme in Kaduna state. This indicates that the adoption of new technologies for irrigation design issues could enhance agricultural productivity in northern Nigeria.

Keywords: decision support system, surface irrigation, basin irrigation, evapotranspiration, advance time, required opportunity time

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Introduction

Problems of inadequate rainfall, water resources scarcity and food security have made irrigation technology a necessity. Besides, the economic importance of agricultural produce and the need to be self-sufficient has brought irrigation technology to the foreground in several parts of the world, including northern Nigeria. Irrigation as defined by Garg [1] is the science of artificial application of water to land, in accordance with the 'crop requirements' through 'crop period' for full-fledged nourishment of the crops. The three broad classes of irrigation systems are pressurised distribution, gravity flow distribution, and drainage flow distribution. The term 'surface irrigation' as defined by Walker and Skogerboe [2] refers to a broad class of irrigation methods in which water is distributed over the field by overland flow. A flow is introduced at one edge of the field and covers the field gradually. The rate of coverage (advance) is dependent almost entirely on the differences between the discharge onto the field and the accumulating infiltration into the soil. Secondary factors include field slope, surface roughness, and the geometry or shape of the flow cross-section.

Irrigated agriculture faces a number of difficult problems in Nigeria. One of the major concerns is the generally poor efficiency with which water resources have been used for irrigation. A comparatively safe estimate of 40% or more of the water diverted for irrigation is wasted at the farm level through either deep percolation or surface runoff. These unfortunately cause problems of erosion and excessive loss of nutrients, which eventually leads to low productivity and sometimes failure of the entire irrigation systems. In addition, shortage of irrigation experts contributes to the failure of many irrigation projects. Having people who are conversant with the soil, water, crop, climate, etc. plays a vital role in the success of irrigation projects. These experts include agriculturists, soil scientists, and engineers. Disappointing results of irrigation development efforts in Nigeria for over 20 years have made it imperative for the incorporation of an intelligent technology such as the use of expert systems. The need to maximise land resources by encouraging all-year-round farming with the aim of boosting agricultural production in the mist of limited water supply has made this technology adaptation expedient for the Nigerian nation. This will help ensure a consistent growth of the development of quality and efficient surface irrigation systems.

Our work presents the usage of a decision support system (DSS) to solve surface irrigation design problems in Nigeria. A DSS is a computer software application designed to assist managers or operators with decision making in a particular field. It facilitates work by decomposing and tackling decision problems into smaller units that may be analysed and/or simulated. An expert system (ES) as defined by Badiru and Cheung [3] 'is an interactive computer-based decision tool that uses facts and heuristics to solve difficult decision problems based on knowledge acquired from an expert'. Agricultural decision support systems and expert systems should combine the analytical, experimental and experiential knowledge with the intuitive reasoning skills of a multitude of specialists to aid farmers in making the best decisions about their farmlands, crops and associated activities.

Related Work

In north-west China, a decision-making support system was designed to improve water management of Jingtia Chuan pumping irrigation scheme at the upper reaches of Yellow River [4].

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The system is based on the application of computer network and specially developed program. Significant benefit and social benefits have been achieved by application of the system. The structure of the system is such that irrigation water management is related to water sources, climate condition, water requirement, canal feature, water distribution, etc. The system was developed with the feature of collecting and processing information dynamically.

Furrow irrigation events conducted under usual farmer management were analyzed to determine the irrigation contribution to deep drainage under surface irrigated cotton in Queensland [5]. Application efficiency was a low 48%. Losses to deep drainage were substantial, averaging 42.5 mm per irrigation. This can lead to significant environmental harm and represents an annual loss of up to 2500 m^3/ha of water that could have been beneficially used to grow more cotton. To tackle this problem, a simulation of each event was carried out using the simulation model SIRMOD; it illustrated simple recipe strategies that would lead to gain in efficiency and reductions in the deep drainage losses. Additional simulations of a selected event showed that further significant improvement in performance could be achieved by the application of more advanced irrigation management practices, involving in-field evaluation and optimisation of the flow rate and irrigation time to suit the individual soil conditions and furrow characteristics. Application efficiency in the range of 85-95% was achievable in all but the most adverse conditions. The relation between deep drainage and irrigation management was demonstrated, confirming that substantial reduction in deep drainage is possible by ensuring that irrigation application does not exceed the soil moisture deficit.

Prasad and Babu [6] surveyed the availability of various expert systems in agriculture dating back 30 years.

Methods

Surface irrigation design is a procedure for matching the most desirable frequency and depth of irrigation with the capacity and availability of water supply. For this, the basin approach was considered wherein the field is levelled in all directions, encompassed by a dyke to prevent runoff, and provided with an undirected flow of water. The design of the user interface of the DSS was accomplished in Matlab by using its graphical user interface development environment (GUIDE). The interactive system allows the user to input data about the farm, soil, crop, location, climate, and type of surface irrigation method to be employed. The resulting output will be the determination of the best configuration that achieves the highest efficiency, estimated water requirement and irrigation scheduling.

The estimation of evapotranspiration (ET) was done using climatological data of the northern region obtained from the Nigeria Meteorological Agency at Lagos. Estimation was chosen owing to difficulties in obtaining accurate direct measurement of ET under field conditions. These data initially include the following:

- Maximum temperature in ^0C (T_1)
- Minimum temperature in ^0C (T_2)
- Solar radiation in $MJm^{-2}\ d^{-1}$ (R_a)
- Air pressure in KPa (P_a)

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- Wind speed in ms^{-1} (U_2)
- Saturated vapour pressure in KPa (e_s)
- Relative humidity (RH)
- Mean daily maximum sunshine hour in hr (N)
- Mean daily actual sunshine hour in hr (n)
- Monthly effective precipitation in mmd^{-1}
- Potential evapotranspiration (ETo) in mm/d

The climatological data obtained were for a period of 7-10 years back. The daily potential evapotranspiration (ETo) for each month from January to December in each year was studied and the extreme value was taken as a representation of ETo value for that particular month. The mathematical model used in estimating crop water requirement is as shown in Figure 1. It is called the FAO-Penman-Monteith evapotranspiration model [7]. The model is preferred because of its combination of energy balance and aerodynamic considerations. Methods outlined by Walker and Skogerboe [2] for calculating required opportunity time and time of advance were used to code the knowledge base.

Knowledge acquisition

The knowledge acquisition technique employed was the protocol analysis techniques, which involves the identification of basic object within a protocol. This was done by highlighting all the concepts that were relevant to the DSS. The knowledge employed was the procedural knowledge as outlined by Compton and Jansen [8]. This knowledge was coded as a set of rules and was stored in the data structure. The knowledge representation used was production rules frames. The knowledge acquisition stage was interesting because we were able to assume responsibility of modelling our own reasoning and expertise in the form of a computer program. An engineer (N.A Ajayi) from the Federal University of Technology at Akure, an expert of over 30 years, was also consulted during the knowledge acquisition process. Design manuals and reports were also used throughout the documentation process. Data related to ET and other parameters were kept in an organised and easily accessible format.

Algorithm for basin irrigation design system [9]

1 Select region
2 Select crop type
3 Select soil type
4 Determine ET_O using FAO-Penman-Monteith model
5 Determine required intake opportunity time
6 Compute the maximum unit-flow associated depth near the basins
7 Select several and appropriate field layouts or configurations
8 Compute advance time
9 Compute cut-off time
10 Determine application efficiency
11 Determine irrigation scheduling

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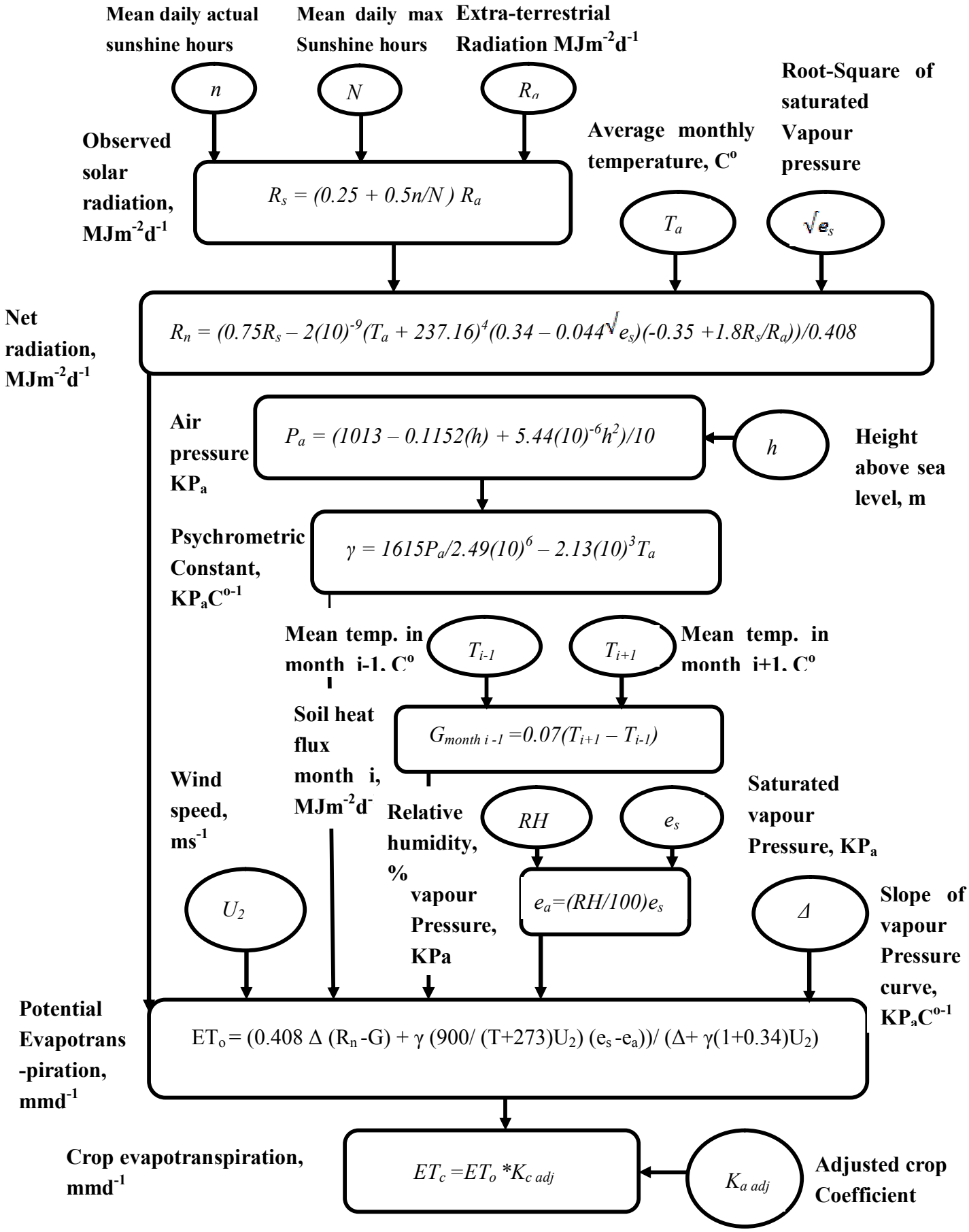

Figure 1. FAO-Penman-Monteith evapotranspiration (ET) model [7]

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Key modelling equations [2]

The *required intake opportunity time* (r_{reg}) can be deduced using the following formulas after a series of numerical iterations:

$$t_2 = t_1 + \frac{(Z_{reg} - Kt_1^a - f_o t_1)}{aK/(t_1^{1-a} + f_o)} \quad (1)$$

$$Z_{reg} = d_r(f_c - m_c)(\lambda_s / \lambda_w)100 \quad (2)$$

$$t_1 = 5A_o L / Q_o \quad (3)$$

where t_1 and t_2 are the initial and final estimate of r_{reg} respectively, Z_{reg} is required application depth, K is the constant coefficient, f_o is the basic intake rate, a is the constant exponent, d_r is the depth of root zone, f_c is the moisture content at field capacity, m_c is the soil moisture content, ℓ_s and ℓ_w are the density of soil and water respectively, A_o is the cross section area of flow at the inlet, L is the length of field, and Q_o is the inlet discharge. If the difference between t_1 and t_2 is within 0.5 minute, then r_{reg} is determined as the result or converged value. If they are not equal, the value of t_1 is replaced by t_2 and the iteration is performed over again.

The maximum *depth of flow* $y_O = (\frac{Q_o{}^2 \mathrm{S_n}{}^2 X}{3600})^{0.23}$ (4)

where S_n is soil roughness factor and X is advance distance from the field inlet. Allowance of up to 20% extra water volume was introduced in the design of the DSS so as to ensure adequate infiltration at the extreme ends of the basin field.

The *advance time* t_L can be deduced after a series of iteration using:

$$t_2 = t_1 - \frac{Q_o t_1 - .77A_o L - S_z K t_1^a L - f_o L t_1/(1+r)}{Q_o - S_z aKL/t_1^{1-a} - f_o L t_1/(1+r)} \quad (5)$$

where Q_o is inlet discharge in m^3/min/basin or unit width, and S_z, the subsurface shape factor, is defined as:

$$S_z = \frac{a + r(1-a) + 1}{(1+r)(1+a)} \quad (6)$$

where a is a constant exponent (soil dependent) and r is the power of advance exponent (ranges between 0.4-0.6). If the difference between t_1 and t_2 is within 0.5 minute in eqn (5), then t_L is determined as the result or converged value.

The cross-section area of flow at the inlet in m^2 is given by

$$A_o = \left[Q_o n/(60P_1 S_o^{.5})\right]^{1/P_2} \quad (7)$$

The parameters P_1 and P_2 are empirical shape coefficient factors for basin and Q_o is inlet discharge in m^3/min/basin or unit width

The *cut-off time* (t_{co}) is computed using $t_{co} = r_{reg} + t_L$ (8)

The *application efficiency* (E_a) can be deduced using

$$E_a = 100Z_{reg}(L/Q_o)60t_{co} \quad (9)$$

Design data (including values to be supplied at run-time) include:

a - constant exponent (soil dependent)

r - power of advance exponent (ranges between 0.4-0.6)

S_n - soil roughness factor

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K - constant coefficient, m^3/min/m of length (soil dependent)
f_o - basic intake rate, m^3/min/m of length (soil dependent)
X - advance distance in metres from the field inlet
S_o - field slope
r_{reg} - required intake opportunity time, min
L - length of field, m
P_1 and P_2 - shape factors

Using the above model, a DSS for solving problems associated with surface irrigation design and management in the northern region of Nigeria was designed at the University of Lagos, Nigeria [9]. The design was based on a large number of theoretical, rational and empirical relationships and heuristic experiences. The system was implemented in Matlab software which allowed the coding of the rules and equations to be easily facilitated in the background while presenting the user with a friendly dialog interface.

Illustrative Example of Software Implementation

Various tests were carried out using the surface irrigation support system for northern Nigeria for different crops, soil types, etc. The following surface irrigation design problem presents a specific case for a design modification of an existing irrigation scheme. The farm under study was situated at Kujama district, Kaduna state of north-central Nigeria and had the following data:

Plantation date: 5/4/2007
Area of field: 2 ha measuring 200 m x 100 m
Drainage system: good
Soil texture: sandy loam (stable)
Field slope: 0.1%
Permanent wilting point: 13%
Field capacity: 27%
Soil moisture content: 18%
Crop to be planted: beans
Total length of crop development: 110 days
Irrigation system: basin

Results using basin irrigation design approach are as shown in the screenshot of the DSS in Figure 2. The test was further carried out for different growth stages of the crop and the results obtained are summarised in Table 1. Analysis of the results of the test showed that out of the five possible configurations for all the stages of growth, the configuration suggested by the DSS was based on the one with highest application efficiency. The design with ten 20-m basins was selected during the initial stage of growth and one with twelve 17-m basins was selected during the medium and final stages. The resulting application efficiency was found to be 82.71 %, 54.49 % and 53.83 % for the initial, medium and final growth stages respectively. Furthermore, a relationship was also established between time of advance and discharge rate for various growth stages. In Figure 3, it can be seen that as advance time increases there is a reduction in basin discharge rate. The finding from the support

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system was then used to prepare a scheduling table for the management of the proposed irrigation system as summarised in Table 2.

