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สำนักงาน : สถาบันวิจัยและพัฒนาแห่งมหาวิทยาลัยเกษตรศาสตร์
มหาวิทยาลัยเกษตรศาสตร์ จตุจักร กรุงเทพฯ 10900

วิทยาสารเกษตรศาสตร์ เป็นวารสารของมหาวิทยาลัยเกษตรศาสตร์ ซึ่งพิมพ์เผยแพร่ผลงานทางวิชาการแขนงต่างๆ ทางด้านวิทยาศาสตร์และสังคมศาสตร์ ทั้งจากภายในและภายนอกมหาวิทยาลัย โดย**วิทยาสารเกษตรศาสตร์ สาขาวิทยาศาสตร์** มีกำหนดออกปีละ 4 ฉบับ คือ เดือนมีนาคม มิถุนายน กันยายน และธันวาคม และ**วิทยาสารเกษตรศาสตร์ สาขาสังคมศาสตร์** มีกำหนดออกปีละ 2 ฉบับ คือ เดือนมิถุนายน และธันวาคม

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Cryopreservation of Kluai Namwa (*Musa* × *paradisiaca* 'Kluai Namwa')

Benchamas Silayoi

ABSTRACT

Cryopreservation encapsulated-vitrified meristem of K.Namwa stored in liquid nitrogen with various loading solutions were investigated. A mixture of 2M glycerol and 0.4 M sucrose could produce 85 percent survival of meristem while 2M ethylene glycol without sucrose gave only 5 percent survival of meristem with pale green colour. After treating the encapsulated-vitrified meristem with loading solution in liquid nitrogen for 1 hour and then transferred to modified MS medium for two weeks, they returned to green colour. All of them were recultured in MS media and proliferation occurred within two months.

The study on shoot development of encapsulated-vitrified meristems, which were dehydrated in PVS2 at various times of 0 10 15 20 25 30 35 and 40 min. at 25°C, before cryopreservation was conducted. The treated encapsulated-vitrified meristems were kept in liquid nitrogen for 1 hour, and transferred to modified MS medium for two months. The result showed that meristems treated with PVS2 for 25 min. gave the highest shoot proliferation of 85 percent followed by 30 min. of 80 percent. The shoot proliferation decreased with increasing times of dehydration which was similar to the one that was not kept in liquid nitrogen.

Key words: cryopreservation, Kluai Namwa, Pisang Awak, *Musa* × *paradisiaca*

INTRODUCTION

Kluai Namwa (K.Namwa) or Pisang Awak, an ancient Thai banana, is a herbaceous pereninal plant belonging to Family Musaceae, Order Zingiberales or Sciataminaceae (Simmonds, 1966) which has been cultivated throughout the country. It is often used as the first solid food fed to infants. K.Namwa provides a more balanced diet than any other fruit and vegetable. It is filling, easy to digest, nearly fat free, rich source of carbohydrate with calorific value of 67/100gm. Flesh of banana is free of sodium, contains various vitamins and has therapeutic value for the treatment of many diseases.

K.Namwa is quite tolerant to arid soil and some diseases. The serious disease of K.Namwa is Fusarium wilt race1 caused by *Fusarium oxysporum* cubense. (Daniells, and Bryde, 2001). It is a soil borne disease destroying many orchards of K.Namwa. Several controls have been employed, including field sanitation, soil fumigation and liming but without success. Crop rotation with paddy has been shown effectiveness in controlling the disease only for 1 to 2 years in the same field. The disease is under controlled by replacing the susceptible cultivar with the resistant one(Hwang,1985). There is an urgent need for conserving K.Namwa *in vitro* because widespread disease of Fusarium wilt is serious threat to germplasm in nature or in the field conservation Method of *in-vitro* storage of germplasm includes development of micropropagation system, slow growth storage and

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cryopreservation.

Cryopreservation is a long-term germplasm storage *in vitro* using cryogenic methods. Storage in liquid nitrogen (liquid N) at –196°C will overcome the problem of repeated subculturing, contamination of cultures and avoiding somaclonal variation (Banerjee and de Langhe, 1985). The success of cryopreservation of *Musa* germplasm using cell suspension and meristem have been reported by several authors. (Panis *et al.,* 1990; Panis and Swenen, 1993). Glycerol, ethylene glycol (EG) and dimethyl sulphoxide (DMSO) were used as loading solution and PVS2 (plant vitrification solution no.2) as dehydrating solution before storing in liquid N for apple, pear, and wasabi (Sakai and Nishiyama, 1978; Sakai *et al* 1990; Matsumoto and Sakai, 1995). The use of meristem encapsuled in a gel and dehydrated using osmoticum were also done. The 3% Na-alginate was reported for encapsulated shoot tip of Basrai Banana (Gunapathi *et al*., 1992). Those beads were directly plunged in liquid N and thus the meristem could theoretically be stored for an indefinite period of time. The plants were recovered by thawing the beads rapidly and placing them on recovery medium. This technique of cryopreservation has also been applied for long-term storage of seed of *Musa balbisiana* (Bhat *et al.,* 1994).