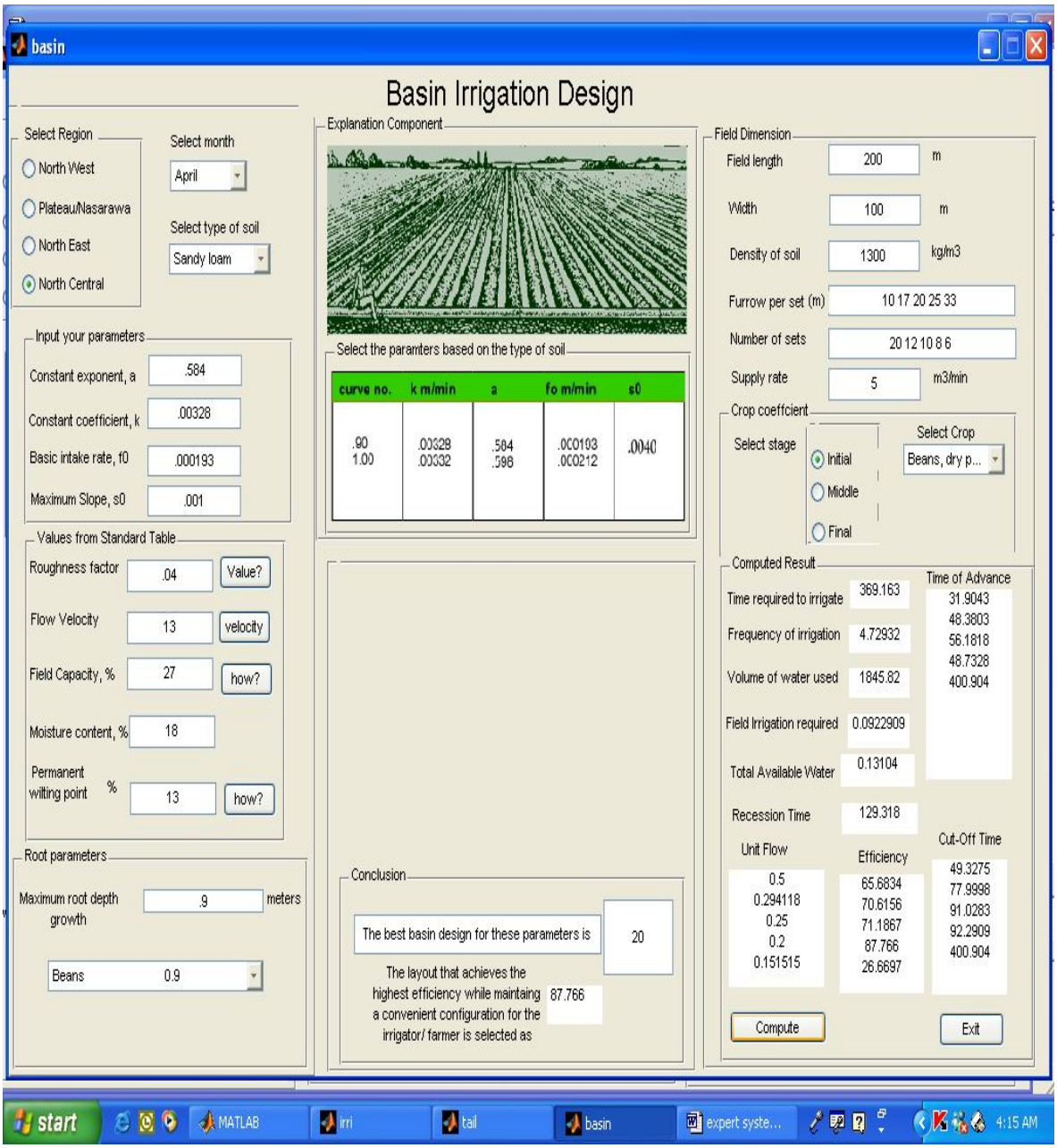

Figure 2. Sample DSS consultation output screen for a basin irrigation problem

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Table 1. Test results for Kujama basin design problem

Initial stage of growth								
No. of basin	Basin width (m)	Discharge (m^3min^{-1})	Advance time (min)	Cut-off time (min)	Irrigation interval (days)	Water required (m^3)	Application time (min)	Application efficiency (%)
20	10	0.5	22.78	42.82	4.7	1958.68	391.74	75.66
12	17	0.29	33.02	67.09				82.1
10	20	0.25	38.27	78.35				*82.71*
8	25	0.2	48.73	98.82				81.96
6	33	0.15	71.84	137.96				77.5
Medium stage of growth								
No. of basin	Basin width (m)	Discharge (m^3min^{-1})	Advance time (min)	Cut-off time (min)	Irrigation interval (days)	Water required (m^3)	Application time (min)	Application efficiency (%)
20	10	0.5	44.29	61.71	1.61	2973.22	594.64	52.5
12	17	0.29	71.47	101.09				*54.49*
10	20	0.25	84.53	119.38				54.28
8	25	0.2	108.75	152.3				53.18
6	33	0.15	155.78	213.37				50.13
Final stage of growth								
No. of basin	Basin width (m)	Discharge (m^3min^{-1})	Advance time (min)	Cut-off time (min)	Irrigation interval (days)	Water required (m^3)	Application time (min)	Application efficiency (%)
20	10	0.5	47.54	63.33	5.3	3009.48	601.9	51.16
12	17	0.29	75.46	102.32				*53.83*
10	20	0.25	88.82	120.42				53.81
8	25	0.2	113.53	153.02				52.93
6	33	0.15	161.32	213.45				50.09

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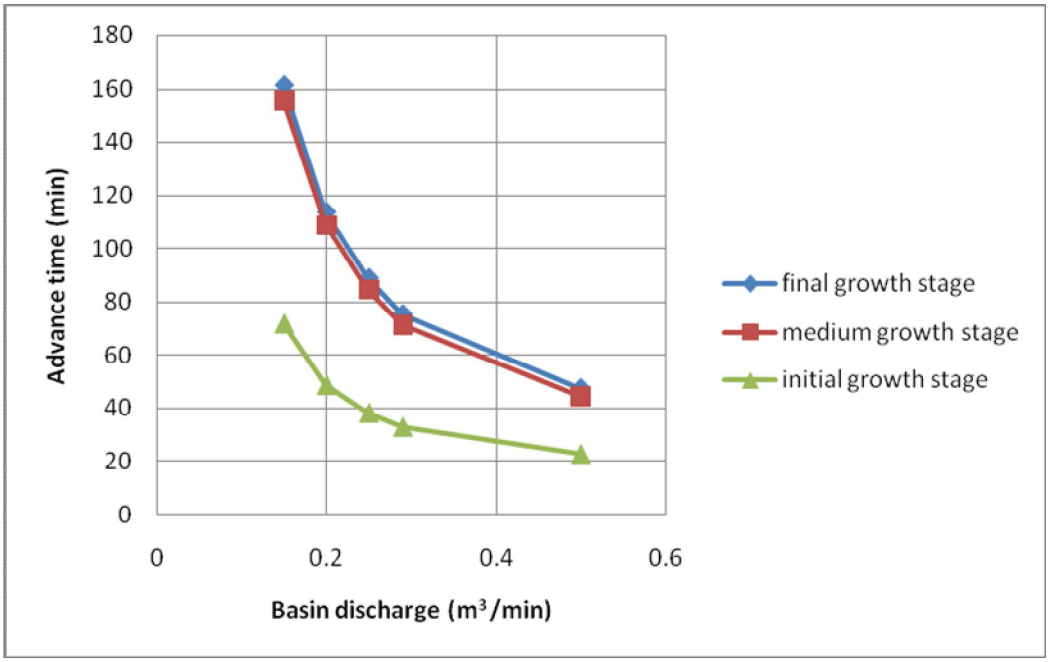

Figure 3. Relationship between inflow rate and advance time at various stages of growth for a basin problem

Table 2. Irrigation schedule for a basin problem

Month	Starting date	Water quantity (m^3)	Interval (days)	Application time (min)
April (5 times)	5/4/2007-30/4/2007	1958.68	5	394.74
May (15 times)	1/5/2007-30/5/2007	2974.22	2	594.64
June (6 times)	1/5/2007-1/6/2007	3000.48	5.3	601.9
July (3 times)	2/6/2007-16/2007	3040.93	5.4	608.19

Conclusions

Surface irrigation design is quite a difficult problem to solve optimally due to the inefficiency of water application arising mainly from deep percolation and surface runoff or tail water. This paper presents the development of a decision support system to address the issue. The decision support system (DSS) allows a much more comprehensive treatment of vital hydraulic processes occurring both on the surface and beneath it. The basin system as modelled and applied has made it possible to

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overcome the high costs associated with the sprinkler and trickle systems while achieving almost the same level of efficiency. Optimal design and management practices can be determined for a variety of conditions, and design historically requiring days of effort can now be made in minutes with this surface irrigation design software application. Also, the effectiveness of existing irrigation schemes or proposed ones can be predicted in a timely manner.

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Maejo International

Journal of Science and Technology

ISSN 1905-7873

Available online at www.mijst.mju.ac.th

Full Paper

Anti-nociceptive activity of *Cansjera rheedii* J. Gmelin (Opiliaceae)

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Received: 11 March 2009 / Accepted: 7 July 2009 / Published: 13 July 2009

Abstract : The ethanolic extract of aerial parts of *Cansjera rheedii* J, Gmelin (Opiliaceae) was screened for its anti-nociceptive property using both chemical and thermal methods of nociception in mice. In the chemical method, acetic acid-induced writhing test, and in the thermal method, tail-flick test was performed. The extract at doses of 250 mg and 500 mg/kg/ip inhibited the abdominal constriction induced by acetic acid and also increased the pain threshold of mice towards thermal source. The activity exhibited by the extract was comparable to that of the standard drugs (Pentozocine, 5 mg/kg/ip, for tail-flick test and Piroxicam, 10 mg/kg/ip, for acetic acid-induced writhing test). From the results it was concluded that the ethanolic extract of the plant exhibits anti-nociceptive activity by central and peripheral mechanisms.

Keywords: *Cansjera rheedii*, anti-nociceptive, writhing test, tail-flick test

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Introduction

Medicinal herbs have been used as a form of therapy for the relief of pain throughout history [1]. Natural products in general, and medicinal plants in particular, are believed to be an important source of new chemical substances with potential therapeutic efficacy. Taking into account that the most important analgesic prototypes (salicylic acid and morphine) were originally derived from plant sources, the study of plant species traditionally used as pain killers should still be seen as a fruitful research strategy in the search of new analgesic drugs. In an earlier study, we have reported analgesic activities of *Toddalia asiatica* Linn [2] and *Helichrysum bracteatum* [3]. The present study was taken up to evaluate the anti-noceptive activity of ethanolic extract of aerial parts of *Cansjera rheedii* J. Gmelin by using acetic acid-induced writhing and tail-flick method.

C. rheedii (Opiliaceae), a climbing shrub, sometimes armed, commonly known as Kalimanakeerai in Tamil, is generally found in India through Malaya to Hong Kong and northern Australia [4, 5]. The tribes of Nilgiris in Tamil Nadu (India) use the plant extract for the treatment of post-natal pain [6] and intermittent fever [7]. The extract of *C. rheedii* has been reported to have a hepatoprotective effect [8], cytotoxic effect [9], anthelminthic activity [10], anti-inflammatory and membrane stabilising properties [11], antipyretic activity [12] and hypnotic activity [13]. Preliminary phytochemical screening of the ethanolic extract of *C. rheedii* reveals the presence of alkaloids, phytosterols, saponins, flavonoids, glycosides, phenolic compounds and tannins [14]. The literature survey revealed that there are no scientific studies carried out regarding anti-nociceptive activity on the aerial parts of *C. rheedii* to substantiate its therapeutic claim, hence the present examination of the plant for its anti-nociceptive property.

Materials and Methods

Collection of plant material

The aerial parts of the plant (*C. rheedii*) were collected in and around Auroville, Puducherry in the month of June 2006, and it was identified and authenticated by Auro Herbarium Sakthi Botanical Survey Department, Auroville. A voucher specimen (VS-12) has been kept in our laboratory for future reference. The aerial parts were cut into small pieces, shade-dried and powdered. The coarse powder was subjected to continuous hot extraction with ethanol (95% v/v) in a Soxhlet extractor. The ethanol was removed by distillation under reduced pressure. The remaining extract was dissolved in water and used for the experiment.

Animals

Wister albino mice (20±5 g) of both sexes were procured from Adhiparasakthi College of Pharmacy, Melmaruvathur, Chengalpet district, Tamil Nadu, South India. They were fed on commercial diet (Hindustan Lever Ltd., Bangalore) and water ad libitum. All the animals were acclimatised for a week before use. The room temperature was maintained at 22±2° C. The institutional Animal Ethical Committee had approved the experimental protocol.

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Drugs and chemicals

Piroxicam (Dr. Reddy's lab, Hyderabad) and Pentazocine (Pure Pharma Pvt. Ltd., Mumbai) were used as reference standards. Ethanol (95%v/v), chloroform (AR), petroleum ether (LR) and acetic acid (AR) were purchased from Ranbaxy Laboratories Ltd., Punjab.

Acute toxicity study

Acute toxicity study was performed according to guidelines 425 of the Organisation for Economic Co-operation and Development (OECD) [15]. No adverse effect or mortality was detected in Swiss albino mice up to 2 g/kg /peroral (po) of the extract during a 24-h observation period.

Anti-nociceptive activity

Two models, viz. acetic acid-induced writhing response (chemical method) and tail-flick assay (thermal method) using albino mice, were employed to study the anti-nociceptive effect [16-18]. The animals were divided into four groups of six animals each. Group I served as normal control and received distilled water (10 ml/kg/po). Group II served as reference group and received Pentazocine (5 mg/kg/ip) in tail-flick method and Piroxicam (10 mg/kg/ip) in acetic acid-induced writhing method. Groups III and IV served as treatment groups and received the ethanolic extract of *C. rheedii* (250 and 500 mg/kg/ip respectively).

Chemical method (acetic acid-induced writhing test)

Acetic acid (1%v/v) was administered intraperitoneally to all the groups at the dose of 1 ml/kg body weight 30 minutes after the administration of the test compounds. Anti-nociception was recorded by counting the number of writhes after the injection of acetic acid for a period of 20 minutes. A writhe is indicated by abdominal constriction and full extension of the hind limb. The pain inhibition percentage (PIP) [19] was calculated according to the following formula: $PIP = \{(T_1-T_0)/T_0\}\times 100$, where T_1 and T_0 are post-drug and pre-drug latency respectively.

Thermal method (tail-flick test)

Basal reaction time of animals to radiant heat was recorded by placing the tip (last 1-2 cm) of the tail on the current source of an analgesiometer (INCO). The tail withdrawal from the heat (flicking response) was taken as the end point. The animals which showed a flicking response within 3-5 sec were selected for the study. A cut-off period of 15 sec was observed to avoid damage to the tail. The measurement of withdrawal time using the tail-flick apparatus was conducted at 0, 15, 30, 60, 90, 120 and 180 minutes after administration of the drugs.

Statistical analysis

The data were expressed as mean $\pm$ SEM of 6 animals. Results were analysed statistically by one-way analysis of variance (ANOVA) test between two groups: test and control groups, followed by student's t-test. P values less than 0.05 ($P<0.05$) were considered indicative of significance.

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Results and Discussion

The anti-nociceptive activity of the ethanolic extract of *C. rheedii* was evaluated in mice using both chemical and thermal methods of nociception. These methods are used to detect central and peripheral analgesia, whereas hot-plate and tail-flick tests are most sensitive to centrally acting analgesics. Intraperitonial administration of acetic acid releases prostaglandins such as PGE_2 and $PGF_{2\alpha}$ and their levels were increased in the peritoneal fluid of the acetic acid-treated mice [20]. Thermal induced nociception indicates involvement of narcotic receptors [21]. Thermal nociceptive tests are more specific to opioid μ receptors and non-thermal tests are to opioid κ receptors [22-23]. Both doses (250 mg and 500 mg) of the plant extract significantly ($P<0.001$) reduced the number of abdominal constrictions and stretching of hind limbs induced by the injection of acetic acid, exhibiting a writhing inhibition percentage of 65.07 and 69.85 respectively (Table 1), which was comparable with that of the standard drug Piroxicam (71.08 %). The abdominal constriction produced after administration of acetic acid is related to sensitisation of nociceptive receptors to prostaglandins. It is therefore possible that the extract exerts its analgesic effect by inhibiting the synthesis or action of prostaglandins.

Table1. Effect of *C. rheedii* on acetic acid-induced nociception

Group	Dose (mg/kg)	No. of writhes (mean ± SEM)	% Inhibition (PIP)
Control	10 ml/kg	81.6 ± 0.89	-----
Standard (Piroxicam)	10	23.6* ± 2.23	71.08
Extract	250	28.5*± 1.78	65.07
Extract	500	24.6*± 2.12	69.85

Note: Values are mean ± SEM (n=6); *$p<0.001$ as compared to control

The centrally acting analgesics generally elevate the pain threshold of mice towards heat. Both doses of the ethanolic extract of *C. rheedii* significantly ($P<0.05$, 0.01 and 0.001) increased the reaction time of the animals towards the thermal source. In the tail-flick test, the drugs showed greatest activity at 60-90 minutes of drug administration (Table 2). The extract at 250 and 500 mg/kg exhibited activity comparable to that of the standard drug Pentazocine at 5 mg/kg.

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Table 2. Effect of *C. rheedii* extracts in tail-flick test in mice

Group	Response time after drug treatment (sec)						
	0 min	15 min	30 min	60 min	90 min	120 min	180 min
Control (2ml/kg)	4.3± 0.72	3.7± 0.72	4.3± 0.47	4.7± 0.27	3.7± 0.55	4.3± 0.73	4.0± 0.47
Standard Pentazocin (5 mg/kg)	4.3± 0.72	14^{*}± 0.03	14^{*}± 0.03	13^{*}± 0.82	12.3^{*}± 0.98	9.7^{*}± 0.27	7.7^{*}± 0.27
Extract (250 mg/kg)	4.7± 0.72	8.7^{***}± 1.52	12^{*}± 0.33	14^{*}± 0.03	13.3^{*}± 0.55	12.7^{*}± 1.09	7.0^{**}± 0.47
Extract (500 mg/kg)	3.7± 0.72	6.3^{***}± 1.19	11.3^{**}± 1.36	13.7^{*}± 0.27	14^{*}± 0.03	11.7^{*}± 0.91	7.7^{***}± 1.19

Note: Values are mean ± SEM (n=6); $^{*}P<0.001$, $^{**}P<0.01$, $^{***}P<0.05$ as compared to control

Conclusions

From the above results it can be preliminarily concluded that the crude extract of *C. rheedii* exhibits anti-nociceptive activity. As indicated by increase in tail-flick latency and decrease in the number of writhing movements following the extract treatment, this extract may possibly have a central as well as peripheral analgesic action.

Acknowledgement

The authors thank the Chairman and Vice-Chairman of Mother Theresa Post-Graduate and Research Institute of Health Sciences (MTPG&RIHS) for providing the facilities to carry out the experiment.

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Maejo International

Journal of Science and Technology

ISSN 1905-7873

Available online at www.mijst.mju.ac.th

Review

Coastal and estuarine resources of Bangladesh: management and conservation issues

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Received: 2 July 2008 / Accepted: 14 July 2009 / Published: 20 July 2009

Abstract : The coastal area of Bangladesh includes a number of bays into which different types of rivers empty, creating an estuarine ecosystem adjacent to the shore. The main estuarine systems are Brahmaputra-Megna (Gangetic delta), Karnaphuly, Matamuhuri, Bakkhali and Naf rivers, which are comprised of mangroves, salt marshes, seagrass, seaweeds, fisheries, coastal birds, animals, coral reefs, deltas, salt beds, minerals and sand dunes. The estuarine environment, which serves as feeding, breeding and nursery grounds for a variety of animals, varies according to the volume of discharge of the river and tidal range. It is highly productive in terms of nutrient input from different sources that promotes other living resources in the estuaries. Drought conditions exist during the winter months, i.e. November to February, and effective rainfall is confined to the monsoon period, i.e. May to June. Changes in salinity and turbidity depend on annual rainfall. The colour of most estuarine waters is tea brown or brown due to heavy outflows during the monsoon. The tidal mixing and riverine discharge governs the distribution of the hydrological parameters. The pH of these waters is reported to be slightly alkaline (>7.66) and dissolved oxygen (<6.0 mg/l) shows an inverse relationship to temperature. Studies of plankton have indicated two periods of maximum abundance, i.e. February-March and August-September. The abundance of fish and shrimp larvae varies in number and composition with season. Many marine and freshwater species are available in various types of coastal brackish water, which depend on monsoonal activities and local environmental conditions.

Keywords: coast, estuary, resources, ecology, Bangladesh

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Introduction

Bangladesh is blessed with an extensive coastline of about 710 Km [1]. The southeastern and southwestern coast of this country is mostly covered by a complex estuarine ecosystem with strong interactions of biotic and abiotic factors. According to Ketchum [2], an estuarine environment is a unique and important part of the aquatic habitat and forms the transition zone between the inland world of freshwater and the seawater lying offshore. Estuaries play a vital role in the life history development of many marine and brackish water coastal animals, and some live out their entire life cycle within the estuarine environment [3-4]. This estuarine ecosystem is dominated by a huge amount of living resources such as aquatic macrophytes (i.e. tropical moist forest, salt marshes, seagrasses and seaweeds), fisheries, avian fauna, animals and coral reefs.

In Bangladesh, the estuarine system is comprised mainly of the Brahmaputra-Megna (Gangetic delta), Karnaphuly, Matamuhuri, Bakkhali and Naf rivers (Figure 1). It is well established that this kind of estuarine environment is highly productive in terms of nutrient input from different sources, which promotes other living resources in the vicinity of the estuaries. These diverse living resources in the estuarine environment play an important role which is economically significant in many ways. In addition, the estuarine resources of this country greatly contribute to the national economy as well as promote the socio-economic well-being of the coastal and often poor communities. However, although coastal and estuarine resources contribute a vital role in terms of both the ecosystem and the economy, study of the estuarine coastal environment in Bangladesh is meagre. To date, no systematic investigations have been carried out on the eco-biology of the estuarine living resources in the country. Few scientific data on hydrology [3], seaweeds [5-7], zooplankton [8-13] and benthos exist [14]. Similarly, the common physical and chemical parameters of water and soil in the estuarine and coastal areas were conducted only recently [15]. Therefore, any form of critical investigation on estuarine living resources and their environment can be considered as an important study in Bangladesh. As part of the estuarine study, this paper deals with the living estuarine resources of Bangladesh and their usefulness, and is the first of a series dealing with estuarine habitats in the country.

Ecological Feature

Climate

The coastal and estuarine environment of Bangladesh has a maritime climate with temperatures buffered by the nearby ocean [16]. The maximum air temperature attained in summer ranges from 31.1-33.3º C and the minimum in winter ranges from 24.8-29.8º C [3, 17]. Drought conditions prevail during the winter months (November-February) and rainfall is confined to the monsoon period. Normally, 80-90% of the annual rainfall occurs during the monsoon months of June-September. Mahmood [17] recorded 3558 mm and 1638 mm annual rainfall in the coastal areas of Cox's Bazar and Satkhira respectively. The entire coastal and estuarine area of Bangladesh is prone to violent storms during the pre-monsoon (March-April) and post-monsoon (October-November) period. Sometimes, tidal bores associated with cyclones cause great loss of property and life.

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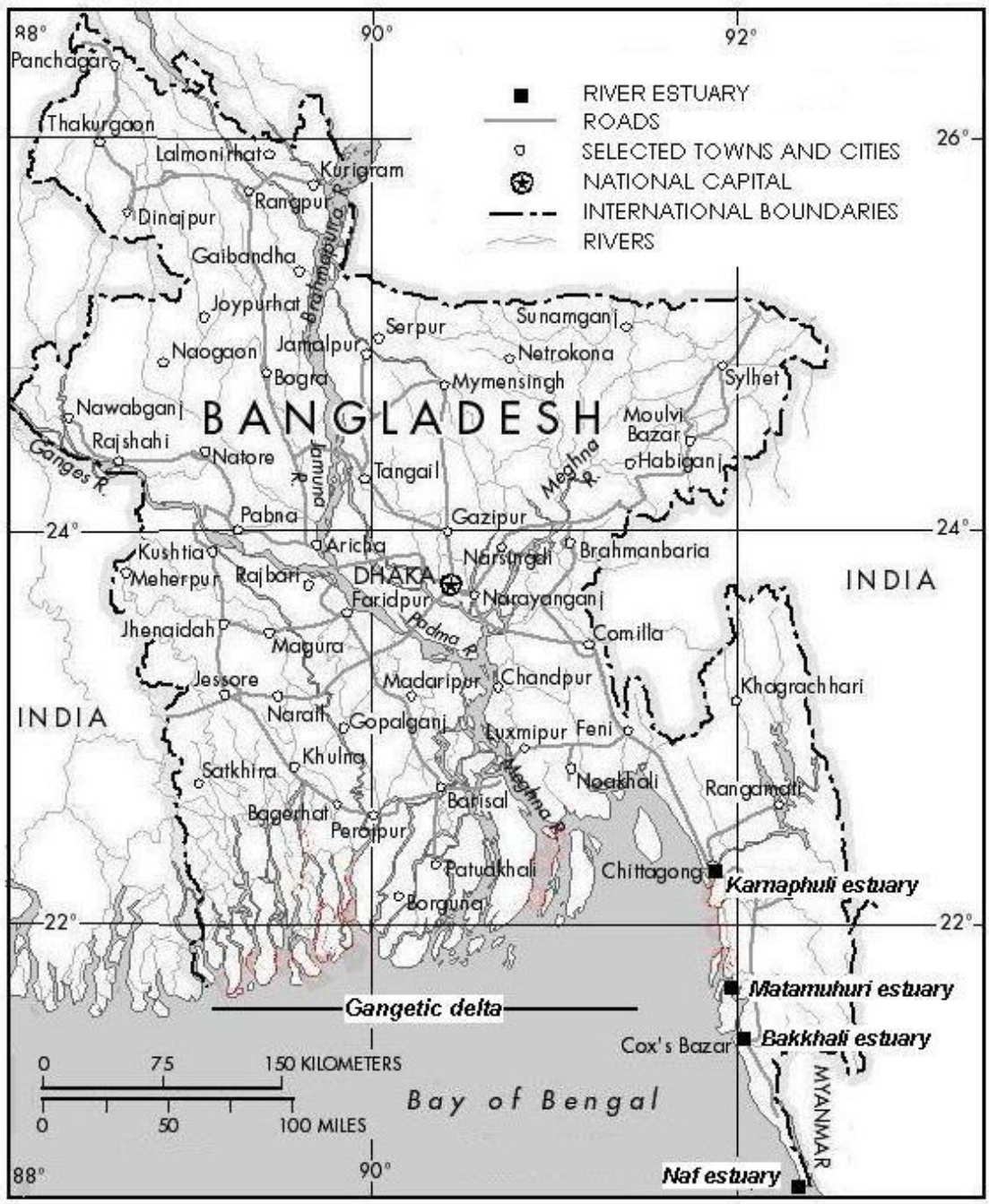

Figure 1. Major coastal and estuarine areas of Bangladesh

Hydrology of the coastal estuaries

The tides in the coastal and estuarine areas are semi-diurnal with two high and two low periods per day and have maximum amplitude of 3-4 m at spring tide [17]. The tidal activity is an important mechanism for the movement of water and nutrients especially in the estuarine areas, and this is probably a reason for the wide range of biodiversity in the estuarine water. The tidal range and river discharge are responsible for the extension of estuarine environment in the sea. The variation of tide level in the coastal areas may be attributed to the depth of the bay and varying topography of coastal water [18]. The tide penetrates up to 170 km in the south-west and 0-50 km in the south-east area of

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Bangladesh during lean period (April-May) depending on the topography and channels in the area [19]. The Bangladesh Inter Water and Transportation Authority [20] recorded 4.27 m neap tides and 6.10 m spring tides in the coastal area of Sandwip Island, Chittagong. However, the tidal range is reduced toward the south along the south-eastern coast of Bangladesh. In 24 years of tidal study, Chowdhury [21] observed that the monthly mean tidal range varies from 1.48 to 4.90 m with the mean value of 3.84 m in the coastline adjacent to the Karnaphuly River estuary.

The physico-chemical parameters of the estuarine environment show regular seasonal patterns of variation (Table 1 and Figure 2). The annual surface water temperature (<30º C) of the estuarine system is closely related to the annual cycle of air temperature. The cycle of dissolved oxygen (<6.0 mg/l) is mainly regulated by air temperature (<33º C) in some of the estuarine areas [22]. Mahmood et al. [3] observed an inverse relationship between dissolved oxygen and water temperature, and direct relationship between dissolved oxygen and pH. These relationships reveal that the influence of biological input/process, water discharge, temperature of neritic water and oxygen uptake in the chemical processes seem to be less important to change and long term variation in the dissolved oxygen and pH cycle in the estuarine environment.