The studies were conducted with the various loading solutions and dehydrating times before storing in liquid N for cryopreservation of K.Namwa.

MATERIALS AND METHODS

One mm. size meristem of K.Namwa sword suckers were used as explants. The explants were precultured in 1/2MS (1962) medium supplemented with 0.3M sucrose for one day. Then the explants were loaded in various solutions having 3%Na-alginate, with and without sucrose. There were 7 treatments and 20 replications (5 explants for one replication) in the experiment.

Experiment 1 Suitable loading solution

The loading solutions were: control or no loading solution, 2M glycerol with and without 0.4M sucrose, 2M ethylene glycol (EG) with and without 0.4M sucrose and 2M dimethyl sulphoxide (DMSO) with and without 0.4M sucrose. After 20 min. in loading solution, the explants were transferred to liquid MS medium with 0.1M $CaCl_2$ and 0.4M sucrose for 30 min. The explants which would be covered with clear gel, was called encapsulated vitrified meristem. Then, the encapsulated-vitrified meristems were soaked in PVS2 which composing of 30% glycerol, 15% ethylene glycol, and 15% DMSO for 20 min. (Sakai *et al.*,1990). The capsules were then plunged directly in liquid nitrogen for 1 hour. The freezed capsules in 40°C water would be thawed and unloaded by soaking in 1.2 M sucrose for 20 min. for 2 times and cultured in semi solid MS medium.

Experiment 2 Proper time for dehydration with PVS2

The procedure of this experiment was similar to experiment1. The best loading solution in experiment1 was used for encapsulation in experiment 2. The encapsulated vitrified meristems were then soaked in PVS2 as dehydration at 25°C for 0,10,15,20,25,30,35, 40 min. and kept in liquid nitrogen for 1 hour. Thawing and unloading were the same as experiment 1.

After cultured in semi solid MS medium, the number of green meristems or survival meristems and number of shoot were recorded.

The experiment was conducted at Department of Horticulture, Faculty of Agriculture, Kasetsart University. Bangkok, Thailand.

RESULTS AND DISCUSSIONS

Experiment 1

When the meristems of K.Namwa were precultured with 0.3M sucrose for 1 day, and then the encapsulated vitrified meristems were made in

Na-alginate having different loading solutions, it was found that the mixture of 2M glycerol and 0.4M sucrose gave the highest survival of 85% followed by 2M DMSO and 2M ethylene glycol (both with sucrose) respectively. The loading solution giving the lowest survival of 5% was noticed in 2M ethylene glycol without sucrose (Table1). The results, which were similar to those reported by Sakai and Nishiyama (1978), Sakai *et al*. (1990) and Matsumoto and Sakai (1995), showed the loading solution with no sucrose to have less survival than those with sucrose. The meristems, when taken out of liquid nitrogen, became pale green, while when cultured in MS media, for 1 day the colour of meristems changed to yellow brown and later to black, the sign of dead tissue. However, the meristems in the loading solution with sucrose especially the one with the mixture of 2M glycerol, when cultured in MS media for 2 weeks was found to regain the greeness (Figure 1). The encapsulated -vitrified meristems transferred to be recultured in the previous formula media were also noticed to be able to grow into normally physiological plants again in 2 months (Figure 2). The use of cryoprotectant, as the loading solution, alone revealed the low survival since the cryoprotectant which acted in dehydrating water from cells, caused them wilted before soaking in liquid nitrogen. If the appropriate concentration was employed, the cell would then be protected from ice-crystal damage. Besides, cell membrane would be protected while the denaturing of protein and nucleic acid would be prevented (Towill,1991). Still, if too much concentration was applied, the chemical toxicity might occur resulting in low survival. It should, thus, be used with other cryoprotectants especially sucrose of low concentration, which would increase greater meristem survival of carnation than the application of cryoprotectant alone (Uema and Sakai,1980). Sucrose, the energy source for cells, its carbon is used in cell wall forming and maintaining osmotic pressure. It is the osmotic active substance which confers vigor to plant, enabling it to tolerate any stress such as dehydration, coldness etc. In comparison of chemical toxicity, glycerol was found to be solution of the least toxicity (Sakai *et al.*, 1990). Therefore, when glycerol is used with low concentrated sucrose, together they enable the plant to tolerate to dehydration as well as freezing. Cryoprotectant will cause changing in cell size making cells to be more flexible. In addition, the application with sugar or other cryoprotectants will prevent the toxicity of the added substances resulting in the highest survival. It was also found that the soaking of meristem in loading solution was of necessity in increasing survival of cells and tissue by giving cells to have dehydration tolerance.

Experiment 2

When the meristems of K.Namwa which went through preculturing and then were made into encapsulated-vitrified meristems in loading solution with the mixture of 2M glycerol and 0.4M sucrose for 30 minutes, after which, the encapsulated-vitrified meristems obtained were soaked in PVS2 solution at 25°C for various different times before being stored in liquid nitrogen and without liquid nitrogen, it was found that the encapsulated-vitrified meristems soaking in PVS2 for 25 minutes gave the highest shoot tip developing of 85% followed by 30

Table 1 Loading solutions affecting the survival of encapsulated -vitrified meristem stored in liquid nitrogen after which transfer to be cultured in MS media for 2 weeks.

Loading solution	Survival(%)
Control	37
2M glycerol+0.4M sucrose	85
2M glycerol	32
2M EG+0.4M sucrose	52
2M EG	5
2M DMSO+0.4M sucrose	63
2M DMSO	12

min. of 80% when stored in liquid nitrogen (Table2). As for the encapsulated-vitrified meristems which had not gone through PVS2 soaking, the meristems turned black after transferred to be cultured in MS media for 1 day, whereas the encapsulated-vitrified meristems which had not gone through storage in liquid nitrogen and was not dehydrated with PVS2, acquired the highest shoot development of 100%. The shoot development decreased with the increasing times of going through dehydration with PVS2. The results were similar to those maintained in liquid nitrogen, that was, the dehydration with PVS2 for longer than 25 min., causing the reduction in shoot tip development.

The 25-30 min. period was appropriate for PVS2 to make balance in cell dehydration and making replacement in plant cells. The nature of PVS2 was that when being stored in liquid nitrogen, instead of forming ice crystal like water, it would create clear crystal covering meristem, preventing cell damage, thus resulting in the highest survival. In less than 25 min. period, the exchange of substance might not be balanced resulting in rather great amount of water remaining in cells. When stored in liquid nitrogen, such water in cell would form ice crystals with greater volume than cell volume, making cell disrupted, followed by death.

As for those soaked in PVS2 longer than 25 min., there would be tendency in shoot developing to decrease, which was quite similar to encapsulated-vitrified meristems that were not stored in liquid nitrogen. It might be due to the chemical toxicity and osmotic pressure, which was harmful to cells, resulting in percent decreasing of shoot development. Therefore, the success in maintaining by encapsulated-vitrification of cryopreservation will depend on cautious cell dehydration, permeability of cryoprotectant into cells and drainage prevention from too much osmotic pressure (Rall,1987).

Figure 1 Encapsulated-vitrified meristems after going through storage in liquid nitrogen and culturing in MS media for 2 weeks.

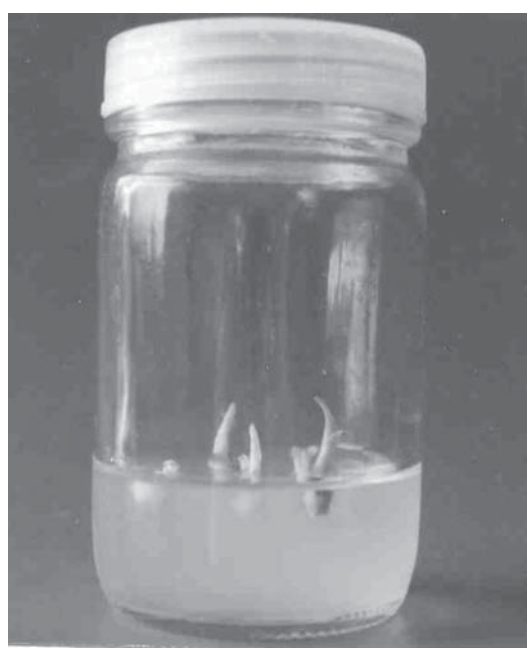

Figure 2 Plantlets developed from encapsulated-vitrified meristems after transferred to be recultured in MS media for 2 months.

Table 2 Dehydration with PVS2 through various times at 25°C with and without going through liquid nitrogen storage affected shoot development after recultured in MS media for 2 months.