Table 1. Physico-chemical parameters of the water of Bakkhali and Karnaphuli river estuary [3, 27]

	MONTH											
Factor	J	A	S	O	N	D	J	F	M	A	M	J
Water temp. (º C)	30.1	29.73	28.60	27.6	25.00	21.70	21.06	22.16	26.06	26.63	28.08	30.06
DO (mg/l)	3.21	3.56	4.23	3.15	3.55	5.37	3.14	2.74	1.89	2.33	2.47	3.79
pH	7.8	7.8	7.7	7.4	6.7	6.2	5.9	5.9	6.3	7.3	7.8	7.7
PO_4- P(mg/l)	0.17	0.13	0.13	0.08	0.11	0.09	0.11	0.13	0.14	0.12	0.17	1.5
MHW (m)	13.5	13.8	13.5	12.7	11.6	10.6	9.9	10	10.3	11	11.75	12.85
MLW (m)	3.07	3.24	3.05	2.62	2.01	1.4	0.92	0.69	0.8	1.33	2	2.65
Salinity (‰)	8.7	7.53	4.53	9.17	11.33	15.04	17.07	18.42	17.42	11.78	5.94	2.63
Air temp. (ºC)	31.7	30.1	29.1	28.26	25.13	23.13	22.03	21.8	26.26	27.18	29.06	30.83
Rainfall (cm)	56.52	29.49	16.43	12.29	2.54	0.076	0	0	0.18	2.77	31.22	51.16
BOD (mg/l)	2.02	1.12	1.04	1.37	1.51	1.61	1.02	1.86	3.38	3.13	3.51	2.22
NH_3 (mg/l)	2.26	2.31	1.44	1.06	1.15	0.49	0.25	1.0	1.17	1.52	2.30	2.58
TDS (mg/l)	300.5	264.3	443.4	385.7	285.7	322.0	408.2	527.4	457.5	474.4	530.24	582.9
TSS (mg/l)	109.81	97.69	99.28	108.74	98.17	124.05	208.15	199.45	147.52	204.93	111.44	104.50

Note: MHW = mean high water, MLW = mean low water, DO=dissolved oxygen, BOD = biological oxygen demand, TDS= total dissolved solids, TSS = total suspended solids

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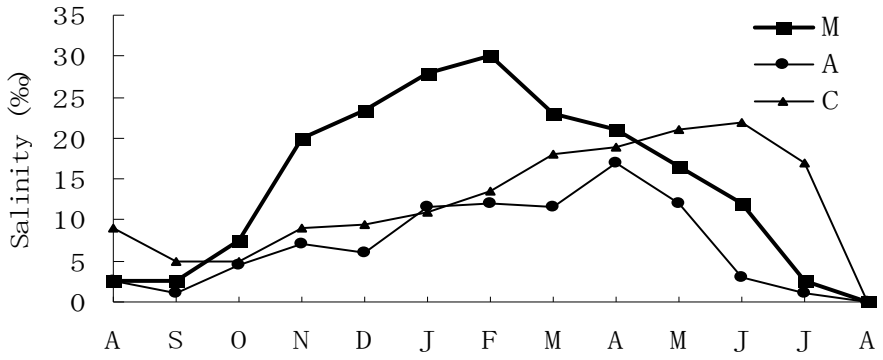

Figure 2. Seasonal variation (August 1982-August 1983) of salinity in the estuaries of the Mathamuhari River (M) of Chakaria, Chittagong; the Andermanik River (A) at Khepupara, Patuakhali; the Coxali River (C) at Satkhira, Khulna [17]

The most notable feature of the hydrology of the estuarine waters is the presence of a prolonged low salinity (<15‰) period mostly during the monsoon and certain period of post-monsoon. During the monsoon season, the water of the estuaries remains comparatively hyposaline due to heavy precipitation and freshwater discharge. This symptom persists for 5 months in the Matamuhuri estuary (Cox's Bazar), for 11 months in the Andhermanik estuary (Patuakhali) and 7 months in the Coxali estuary (Khulna) throughout the monsoon and post-monsoon period (Table 2 and Figure 2). The FAO of United Nations [23] recorded a wide variation in salinity (0-29%) at the estuarine system of Sunderban mangrove forest, Khulna. Salinity stratification is comparatively less during the monsoon than the other seasons of the year. Spencer [24] stated that the development of intensive chlorinity stratification under the influence of freshwater discharge is common to all estuarine tropical and subtropical systems. Similar results were observed by Ramanadham and Varadarajulu [25] in India. Based on the climatic factors, however, in the estuarine environment of Bangladesh, considerable salinity stratification exists during the non-rainy days when the evaporation rate is high during the dry seasons (November-February and April-May) and freshwater movement is not strong enough to create turbulent mixing with the tidal water.

Table 2. Variation in hydrological parameters of some of the estuaries of Bangladesh [17]

Parameter	Matamuhuri River (Chittagong)	Andhermanik River (Patuakhali)	Coxali River (Khulna)
Water temp. (° C)	21.38-31.60	22.50-31.88	21.07-31.16
DO (mg/l)	1.76-6.09	5.01-7.37	3.59-5.49
Salinity (%)	0.40-32.6	0.54-17.58	5.10-21.59
Transparency (cm)	22.8-97.5	9.0-31.0	7.4-14.3

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Horizontally, salinity variation is observed at the upper portion of the estuary [22] where fresh water flow dilutes with high tide. The vertical salinity distribution is always observed at the bottom; it appears that tidal water is more saline and heavier than the fluvial water-mass of the estuary, the probable reason for the heavier saline bottom water and the less saline surface water of the estuary. This phenomenon probably exists in all the coastal estuarine water systems in Bangladesh.

Based on universal relationships between climate and tide, the estuaries of Bangladesh are typical in that both freshwater discharge and tidal oscillation act together in creating a high mixing of neritic and fluvial water. This type of phenomenon occurs in the other estuaries elsewhere [2, 25, 26]. According to Mahmood et al. [3] the high circulation pattern is the dominant factor in the distribution of organisms especially the drifting forms in the estuarine systems of Bangladesh. Turbidity and total suspended solids (TSS) also depend on circulation pattern and freshwater discharge, and the range of TSS is 91.17-213.25 mg/l in the Bakkhali River estuary [27].

Biological Feature

Mangroves

Mangrove forests are usually found in the tropical and sub-tropical riverbanks, estuaries and along the coastlines, adapting to anaerobic conditions of both salt and freshwater environment. A mangrove community plays an important role to the stabilisation and maintenance of various closely linked ecosystems, such as seagrass, coral reef and marine ecosystems. It represents a unique ecological niche and habitat for a variety of marine and terrestrial animals. The amount of organic matter produced by a mangrove community supports not only the mangrove ecosystem itself but also its related ecosystems. Apart from providing an important coastal habitat for many types of species, a mangrove forest forms a community which helps to stabilise river banks and coastlines. Mangroves export detritus and nutrients into nearby systems that form a complex food chain which in turn supports valuable near-shore fisheries. In general, the mangrove forest of Bangladesh is divided into three zones, namely the Sunderban (largest continuous single productive forest of the world with an area of 577,040 ha), the Chakaria Sunderban in Cox's Bazar with an area of 8540 ha, and the planted coastal mangrove forests. The plantation of mangroves was introduced in the coastal area of Bangladesh in 1964 and is still carried out in the coastal belt of Cox's Bazar, Chittagong, Barisal, Patuakhali and off-shore islands, and now covers an area of 100,000 ha [28].

Small patches of mangroves are also found along the belt of nearly all coastal sub-districts. Different types of mangrove species dominate in different places of the coastal and estuarine areas of Bangladesh (Table 3). Fishing within the mangroves is one of the major activities in the coastal area. Several species of fish are found; common ones are mullet (*Mugil* spp.), marine catfish (*Mystus* spp.), seabass (*Lates calcarifer*) and black tiger shrimp (*Penaeus monodon*). Other species of shrimps are *Metapenaeus monoceros*, *M. brevicornis*, *P. indicus* and *Macrobrachium rosenbergii*. However, ever-expanding traditional culture of tiger shrimp has already led to the destruction of mangroves in Chakaria Sunderban, Moheskhali, Teknaf and Sonadia Island at the south-east coast of the country. In 2002-3, the destruction of mangroves for traditional shrimp culture in the south-eastern area was much higher than the previous years [Abu Hena, unpublished data]. The environmental damage and destruction of fishery resources is still unknown in those areas. According to available data of

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Mahmood [28] and Ahmad [29], the loss of mangrove wetland in Bangladesh during the last 25 years is about 50-70%. Similarly, in Sri Lanka an 11-65% [30] and in Thailand a 12-25% loss were estimated [31]. However, not all the blame for mangrove destruction lies in coastal aquaculture. Depending on the locality, the impact of the destructive uses is highly variable, though the scale of impact commonly found elsewhere [32] is similar to that found in Bangladesh, i.e. from (in descending order) clear cut for firewood and pool, conversion to agriculture (salt bed and aquaculture), conversion to human settlement, and diversion of fresh water or water quality changes.

Table 3. Mangroves of the coastal and estuarine areas of Bangladesh [61]

Family	Species
Acanthaceae	*Acanthus ilicifolius*
Pteridiaceae	*Acrosticum aureum*
Plumbaginaceae	*Aegialitis rotundifolia*
Myrsinaceae	*Aegiceras corniculatum*
Avicenniaceae	*Avicennia alba, A. marina, A. officinalis*
Rhizophoraceae	*Rhizophora mucronata, R. apiculata*
	Bruguiera grymorrhiza, B. seangula
	Ceriops decandra, C. tagal
	Kandelia candel
Euphorbiaceae	*Excoecaria agallocha, E.indica*
Sterculiaceae	*Heritiera fomes, H. littoralis (Extinct)*
Combretaceae	*Luminitzera racemosa*
Sonneratiaceae	*Sonneratia caseolaris, S. apetala*
Meliaceae	*Xylocarpus granatum, X. mekongensis*
Palmae	*Nypa fruticans*

Seaweeds

Sub-tidal macroalgal beds, i.e. *Sargassum*, *Dictyota* and *Codium*, play an important role in the life cycle of numerous important commercial species. Benthic forms of seaweeds are attached to the pneumatophores of the mangrove in inter-tidal areas of the coast. The rocky substratum of Saint Martin's Island is also a favourable place for seaweed growth and propagation. About 165 species belonging to 77 genera of seaweeds have been recorded in the coastal and estuarine areas (Table 4), whereas 1500 metric ton of red seaweed biomass are available around Saint Martin's Island [33]. However, they are often consumed elsewhere in the world and are not part of the daily tranditional diet in Bangladesh.

Table 4. Different groups of benthic marine algae of the coastal and estuarine areas of Bangladesh [33]

Division	No. of genera	No. of species
Chlorophyta	15	38
Chrysophyta	01	05
Phaeophyta	14	46
Rhodophyta	35	49
Cyanophyta	12	27
Total	77	165

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Salt marshes and seagrass

Salt marshes and seagrass are well recognised as important components of coastal productivity all over the world. These systems serve as feeding area for a variety of species including avifauna (birds), and contribute considerable quantities of leaf detritus to the water column. The detritus from seagrass plays an active part in nitrogen and phosphorus cycles that provide essential elements to the primary producers of all ecosystems. Seagrass also serves as a protective canopy, shielding the inhabitants of the bed from the effects of strong sunlight. When the bed occurs in the inter-tidal zone, the leaves may cover the bottom substrate during low tide, protecting the inhabitants from desiccation. Information on the existence of salt marsh and seagrass beds is lacking. No inventories have been conducted so far on salt marsh and seagrass resources in the coastal area of Bangladesh. Only 5 salt marsh plants (*Porteresia coarctata, Imperata cylindrica*, *Eriochloa procera*, *Myriostachya wightiana* and *Phragmites karka*), and five types of seagrass have been reported in the coastal and estuarine areas (Table 5) compared to nine species in Sabah, Malaysia.

Table 5. Seagrass of the coastal and estuarine areas of Bangladesh [36, 62-63] and Sabah, Malaysia [64]

Coastal and estuarine area, Bangladesh	Sabah, east Malaysia
Hydrocharitaceae	Hydrocharitaceae
Halophila decipiens	*Enhalus acoroides*
Halophila beccarii	*Halophila ovalis*
	Halophila minor
	Thalassia hemprichii
Cymodoceaceae	Cymodoceaceae
Halodule uninervis	*Cymodocea serrulata*
*Halodule pinifolia**	*Syringodium isoetifolium*
*Ruppia maritima**	*Halodule pinifolia*
	Halodule uninervis
	Cymodocea rotundata

* unpublished and newly recorded

Estuarine phytoplankton

Phytoplankton is a primary producer in the estuarine water. Phytoplankton produces organic compounds by utilising the solar energy during the photosynthesis process and releases oxygen into the estuarine water. This system controls the oxygen balance in any aquatic environment and mainly near the surface (0.4-0.6 m) where productivity is high [34]. It is well known that in any aquatic environment, the food chain starts with the phytoplankton. Thus, it plays a vital role in the estuarine food chain. Very little is known about the phytoplankton of the estuarine systems of Bangladesh. A few studies such as those of Salam and Khan [6], Ali et al. [35], Islam and Aziz [36], Hoque et al. [37] and Zafar [38] have been conducted on the seasonal abundance and distribution of phytoplankton in the estuarine environment (Table 6). The studies recorded that the major groups of phytoplankton are diatoms (88.60%), followed by dinophyceae (6.32%) and myxophyceae (5.10%). According to Ali et

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al. [35] higher production of phytoplankton was found in the dry season while the lowest one was recorded during the monsoon. However, its distribution and abundance in any estuarine environment is also influenced by the wind direction, current, nutrient input and freshwater discharge, which are not well studied in Bangladesh.

Table 6. Phytoplankton of the estuarine environment of Bangladesh [35, 66]

Group	Species
Diatoms	*Chaetoceros atlanticus, C. costatus, C. curvisetus*
	Coseinodiscus centrales, C. lineatus, C. excentricus, C. nitidus, C. radiatus, C. curvatulus, C. marginatus, C. gigas, C. perforatus, C. granii
	Biddulphia granulata, B. mobiliensis
	Lauderia sp.
	Nitzschia closterium, N. pacifica, N. longissima, N. pungens, N. sigma, N. seriala, N. paradoxa
	Thalasionema nitzschioides
	Rhizosolenia sp.
	Thalasiothrix longissima, T. frauenfeldi
	Encampia zoodiacus
	Melosira sulcata, M. nummuloides, M. moniliformis, M. varians
	Asterionella japonica
	Hemidiscus sp.
	Hemiaulus hauckii
	Anabaena circinalis
Myxophyceae	
	Oscillatoria tenuis, O. limosa
	Dinophysis sp.
Dinophyceae	
	Ceratium sp.

Estuarine fish and shellfish

The estuarine coastal and adjacent areas of Bangladesh support a variety of economically important fishes. The fishes are those that spend all or a major part of their lifetime in the estuarine environment; marine or freshwater species migrate seasonally into or through the estuaries. In the present state of investigation, a proper classification of fish species based on their period of life and availability in the estuaries is difficult. Mahmood and Khan [12], Ahmed [39-40] and Mahmood [28] described a tentative list of fishes and shrimps of the estuaries (Tables 7-8), while the FAO [23] gave a generalised list of the most common finfish for the estuaries.

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Table 7. Some of the shrimp fauna of the estuarine and mangrove ecosystem of Bangladesh [12, 28]

Family	Species
Penaeidae	
	Penaeus monodon, P. merguiensis, P. indicus. P. uncta
	Metapenaeus monoceros, M. lysianass, M. spinulatus, M. brevicornis, M. affinis
	Parapenaeopis sculptilis, P. stylifera, P. hardwickii, P. semisulcatus
Solenoceridae	
	Solenocera subnuda
Sergestidae	
	Acetes erythraeus, A. japonicus, A. indicus
Palaemonidae	
	Macrobrachium rosenbergii, M. lamarrei, M. rude, M. villosimanus,
	M. mirabile, M. birmanicum
	Palaemon Styliferus, P.(Nematopalaemon) tenuipes, P.(N) karnafuliensis
Alpheidae	
	Alpheus euphrosyne, A. crassimanus

Table 8. List of some ichthyofauna of coastal and estuarine environment of Bangladesh [12, 28]

Family	Species
Carcharhinidae	
	Scolidon lacticaudus
	Eusphyra blochii
Sphyrnidae	
	Carcharhinus melanopterus
Rhinobatidae	
	Rhynchobatus djiddensis
Dasyatidae	
	Dasyatis zugei
	Himatur uarnak, H. imbricata, H. fluviatilis
	Pastinachus sephen
Clupeidae	
	Escualosa thoracata
	Gudusia chapra
	Hilsa (Hilsa) kelee, H. (Tenualosa) ilisha, H. (T.) toil
	Anodontosoma chacunda
	Gonialosa manmina
	Dussumieria acuta
	Sardinella gibbosa, S. melanura, S. fembriat

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Table 8. (cont'd)

Family	Species
Pristigasteridae	
	llisha megaloptera, l. melastoma
	Rconda russelliana
Engraulidae	
	Thryssa dussumieri, T. hamiltonii
	Setipinna phasa, S. taty
	Coilia dussumieri, C. neglecta, C. ramcarati
	Stolephorus tri
Chirocentridae	
	Chirocentrus dorab, C. nundus
Elopidae	
	Elops machnata
Muraenesocida*e*	
	Congresox talabon
	Muraenesox cinereus
Cuchidae	
	Cuchia cuchia
Bagridae	
	Mystus gulio
Schilbeidae	
	Silonia silondia
Pangasiidae	
	Pangasius pangasius
Ariidae	
	Arius sona, A. gogora, A. maculatus, A. buchanani, A. caelatus, A. thalassinus, A. dussumieri, A. arius, A. nenga
Plotosidae	
	Plotosus canius, P. lineatus
Synodontidae	
	Saurida tumbil
Harpadontidae	
	Harpodon nehereus
Hemiramphidae	
	Hemiramphus georgii
Fistulariidae	
	Fistularia villosa
Syngnathidae	
	Hippocampus kuda

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Table 8. (cont'd)

Family	Species
Scorpaenidae	
	Pterois russeli, P. miles
Platycephalidae	
	Platycephalus crocodilus, P. scaber
	Rogadius asper
Ambassidae	
	Chanda nama
	Pseudambassis baculis, P. ranga
Centropomidae	
	Lates calcarifer
Serranidae	
	Cephalopholis miniatus
	Epinephelus fasciatus, E. tauvina
	Promicrops lanceolatus
Theraponidae	
	Therapon jarbua, T. theraps
Apogonidae	
	Apogon novemfasciatus, A. septemstiastus
Sillaginidae	
	Sillago domina, S. shihama
	Sillaginopsis panijus
Lactariidae	
	Lactarius lactarius
Carangidae	
	Alectis indica, A. melanoptera
	Alepes djedaba
	Megalaspis cordyla
	Atropus atropus
	Scomberoides commersonianus
	Carangoides malabaricus
	Selar boops, S. crumenophthalmus
Formionidae	
	Formio niger
Menidae	
	Mene maculata
Leiognathidae	
	Gazza minuta
	Leignathus bindus, L. equulus, L. fasciatus

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Table 8. (cont'd)

Family	Species
	Secutor ruconius, S. insidiator
Lutjanidae	
	Lutjanus johnii, L. sanguineus
	Pinjalo pinjalo
Nemipteridae	
	Nemipterus japonicus, N. nematophorus
Lobotidae	
	Lobotes surinamensis
Gerreidae	
	Gerres filamentosus
	Pentaprion longimanus
Haemulidae	
	Pomadasys argentus, P. maculates, P. hasta
Lethrinidae	
	Lethrinus ornatus
Sparidae	
	Acanthopagarus latua
	Argyrops spinifer
Sciaenidae	
	Atrobucca nibe
	Dendrophysa russelli
	Macrospinosa cuja
	Protonibea diacanthus
	Pama pama
	Panna microdon
	Johnius argentatus
	Johnius dussumieri
	Pterotolithus maculatus
	Otolithes ruber
	Pennahia macropthalmus
Mullidae	
	Upeneus sulphureus
	Parupeneus heptacanthus
Drepanidae	
	Drepane longimanus, D. punctatus
	Ephippus orbis
Scatophagidae	
	Scatophagus argus

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Table 8. (cont'd)

Family	Species
Mugilidae	
	Liza parsia, L. subviridis, L. tade
	Mugil cephalus, M. cascasia
	Valamugil speigleri
	Rhinomugil corsula
Sphyraenidae	
	Sphyraena barracuda, S. putnamiae
Polynemidae	
	Eleutheronema tetradactylum
	Polynemus paradiseus
	Polydactylus indicus, P. sexfilis, P. sextarius
Uranoscopidae	
	Uranoscopus quttatus
	lchthyscopus inermis
Eleotrididae	
	Eleotris fusca
	Butis melanostigma
Gobiidae	
	Brachygobius nunus
	Glossogobius giuris
	Pogonogobius planiformes
	Stigmatogobius sadanundio
	Apocryptes bato
	Boleopthalmus boddarti
	Parapocryptes batoides
	Pseudapocryptes lanceolatus
	Scartelaos viridis
	Periopthalmodon schlosseri
	Periopthalmus koelreuteri
Gobioididae	
	Odontamblyopus rubicundus
Trypauchenidae	
	Trypauchen vagina
Kurtidae	
	Kurtus indicus
Trichiuridae	
	Eupleurogrammus muticus
	Lepturacanthus savala

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Table 8. (cont'd)

Family	Species
	Trichiurus lepturus
Scombridae	
	Euthynnus affinis
	Rastrelliger brachysoma, R. kanagurta
	Sarda orientalis
	Scomberomorus commerson, S. quttatus
Stromateidae	
	Pampas argentaeus, P. chinensis
Psettodidae	
	Psettodes erumei
Bothidae	
	Pseudorhombus arius, P. elevatus, P. malayanus
Soleidae	
	Synaptura pan
	S.orientalis
	Zebreas altipinnis
	Cynoglossus bilineatus, C. cynoglossus, C. lingua, C. macrolepidotus,
	C. versicolor
	Paraplagusia bilineata
Triacanthidae	
	Triacanthus brevirostris
Balistidae	
	Abalistis stellatus
Tetraodontidae	
	Arothron stellaris
	Tetradon cutcutia
	Chelonodon fluviatilis, C. patoca

In the estuarine area of Sundarban, over 187 species are caught by commercial fishermen [28]. Out of 187 species only 22 species are most common throughout the year in the estuarine waters of Bangladesh (Table 9) [23]. Pillay [41-42] recorded over 120 species in the estuarine area of Sunderban, India and 128 species from 45 families in West African estuaries. About 138 species are recorded in Chilka Lake where many are only migrants or stray visitors [42]. In the estuary of the Rokan River in Sumatra, Hardenberg [43] recorded more than 150 species including occasional migrants. The number of individuals is, however, comparable.

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Table 9. General list of the most common fish and shrimp fauna available in the coastal environment of Bangladesh [23]

Family	Species
	Shrimp
Penaeidae	*Penaeus indicus* (H. Milne-Edw.), *P. monodon* (Fabricius)
	Metapenaeus brevicornis (H. Milne-Edw.), *M. monoceros* (Fabricius)
	Parapenaeopsis sculptilis (Heller), *P. stylifera* (H. Milne-Edw.)
Palaemonidae	*Macrobrachium dolicodaetylus* (Hi. lgendorf), *M. dyanus* (Henderson),
	M. rosenbergii (De Man), *M. villosimanus* (Tiwari)
	Palaemon karnafullensis (Khan-Fincham-Mahamood), *P. styliferus* (H. Miline-Edw.)
Sergestidae	*Acetes indicus* (H. Milne-Edw.)
	Fin fish
Polynemidae	*Eleutheronema tetradactylum* (Shaw)
	Polynemus paradiscus (Linn.)
Mugilidae	*Liza tade* (Forskal)
	Rhlnomugli corsula (Ham.)
Bagridae	*Mystus golio* (Ham.)
Clupediae	*Gonialosa manminna* (Ham)
	Tenualosa ilisha (Ham.)
	Ilisha megalopetra (Swainson)
Engrnulidae	*Collia ramcarati* (Ham. Buch)
	Septipinns phasa (Ham.)
	Stolephorus tri (Bleeker)
	Thrysssa purava (Ham.)
Gobiidae	*Apoeryptes bato* (Ham. Buch)
	Glossogoblus gluris (Ham.)
Taenioidae	*Odontamblyopus rubicundus* (Ham.)
Scatophagidae	*Scatophagus argus* (Linn.)
Centropomidae	*Lates calcarifer* (Bloch)
Sciaenidae	*Otolitholidaes pama* (Ham)
Cynoglossidae	*Cynoglossus cynoglossus*
Harpadontidae	*Harpodon nehereus* (Ham. Buch)
Sillaginidae	*Sillago domina* (Cuv. Val.)
Trichiuridae	*Lepturacanthus aavala* (Cuv.)

The fishery resources of the estuarine and coastal regions of Sunderban mangrove forest constitute 2-5% of the total capture fisheries of Bangladesh [44]. The common fishing method in almost all of the estuarine areas is by means of a fixed estuarine set bag net. Different sizes of the net are set against the current in shallow water areas by fastening the wings to bamboo poles driven into the bottom; the mouth of the net is kept open by means of bamboo poles. Other types of net used for

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fishing are gill net, drift net, larval net, trammel net, beach seine, marine set bag net, cast net, encircling net and drag net (Figure 3). In the estuarine areas of Bakkhali, Matamuhuri and Sunderban, narrow creeks or inter-tidal mud flats are often fenced off at high tide with walls of bamboo slats or netting. When the mud flat is drained dry at low tide, the fish are caught by hand. Cast nets are extensively used in the estuarine rivers in the country and catch a good amount of shrimps and fishes.

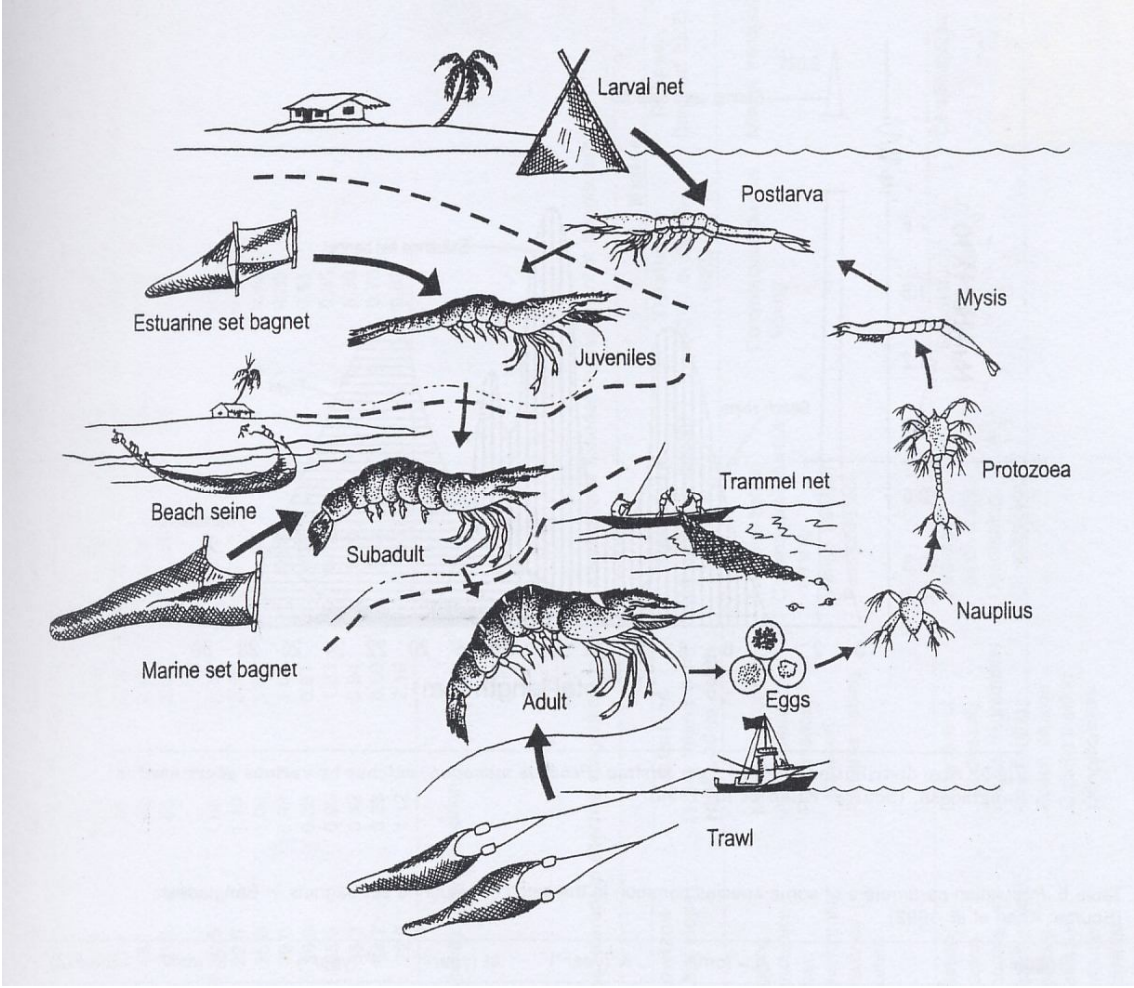

Figure 3. Fishing gears used at various stages in the coastal and estuarine fisheries of Bangladesh [66]

In the coastal and estuarine fisheries of Bangladesh, the increase in overfishing is a serious problem due to the use of huge numbers of push or larval nets and estuarine set bag nets (Table 10). Push and larval nets collect shrimp fry for the expanding shrimp aquaculture industry. In recent years, although a considerable number of shrimp hatcheries have been established, the wild fry collection is still practiced in the coastal area of the country. The loss of other species during the collection of tiger shrimp (*P. monodon*) post-larvae is well documented. It is notable that in catching a single species of tiger shrimp fry, about 26 other species, 29 finfish species and 70 other zooplankton were simultaneously destroyed [45] (Figure 4). The fry catchers carefully sort out *P. monodon* fry from the mixed catch and the rest of the plankton including fish and shellfish larvae are discarded anywhere on

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the shore. These activities cause great loss of the biodiversity and valuable fishery resources. In the case of estuarine set bag nets, area and seasonal closures for operation of the gears and the establishment of alternative livelihood projects in the context of integrated coastal community development projects have been proposed [46].