Time (min.)	Percentage of shoot	
	Liquid nitrogen	Without liquid nitrogen
0	0	100
10	25	95
15	40	92
20	72	90
25	85	92
30	80	73
35	60	60
40	45	54

CONCLUSIONS

1. The appropriate loading solution for storage of meristem of K.Namwa in liquid nitrogen was the one with the mixture of 2M glycerol and 0.4M sucrose which gave the highest survival rate of 85% followed by 2M DMSO and 2M EG (both with sucrose) respectively.

2. The soaking of encapsulated-vitrified meristems in PVS2 at different periods of time found the 25-30 min.to be the most appropriate in storing the meristems of K.Namwa due to the highest shoot development of 80-85% obtain.

3. The kind of loading solution and the most suitable period in dehydration with the essential factors in the success of storing meristems of K.Namwa in liquid nitrogen was found to be the employing encapsulated vitrification of cryopreservation.

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Some Chemical Treatments on Kluai Khai Through Tissue Culture for Mutation Breeding

Parson Saradhuldhat and Benchamas Silayoi

ABSTRACT

Polyploidy of *in vitro* Kluai Khai plantlets were induced by different concentrations of the two mutagens : 0, 0.5, 0.75 and 1% colchicine and 0,15, 30, 45mM oryzalin containing 2% DMSO for 2.5, 5.0, 7.5 hours. It was found that the higher the mutagen doses and the longer the treatment duration, the less the survival rates. After treating plantlets with colchicine and oryzalin at 0.4, 0.5, 0.75% and 13, 15, 22mM, respectively the survival rate of each one was found to be 50%. In MV_1, adventitious bud initiation of mutagenic treatment yielded 0.2-1.2 shoots which was lower than those in the control while those of MV_2 and MV_3 were not significantly different from each other. Treated plantlets were revealed to be abnormal in chimera and stomata sizes were larger than those of the controls. The plantlets with stomata over 28mm were selected for chomosome count. The controls were diploid (2n = 22) whereas three selected clones were tetraploid (2n = 44) derived from 1% colchicine at 7.5 hours and 45mM oryzalin at 2.5 hours. The mutants were then subcultured. The number of suckers of mutants was less than those of the control. MS medium without plant growth regulator was observed to give the best rooting of all treatments. After transfered to the greenhouse, the height of the controls and the colchicine treated plants were revealed to be significantly different from the oryzalin treated ones which were shorter with low survival rate. The results obtained in the field were similar to those in the greenhouse. The foliage of all mutants appeared more drooping with larger stomata cell, lower in stomata number per area and thicker than those of the controls. The numbers of leaf and sucker of each mutant were not significantly different from the controls.

Key words: Kluai Khai, tissue culture, mutation breeding, colchicine, oryzalin, Pisang Mas

INTRODUCTION

Kluai Khai (*Musa acuminata* 'Kluai Khai') or Pisang Mas belongs to AA Group with plant height under 2.5 m.and fruit size quite small of 3.5 width and 10.5 cm. long. Flesh color is yellow-orange and seedless (Silayoi and Babpraserth, 1983). K.Khai can grow well all year round. The most production occurs in the northern provinces, such as, Kamphangphet, Tak, Sukhothai, Pichit and Nakhonsawan. The quality of flesh is quite excellent, fragrant and sweet but the fruit is quite small with thin peel. According to these problems, the improvement of K.Khai, should therefore, be brought into consideration.

The genetic system of *Musa* is very much complicated owing to the inherent problems of sterility, heterozygosity and polyploidy in most of the clones. Parthenocarpy, sterility are very difficult to obtain viable seeds. These are limiting factors for conventional breeding of K. Khai. Alternative technique to select conventional recombinant

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phenotypic is to explore plant tissue to chemical mutagens and *in vitro* culture, to induce mutation. The degree of mutation depends on the level and duration of the treated explant and potential increasing variation as the size of the exposed explant is reduced (Krikorian, 1987). Many chemicals can be used as mutagens. Colchicine is remarkable in the sense that it will arrest metaphase. All plant organs respond to its treatment. In plants, the somatic tissue responds more readily to colchicine action than the meiotic cells. For the study of haploid mitosis, pollen tube culture, with colchicine added in the medium, is considered to be most satisfactory to dihaploid (Sharma and Sharma, 1980). Chen and Coedem-Kallemeyn (1979) induced tetraploid Day lily by using 20 mg/l of colchicine to their callus. It has been reported that, colchicine at 0.5% could produce hexaploid banana from triploid one (Nukulkarn, 1983)