Table 10. Population parameters of some common species in the estuarine set bag net [65]

Species	L∞ (cm)	K ($year^{-1}$)	M ($year^{-1}$)	F ($year^{-1}$)	L_c (cm)	E (=F/Z)
Crustaceans						
Peneus monodon	31.4	0.72	1.42	8.38	13.8	0.86
Peneus indicus	22.8	0.55	1.30	3.70	5.9	0.74
Metapenaeus monocerous	19.8	0.44	1.17	3.65	5.9	0.76
Metapenaeus bervicornis	15.6	0.31	1.00	4.24	4.8	0.81
Metapenaeus spinulatus	20.1	0.39	1.08	5.90	5.3	0.84
Perapenaeopsis sculptilis	16.9	0.76	1.75	4.15	15.3	0.70
Perapenaeopsis stylifera	14.4	1.66	3.06	3.00	2.8	0.50
Acetes indicus	5.0	0.73	2.40	1.10	2.0	0.31
Macrobracium rosenbergii	35.5	0.34	0.84	1.96	7.3	0.70
Palaemon styliferous	15.4	0.63	1.59	3.20	3.74	0.67
Fish						
Raconda russelliana	23.6	0.43	1.10	2.10	2.90	0.66
Septinna taty	21.3	0.53	1.28	0.80	15.8	0.28
Stolephorus tri	16.8	0.65	1.59	9.00	3.4	0.85
Harpadon nehereus	34.9	0.38	0.91	3.75	6.3	0.80
Lepturacanthus savala	93.0	0.29	0.58	2.62	22.60	0.82
Eleutheronema tetradactyldum	38.1	0.1	0.85	3.50	5.3	0.87
Polynemous paradiscus	21.6	0.52	1.28	4.72	2.7	0.79
Sillaginopsis panijus	43.3	0.38	0.86	2.70	13.1	0.76
Sillago sihama	27.4	0.39	0.99	3.00	5.1	0.75

Note: L∞ = asymptotic length, K = growth co-efficient, M = natural mortality, F = fishing mortality, Lc = length at first capture, E= exploitation rate, Z = total mortality

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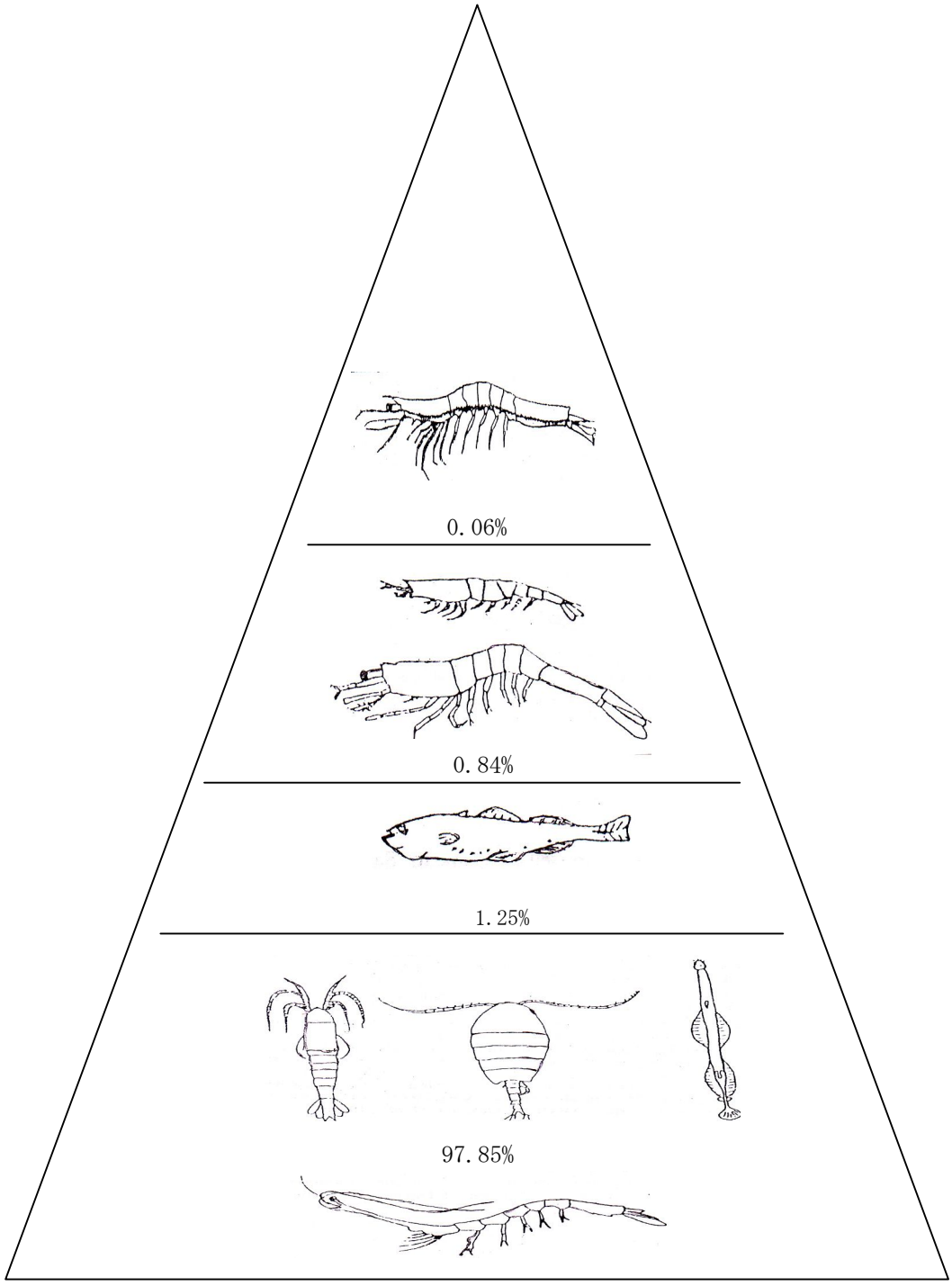

Figure 4. From top, showing average distribution (in percentage) of postlarvae of *P. monodon,* other shrimps, finfishes and all other zooplankton in the estuaries of Bangladesh (adopted from Mahmood [67])

Crabs belonging to different genera and species are widely distributed along the estuarine and coastal shores and swamps. Crabs, which are caught in cage traps or by a fishing rod, are one of the most important hidden living resources of Bangladesh. Siddiqui and Zafar [47] gave a check list of the coastal estuarine crabs of Bangladesh (Table 11). Due to religious prohibition on eating crabs, the mud

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crab (*Scylla serrata*) and swimming crab (*Portunus pelagicus*) fisheries are not popular in Bangladesh even though they have commercial importance in the world market. However, some rural Buddhists and Hindu people consume crabs as food locally. In some parts of the coastal areas, crabs are harvested naturally from the mangrove area and shrimp ponds as a by-product. The annual wild production of crabs in Bangladesh was about 2159 metric ton [48].

Table 11. Crabs of the estuarine and coastal areas of Bangladesh [44]

Family	Species	Economic importance
Portunidae	*Scylla serrata*	Used as food
	Portunus sanguinolentus	do.
	Portunus pelagicus	do.
Grapsidae	*Metopograpsus thukuhar*	No value
	Metopograpsus messor	do.
	Prasesarma plicatum	do.
	Sesaema lanatum	do.
	Episesarma versicolor	do.
Potamoniade	*Potamon wood-masoni*	As feed in aquaculture
	Potamon martensi	do
	Paratelphusa lamellifrons	Used for poultry feed
Ocypodidae	*Uca urvillei*	No value
	Uca annulipes	do.
	Ocypode ceratophthalmus	do.
Paguridae	*Anapagurus laevis*	do.
Leucosiidae	*Ebalia cranchii*	do.

Among the shellfishes, mollusks belonging to 187 genera of bivalves, clams, mussels and oysters have been recorded in the south-eastern coastal and estuarine areas of Bangladesh [49-51]. Three species of cephalopods (octopus, i.e. *Octopus aegina*, cuttlefish, i.e. *Sepia eculeata* and squid, i.e. *Loligo edulis*) are found in these coastal areas [52]. Some important gastropods (*Conus striatus, C. textile* and *C. geogrphes*) are abundant, and two economically important gastropods (*Trochus niloticus* and *Turbo marmoratus*) that are heavily depleted worldwide are also present in these coastal areas. One species of star fish, 5 species of lobster and a number of coelenterates are found in some of the estuarine parts [18, 50]. Seven species of edible oysters are found in the coastal areas of the country [18]. The green mussel *Perna viridis* is common in the estuarine waters, growing attached on any hard substrates or fishing poles. One of the most important shellfish, the pink pearl bearer bivalve *Lamelliden jenkinsianus var.obesa*, is also obtained around the coastal bays of the south-eastern area [18]. Shellfish grow and breed well when the water quality is good enough during the winter/dry season, because, as a filter feeder by nature, oyster or any other types of bivalves does not grow well in silted areas or turbid water environment. The *Meretix* spp. clams are found abundantly in the muddy coast of nearly all estuarine areas. Shells of these animals are collected in large quantities for lime production and manufacture of handicrafts which sell commonly in the local tourist markets.

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Estuarine and coastal water zooplankton

Zooplankton, the secondary consumer in an aquatic food chain, plays an important role in the estuarine and coastal water environment. The net fluxes of the water in the estuarine environment play an important role in the distribution of planktonic animals [53]. It is noted that there have been very limited published studies on the abundance of zooplankton and their ecology in the coastal and estuarine environment of Bangladesh [35]. On the other hand, a number of valuable investigations have been made in Indian waters by Krishnamurathy et al. [54], Santhanan et al. [55-56] and Menon and George [57]. Ali et al. [35] recorded a periodic variation of zooplankton in the coastal estuarine water of the south-eastern part of Bangladesh. The major groups of zooplankton, i.e. copepoda, decapoda, chaetognatha, cladocera, and fish and shellfish larvae were recorded by Ali et al. [35] and Ali [58], and are presented in Table 12. Studies have revealed a negative relationship between zooplankton abundance and NO_2-N concentration [35]. Furthermore, an inverse relationship between the abundance of phytoplankton and zooplankton has also been observed in Bangladeshi coastal waters [35]. Similar observations in Indian marine waters were also noted by Goswami and Singbal [59], who reported that tidal current, wind direction and river discharge can contribute to controlling the diversity of zooplankton in the estuarine environment [53].

Table 12. Zooplankton composition in the coastal and estuarine waters of Bangladesh [35, 55]

Group	Species
Copepoda	*Calanus* sp.
	Microsetella sp.
	Oncaea sp.
	Calanopia sp.
	Coryeacus sp.
	Oithona sp.
Crustacean	*Penaeus monodon, P. merguiensis*
	Metapenaeus monoceros, M. brevicornis
	Penaeus indicus
	Macrobrachium rosenbergii
	Acetes erythraeus, A. indicus, A. japonicus
Decapoda	*Lucifer* sp.
Chaetognatha	*Sagitta* sp.
Cladocera	*Evadue* sp.
Meropalankton	Nauplius
	Copepodite
	Zoea
	Hydromedusae

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Estuarine and coastal animals/wildlife

The estuarine and coastal environment supports a considerable size and variety of animal population. These animals spend at least a part of their life cycle in the coastal forest swamps which are subject to periodic tidal inundation. The wildlife of the coastal moist forest has been described by Blower [60], but the record for coastal forest faunas is still lacking. There are animals ranging from protozoa to birds, reptiles and mammals including tigers, deer and monkeys. Among the avian fauna, both migratory and indigenous birds are present. The common coastal and estuarine birds are gulls, terns, egrets, herons, bitterns, plovers, sandpipers and cormorants (Table 13). However, due to the population load on the coastal areas and severe destruction of coastal forests, almost all of the animal species are in danger, in particular, *Betagus basca* (River terrapin), *Lissemys punctata* (Indian flap-shelled turtle), *Panthera tigris* (Bengal tiger), *Python molorus* (Indian python), *Trionyx hururm* (Peacock soft shelled turtle) and *Vanaus flavescens* (Yellow monitor). However, a few of these together with some other reptiles have been protected by the Bangladesh Wildlife (Preservation) Act of 1973. It is worth mentioning that five major animal species, viz. *Axis procinus* (Hog deer), *Bubalis bubalis* (Water buffalo), *Cervus duvauceli* (Swamp deer), *Rhinoceros sondaicus* (Javan rhinoceros) and *Crocodylus palustris* (Mugger crocodile), have become extinct in the Bangladeshi coastal estuarine forests. The tidal waterways, varying in width from a few metres to 5 km, are inhabited by dolphin (4 species), otter and the saltwater crocodile (*Crocodylus porosus*), which are not well protected and conserved.

Table 13. Coastal and estuarine migratory and indigenous birds of Bangladesh

English name	Family	Generic name
	Dendrocygnidae	
Fulvous Whistling-duck		*Dendrocygna bicolor*
Lesser Whistling-duck		*Dendrocygna javanica*
	Anatidae	
Bar-headed Goose		*Anser indicus*
Ruddy Shelduck		*Tadorna ferruginea*
Common Shelduck		*Tadorna tadorna*
Comb Duck		*Sarkidiornis melanotos*
Cotton Pygmy-goose		*Nettapus coromandelianus*
Gadwall		*Anas strepera*
Falcated Duck		*Anas falcata*
Eurasian Wigeon		*Anas Penelope*
Mallard		*Anas platyrhynchos*
Indian Spot-billed Duck		*Anas poecilorhyncha*
Northern Shoveled		*Anas clypeata*
Northern Pintail		*Anas acuta*
Garganey		*Anas querquedula*
Common Teal		*Anas crecca*

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Table 13. (cont'd)

English name	Family	Generic name
Red-crested Pochard		*Netta rufina*
Common Pochard		*Aythya ferina*
Ferruginous Pochard		*Aythya nyroca*
Tufted Duck		*Aythya fuligula*
Greater Scaup		*Aythya marila*
	Rallidae	
White-breasted Waterhen		*Amaurornis phoenicurus*
Eastern Baillon's Crake		*Porzana pusilla*
Ruddy-breasted Crake		*Porzana fusca*
Watercock		*Gallicrex cinerea*
Purple Swamphen		*Porphyrio porphyrio*
Common Moorhen		*Gallinula chloropus*
Common Coot		*Fulica atra*
Spotted Crake		*Porzana porzana*
Water Rail		*Rallus aquaticus*
	Scolopacidae	
Pintail Snipe		*Gallinago stenura*
Common Snipe Fantail Snipe		*Gallinago Gallinago*
Greater Painted-snipe		*Rostratula bengalensis*
Eastern' Black-tailed Godwit		*Limosa melanuroides*
Bar-tailed Godwit		*Limosa lapponica*
Whimbrel		*Numenius phaeopus*
Eurasian Curlew		*Numenius arquata*
Spotted Redshank		*Tringa erythropus*
Common Redshank		*Tringa tetanus*
Marsh Sandpiper		*Tringa stagnatilis*
Common Greenshank		*Tringa nebularia*
Nordmann's Greenshank		*Tringa guttifer*
Green Sandpiper		*Tringa ochropus*
Wood Sandpiper		*Tringa glareola*
Terek Sandpiper		*Xenus cinereus*
Common Sandpiper		*Actitis hypoleucos*
Ruddy Turnstone		*Arenaria interpres*
Asian Dowitcher		*Limnodromus semipalmatus*
Red Knot		*Calidris canutus*
Great Knot		*Cakidris tenuirostris*
Sanderling		*Calidris alba*
Spoon-billed Sandpiper		*Calidris pygmea*

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Table 13. (cont'd)

English name	Family	Generic name
Little Stint		*Calidris minuta*
Red-necked Stint		*Calidris ruficollis*
Temminck's Stint		*Calidris temminckii*
Long-toed Stint		*Calidris subminuta*
Curlew Sandpiper		*Calidris ferruginea*
Broad-billed Sandpiper		*Limicola falcinellus*
Ruff		*Philomachus pugnax*
	Rostratulidae	
Greater Painted-snipe		*Rostratula benghalensis*
	Jacanidae	
Pheasant-tailed Jacana		*Hydrophasianus chirurgus*
Bronze-winged Jacana		*Metopidius indicus*
	Charadriidae	
Black-winged Stilt		*Himantopus himantopus*
Pied Avocet		*Recurvirostra avosetta*
Pacific Golden Plover		*Pluvialis fulva*
Grey Plover		*Pluvialis squatarola*
Common Ringed Plover		*Charadrius hiaticula*
Little Ringed Plover		*Charadrius dubius*
Kentish Plover		*Charadrius alexandrinus*
Lesser Sand Plover		*Charadrius mongolus*
Greater Sand Plover		*Charadrius leschenaultia*
Grey-headed Lapwing		*Vanellus cinereus*
Red-wattled Lapwing		*Vanellus indicus*
Yellow-wattled Lapwing		*Vanellus malabaricus*
River Lapwing		*Vanellus duvauceli*
	Glareolidae	
Small Pratincole		*Glareola lactea*
	Laridae	
Indian Skimmer		*Rynchops albicollis*
Heuglin's Gull		*Larus heuglini*
Steppe Gull		*Larus* [*heuglini*] *barabensis*
Pallas's Gull		*Larus ichthyaetus*
Brown-headed Gull		*Larus brunnicephalus*
Black-headed Gull		*Larus ridibundu*
Gull-billed Tern		*Gelochelidon nilotica*
Caspian Tern		*Sterna caspia*
River Tern		*Sterna aurantia*

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Table 13. (cont'd)

English name	Family	Generic name
Lesser Crested Tern		*Sterna bengalensis*
Great Crested Tern		*Sterna bergii*
Sandwich Tern		*Sterna sandvicensis*
Common Tern		*Sterna hirundo*
Little Tern		*Sterna albifrons*
Whiskered Tern		*Chlidonias hybridus*
White-winged Tern		*Chlidonias leucopterus*
Black belied Tern		*Sterna acuticauda*
Black Naped Tern		*Sterna sumatrana*
Lesser Noddy		*Anous tenuirostris*
Black Noddy		*Anous minuts*
	Phalacrocoracidae	
Little Cormorant		*Phalacrocorax niger*
Great Cormorant		*Phalacrocorax carbo*
	Ardeidae	
Little Egret		*Egretta garzetta*
Grey Heron		*Ardea cinerea*
Goliath Heron		*Ardea goliath*
Purple Heron		*Ardea purpurea*
Great Egret		*Casmerodius albus*
Intermediate Egret		*Mesophoyx intermedia*
Cattle Egret (Eastern)		*Bubulcus* (*ibis*) *coromandus*
Pond Heron		*Ardeola grayii*Indian
Little Heron		*Butorides striatus*
Black-crowned Night Heron		*Nycticorax nycticorax*
Cinnamon Bittern		*Ixobrychus cinnamomeus*
Little Bittern		*Ixobrychus minutus*
Yellow Bittern		*Ixobrychus sinensis*
Black Bittern		*Dupetor flavicollis*
	Threskiornithidae	
Black-headed Ibis		*Threskiornis melanocephalus*
Eurasian Spoonbill		*Platalea leucorodia*
	Ciconiidae	
Asian Openbill		*Anastomus oscitans*
Lesser Adjutant		*Leptoptilos javanicus*

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Conclusions

The coastal and estuarine areas of Bangladesh are covered by extensive mud flats, saline water, brackish water and protected bays. These places support huge numbers of important living resources, which are suitable for marine ranching. Local people indiscriminately utilise these natural resources and some are now completely destroyed, for example, the Chakaria Sunderban mangrove forest in Cox's Bazar. Most of the resources are being over-utilised (e.g. fish stock and shrimp fry) while some remain untouched or under-utilised (molluscs, seaweeds, crabs and offshore fishes). Therefore, sustainable practices, management and conservation of the estuarine and coastal resources and their related ecosystems are needed. The coordination of future research projects on ecology and conservation science of the coastal resources is required. The main problem facing Bangladesh is the limited capacity to enforce regulation and monitoring, as well as lack of knowledge on sustainable uses of coastal and estuarine resources. There are some management approaches and rules for coastal forests and fisheries of Bangladesh, which are not effective. Apart from this, there is a need for strong protection and conservation policies, which have to be incorporated into a management plan. Some of the plans should include: i) Major identification of coastal resources, ii) Monitoring activities, which contribute to the understanding of changes in coastal resources, iii) Improved management of existing reserves to correspond with their multipurpose usefulness, iv) Coordination of research project on conservation science and ecology of coastal resources, v) Creation of public awareness and promotion of local participation in managing natural coastal resources, vi) Strengthening and providing the required expertise on coastal zone management for existing coastal resources, vii) Participation by public bodies (non-government or trade organisations) in the planning of conservation strategies, and viii) Research on natural and human-induced treats to coastal resources and implementation of national coastal resource management programmes.

Acknowledgements

This is the contribution no. 7 of the Laboratory of Estuarine, Coastal and Aquaculture Research (LECAR) Institute of Marine Sciences and Fisheries, University of Chittagong. The authors are grateful to UNESCO/IOC and AWARE Foundation, Australia for their financial support for the present work. The authors also express their gratitude to the Director, IMSF, University of Chittagong for providing the necessary facilities pertaining to the work. We thank Professor Dr. Frederick Short, University of New Hampshire (USA) for assistance and for editing the manuscript.

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Maejo International

Journal of Science and Technology

ISSN 1905-7873

Available online at www.mijst.mju.ac.th

Full Paper

Determination of proportionality constants from cutting force modelling experiments during broaching operation

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Received: 14 March 2009 / Accepted: 13 August 2009 / Published: 17 August 2009

Abstract: Mechanistic model assumes that the types of cutting force acting on the broach teeth, namely axial force, normal force and lateral force, are proportional to chip-thickness area. In this paper, the proportionality constants related to the cutting force and chip-thickness area were obtained through experimentation. The shaping process was used to determine the proportionality constants in terms of specific cutting energy constants. The paper also includes static force modelling for broaching operation and graphical presentation of the experimental and simulated results.

Keywords: metal cutting, cutting force, broaching operation

Introduction

Metal cutting is one of the basic operations in manufacturing industries to produce the parts of desired dimensions and shape. Metal cutting constitutes a complex process involving the diversity of physical phenomena such as large plastic deformation, frictional contact, thermo-chemical coupling, and chip and burr formation mechanisms. A great deal of research [1-9] has been devoted to understanding the mechanism of machining with the objective of obtaining more effective tools and manufacturing operations.

Over the last several years, research has been carried out to develop mechanistic force models for a variety of machining processes, including end milling, face milling, boring, turning and broaching. These models have been employed in a number of designs, operation plannings and process control settings to predict both the cutting force and the resulting machine surface error.

In the literature, two different approaches have been adopted for the prediction of the cutting force system. The first method is based on the work done by Merchant [1] and involves a study of the cutting mechanics and the prediction of the shear angle in metal cutting. Both analytical and empirical models for shear angle prediction have been attempted. Lee and Shaffer [6] applied slip line theory to machining to develop the equation for predicting the shear angle. Usui et al. [10] developed a model that was based on the minimum energy criterion for predicting the chip flow angle and empirical models were used for predicting both the friction angle and the shear angle. This approach generally requires experimentation of a more fundamental nature of cutting mechanics to achieve the measurement and prediction of the shear angle.

In this paper, an attempt has been made to determine the specific energy constants experimentally for mild steel, aluminium and cast iron. Shaping process was used for the experimental purpose of determining specific energy constants and proportionality constants which are used in mechanistic modelling during broaching operation. A mechanistic model has been developed to compute static forces. The experimental results and simulated results by mechanistic modelling are presented graphically.

Methods

Computation of specific cutting energy constant for predicting the cutting forces–mechanistic modelling approach

In the mechanistic modelling approach, for any machining process the basic equations that relate the axial force F_a, normal force F_n and lateral force F_t to the chip-thickness area are given by [10]:

$$\begin{aligned} F_a &= K_a A_c \\ F_n &= K_n A_c \\ F_t &= K_t A_c \end{aligned} \qquad (1)$$

where F_a, F_n and F_t are the three-dimensional forces acting on the tool tip. A_c is the chip-thickness area and K_a, K_n and K_t are the proportionality constants corresponding to three-directional cutting forces.

The proportionality constants depend on the chip thickness t_c, cutting velocity v_c and rake angle γ_a of the cutting tool. Mathematically [10],

$$\begin{aligned} K_a &= e^{a_0 + a_1 \log t_c + a_2 \log v_c + a_3 \log t_c \log v_c + a_4 \log \gamma_a} \\ K_n &= e^{b_0 + b_1 \log t_c + b_2 \log v_c + b_3 \log t_c \log v_c + b_4 \log \gamma_a} \\ K_t &= e^{c_0 + c_1 \log t_c + c_2 \log v_c + c_3 \log t_c \log v_c + c_4 \log \gamma_a} \end{aligned} \qquad (2)$$

The coefficients a_i, b_i and c_i ($i = 1, 2, 3, 4$) are called the specific cutting energy constants. These constants depend upon the tool, work piece material, range of cutting speed and chip thickness. They are independent of the machining process. These constants are determined from calibration test for a given tool and work piece combination and a given range of cutting conditions.

Keeping the rake angle and the velocity of the tool movement constant corresponding to the broaching tool, the equations (2) reduce to:

$$K_a = e^{a_0 + a_1 \log t_c}$$
$$K_n = e^{b_0 + b_1 \log t_c} \quad (3)$$
$$K_t = e^{c_0 + c_1 \log t_c}$$

The values of specific cutting energy constants can be determined using a simple calibration experiment. The experiment was conducted on a shaping machine and the specific cutting energy constants were determined.

Experimental investigation

Several sets of shaping operation experiments were conducted in order to determine the specific cutting energy constants. Three sets of experiments were conducted on each set of processes. The first set of experiments was used to ascertain which tool and cutter geometry variables affected the proportionality constants. The second set of experiments was used to develop an adequate model for proportionality constants based on important tool and cutter geometry variables determined from the first set of experiments. The third set of experiments was used to determine the specific cutting energy constants. Experiments were repeated for aluminum, cast iron and mild steel at different depths of cut. Chip-thickness area for calibration purpose was measured from the chip curl. The volume of the chip was measured using water displacement method. After that the chip curl was heated and elongated. The chip width (b) and length (ℓ) were measured using a micrometer and vernier calliper respectively. Then the actual chip thickness (t_c) was obtained by dividing the volume by the product of length and width, i.e. $t_c = v / b\ell$, where v is the volume of chip curl. Knowing the chip thickness and width, the chip-thickness area could be computed as the product of t_c and b.

Static model

Figure 1 illustrates the static force measurement set-up during the broaching operation. The cutting forces in three directions were measured using an accelerometre and the root mean square (RMS) value was taken as the static cutting force. A static analysis calculated the effects of steady loading conditions on a structure while ignoring inertia and damping effects such as those caused by time-varying loads. A static analysis can, however, include steady inertia loads (such as gravity and rotational velocity) and time-varying loads that can be approximated as static equivalent loads.

Chip-thickness area measurement

A tool-maker's microscope was used to obtain the coordinates of the tooth profile of a broaching tool. AutoCAD drawing of the broaching tool was drawn to procure the chip load area and the dimensions of the tooth profile. Figure 2 shows the coordinates of a single tooth of the broach obtained through the tool-maker's microscope and AutoCAD drawing. Figure 3 shows three planar angles of the cutting edge of a broach tooth where γ_R is the rake angle and γ_L is the release angle. Figure 4 shows the coordinates of the full broach, which gives pitch = 6.78 mm, rake angle = 30^0, rise per tooth = 0.05 mm and width = 8 mm; hence, chip-thickness area = width x rise/tooth = 0.4 mm^2.

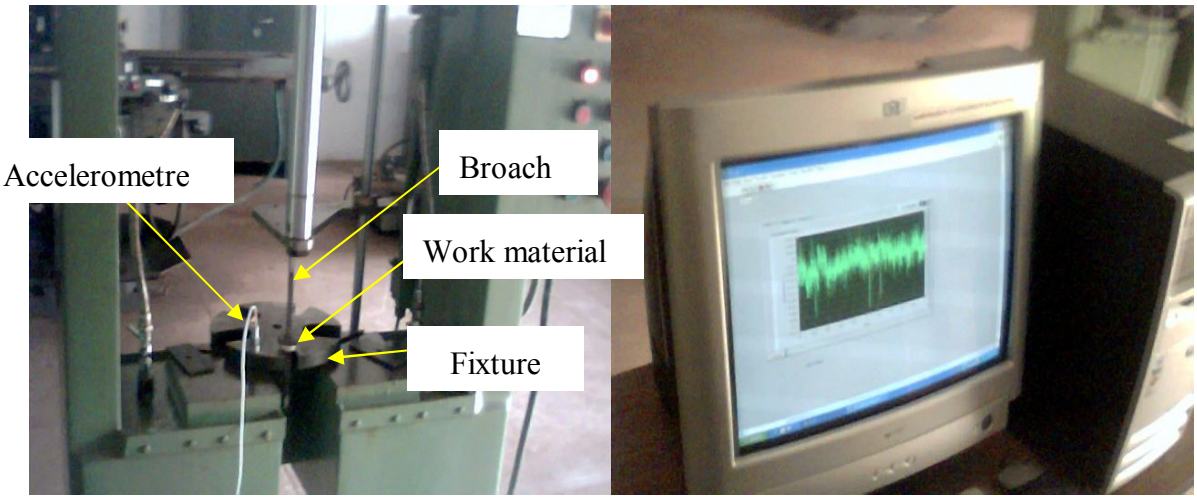


Figure 1. Experimental set-up to obtain the force pattern during the broaching process

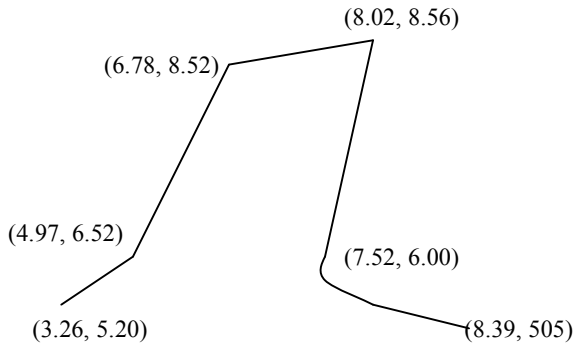
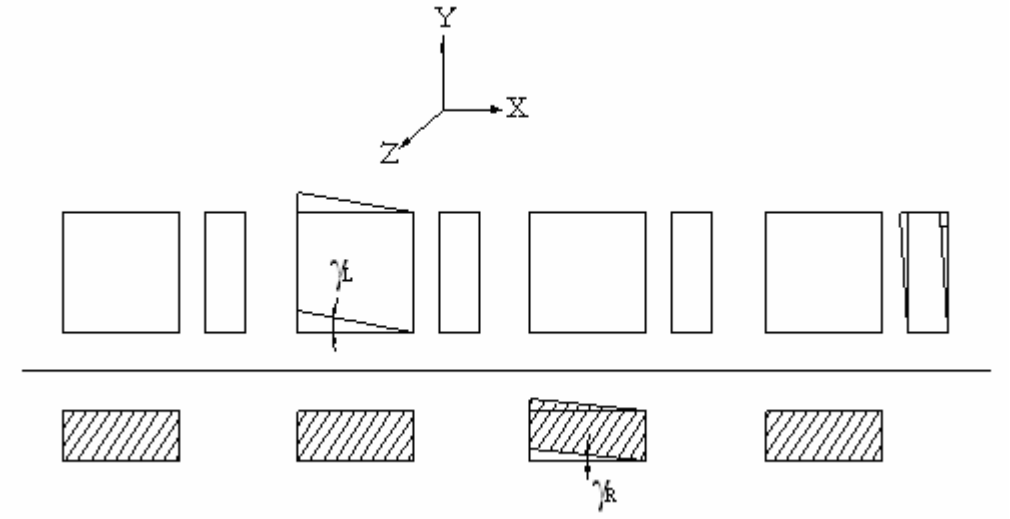


Figure 2. Broach tooth profile as obtained by tool maker's microscope



a) Normal position. b) Rotated about z axis. c) Rotated about y axis d) Rotated about x axis

Figure 3. Planar angles of the cutting edge of broach tooth

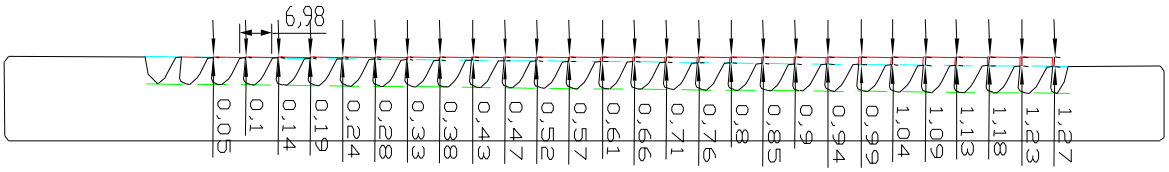
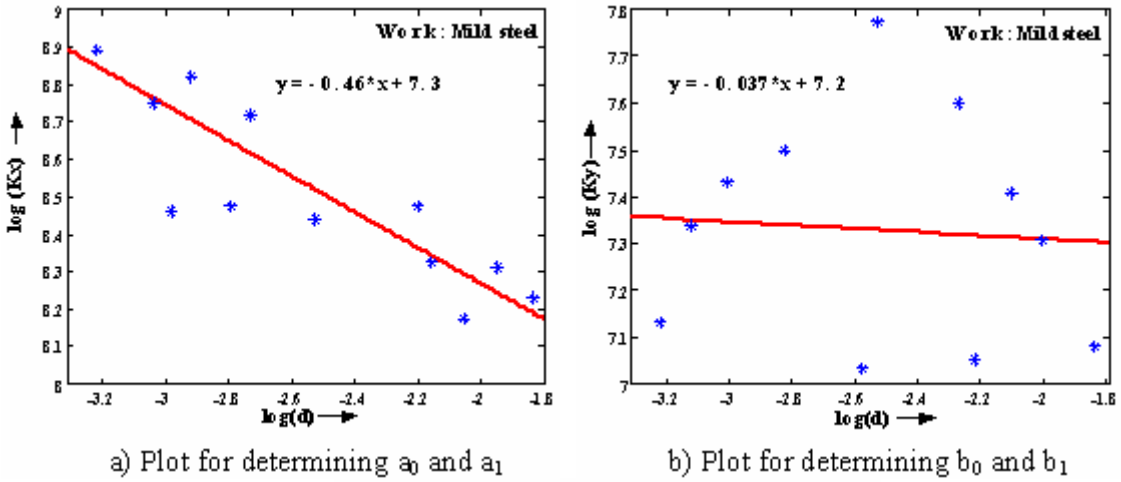


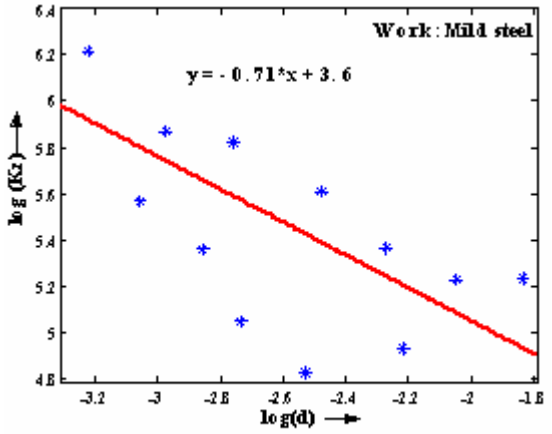
Figure.4. AutoCAD drawing of a broach tool

Results

Figures 5, 6 and 7 illustrate the cutting force versus chip thickness during shaping operation for mild steel, aluminium and cast iron respectively. In the plot, the x-axis is the logarithm of chip thickness (marked as d) and y-axis is the logarithm of the cutting forces in Newton (marked as Kx, Ky and Kz) along x, y and z directions respectively. Chip thickness corresponds to depth of cut which varied from 0.04 mm to 0.16 mm. The calibration test was performed for a small depth of cut to avoid error due to impulsive cutting force coming on the work piece at greater depth of cut. A linear curve fitting was made using Matlab software to determine the specific energy constants. Negative-slope linear curves were obtained and the coefficients of the linear curve fitting gave the specific energy constants. The proportionality constants K_a, K_n and K_t were determined using equation (3). Tables 1 and 2 give the values of specific cutting energy constants and proportionality constants respectively.

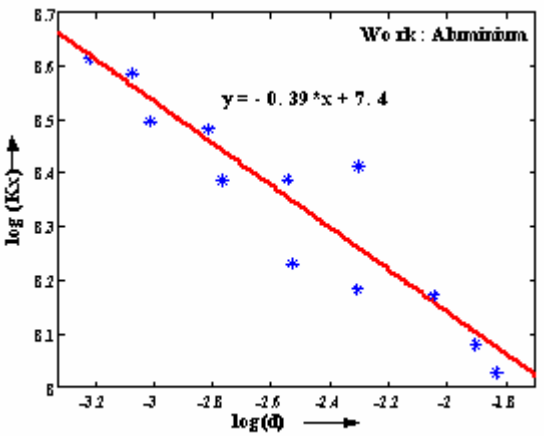


a) Plot for determining a_0 and a_1 b) Plot for determining b_0 and b_1

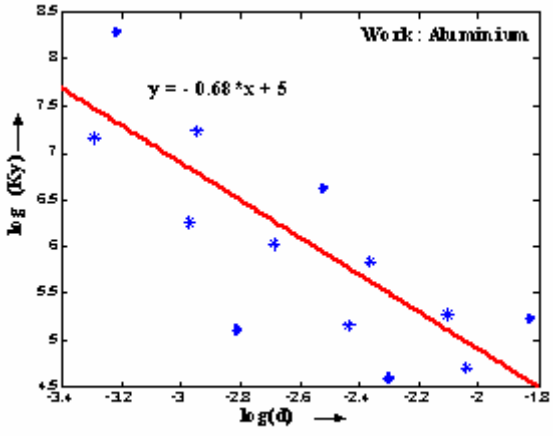


c) Plot for determining c_0 and c_1

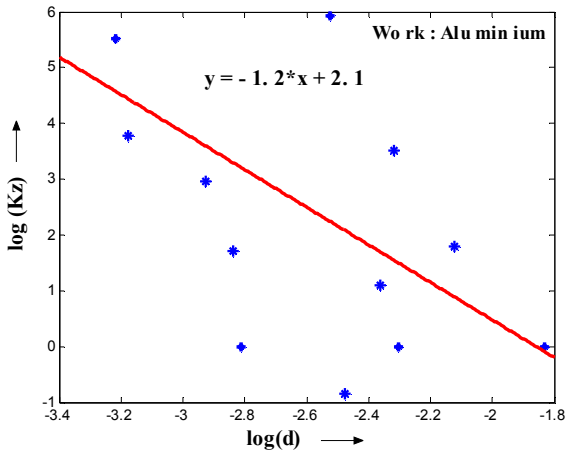
Figure 5. Specific cutting energy constants for mild steel work material during shaping operation



a) Plot for determining a_0 and a_1



b) Plot for determining b_0 and b_1



c) Plot for determining c_0 and c_1

Figure 6. Specific cutting energy constants for aluminium work material during shaping operation

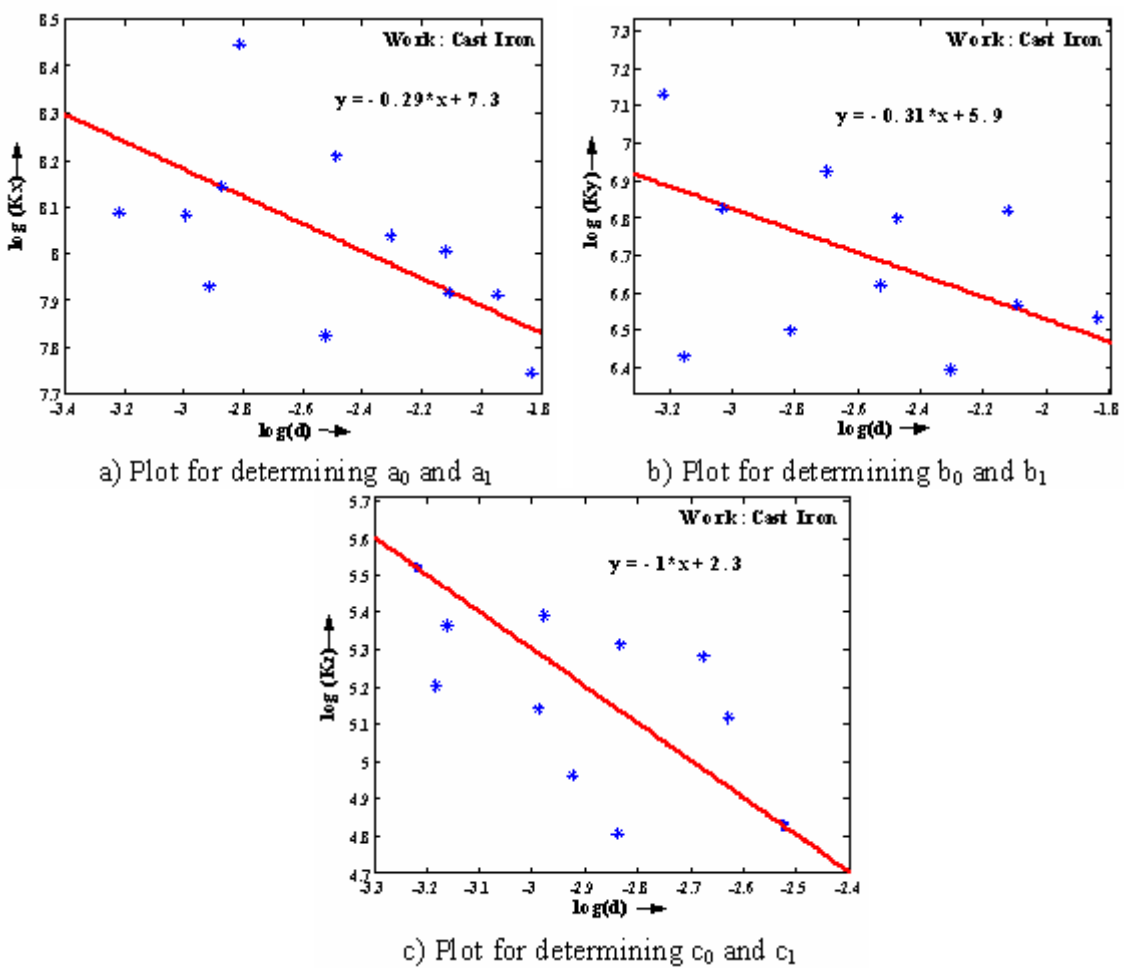


Figure 7. Specific cutting energy constants for cast iron work material during shaping operation

Table 1. Specific cutting energy constants obtained during shaping operation

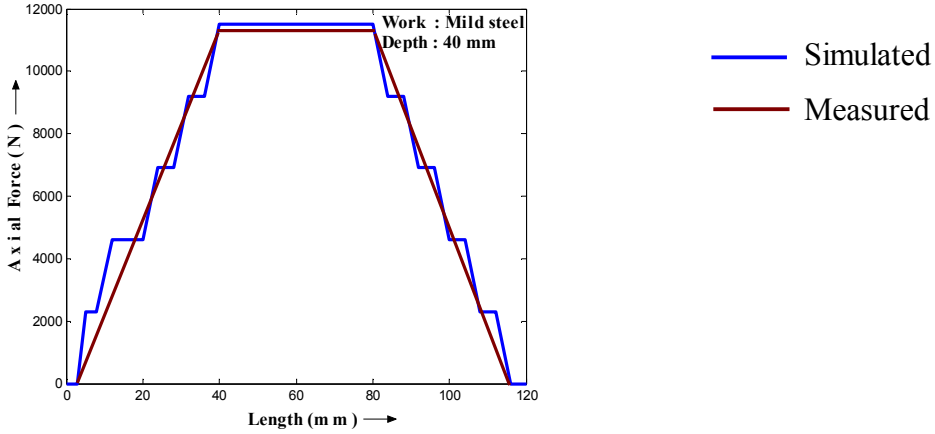
Material	a_o	a_1	b_o	b_1	c_0	c_1
Mild steel	7.3	-0.46	7.2	-0.037	3.6	-0.71
Aluminium	7.4	-0.39	5.0	-0.68	2.1	-1.2
Cast iron	7.3	-0.29	5.9	-0.31	2.3	-1.0

Table 2. Proportionality constants determined from specific cutting energy constants

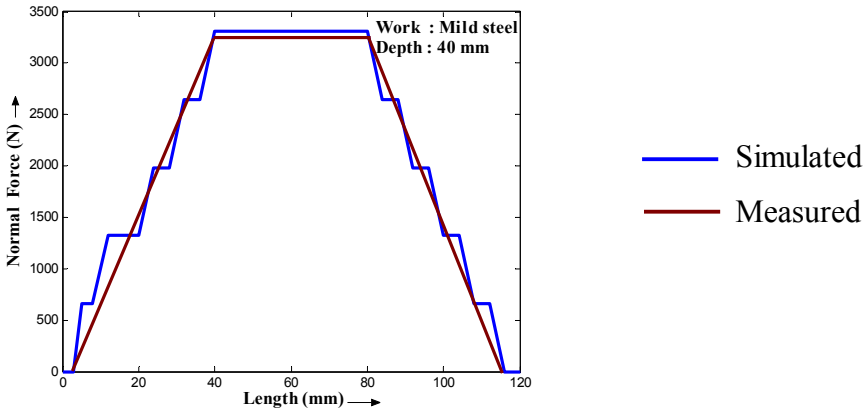
Material	K_a (N/mm^2)	K_n (N/mm^2)	K_t (N/mm^2)
Mild steel	5732.3	1654.0	307.1
Aluminium	5370.0	1088.0	279.4
Cast iron	3562.0	924.4	199.5

Static forces during broaching operation

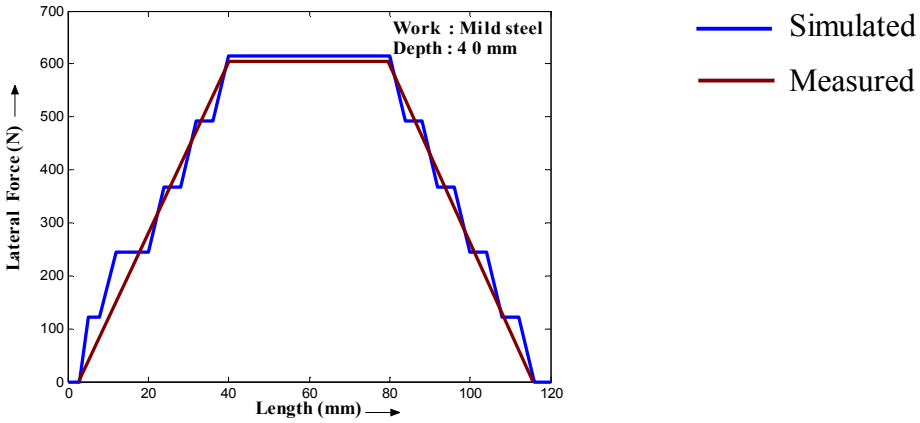
A MatLab program was written to simulate the static forces during broaching for different materials at different depths. The simulation results were plotted as shown in Figure 8 for mild steel work material at 40-mm depth. The cutting force progressively increased to a steady-state force when all the teeth were engaged in the work piece.



a) Axial force acting on the broach



b) Normal force acting on the broach



c) Lateral force acting on the broach

Figure 8. Comparison of measured and simulated static forces for mild-steel work material at 40-mm depth

Conclusions

This paper presents the determination of proportionality constants, which are a determining factor in cutting force modelling. These proportionality constants were determined experimentally using shaping as a fundamental calibration process. The experiment was repeated for different materials, i.e. mild steel, aluminium and cast iron, and results are presented graphically. After determining the proportionality constants, mechanistic modelling for static force computation was carried out for the broaching operation. The comparison of the experimental and simulated results for static forces are presented graphically and are closely in agreement.

Acknowledgements

The authors thank the Head of the Mechanical Department of NITK, Surathkal and the Director of MIT, Manipal for providing the facilities to carry out the experiments.

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