Another interesting chemical is oryzalin whose trade names include Dirimal, EL-119, Rycelan, Ryzelan, Ryzelon and Surfran. Oryzalin is a selective pre-emergence surface applied herbicide, which is used for control of annual grasses and broadleaf weed seeds by blocking cell division in the meristem. It is also an antimitotic agent the same as colchicine (Hassawi and Ling,1991; Verhoeven *et al*., 1990). It loses centrosomal function as well as spindle fiber function. The mode of loss of the spindle function and spindle fiber is more effective than colchicine in *Nicotiana plembaginifolia* (Verhoeven *et al.*, 1990). Ramulu *et al.* (1991), reported that, 15-35 mM oryzalin could break the cell division at metaphase and made 4X cell of potato. Double haploid could also be done in wheat and corn by using oryzalin (Wan *et al.*, 1991; Hassawi and Ling, 1991).

The objective of this study was to produce the 4X Kluai Khai mutants by using colchicine and oryzalin solution at different concentrations.

MATERIALS AND METHODS

The experiment was carried out at the Department of Horticulture, Kasetsart University. Tissue culture plants of K.Khai were cultured on MS media supplementing with 4 mg/l BA for each treatment. One hundred of K.Khai plantlets were soaked in 0.5, 0.75, 1% colchicine and 15 mM, 30 mM, 4 mM of oryzaline containing DMSO for 2.5, 5.0 and 7.5 hours while the control was soaked in distilled water. The explants were washed after soaked in distilled water and then cultured in modified MS medium with 4 mg/l BA. Subcultures of selected plants were prepared every month.

Observations were made on survival rate in MV_1, and stomata size measuring with ocular micrometer in MV_2. The plantlets with stomata about 2 times the normal size were selected for chromosome number counting. The plantlets that possessed the tetraploid chromosome were later selected for further study. 100 plantlets from each treatment of tetraploid plant were multiplied for 4 generations. They were rooted on MS media without hormone (Silayoi, 1985). The rooted plantlets were then transplanted to pots and to field respectively. In the field, the height, circumference of pseudostem, number of leaf and number of sucker were observed.

RESULTS AND DISCUSSION

Survival percentage

After one month, the survival percentage of the control was noticed to be 100 per cent but at higher concentrations of either colchicine or oryzalin, they decreased as shown in Table 1. It was also found that LD_{50} of colchicine treatments at 2.5, 5.0 and 7.5 hrs. were 0.4, 0.5, 0.75 per cent while those of oryzalin at 2.5, 5.0 and 7.5 hrs were 13, 15 and 20 mM respectively (Figure 1 and 2).

The growth of treated plantlets in MV_1-MV_3 was noticed to be not so good as in the control. The treated plants appeared albino, dwarf with leaf thickness (Figure 3). The control of MV_1 gave

Table 1 Survival plantlets (as % of control) after 1 month treatment.

Chemical	Doses	Treated time (hrs)			Average
		2.5	5	7.5	
Control	0	100.00	100.00	100.00	100.00
Colchicine	0.50 %	61.11	50.00	39.89	50.33
	0.75%	50.00	44.44	33.33	42.59
	1.00%	38.89	27.78	16.67	27.78
	Average	50.00	40.74	29.96	
Oryzalin	15mM	66.67	38.89	50.00	51.85
	30mM	27.78	33.33	16.67	25.93
	45mM	38.89	16.67	11.11	22.22
	Average	44.45	29.63	25.93	

significantly more shoots than the other treated explants. At MV_2, the 45 mM, 7.5 hrs. oryzalin treated plants died because of high dose and too long time treating (Table 2). The results agreed with Keawsompong (1993) who reported that 20% Kluai Khai died after soaked in 500-1500ppm colchicine at 24-72 hrs.

Stomata sizes were measured in MV_3. The stomata of 9 colchicine and 7 oryzalin treated plants were longer than 28 mm while the control one was 19 mm (Table 3, Figure 4). They were then selected for chromosome counting. The polyploid were selected for further study in the field. Only 2 plants of C3 (explant treated with 1% colchicine) or plant no. 5 and 6 (C3-5 and C3-6) and 1 plant of Z1 (explant treated with 45mM oryzalin) or plant no.6 (Z1-6) were selected as tetraploid (Figure 5). The plants are shown in Figure 6. These should imply that both chemicals, colchicine and oryzalin, could double chromosome of K. Khai. The results agreed with Chen and Coeden-Kallemeyn (1979) who reported that colchicine could induce tetraploid Day lily and with Wan *et al.* (1991) on doubled haploid of wheat and corn by using oryzalin.

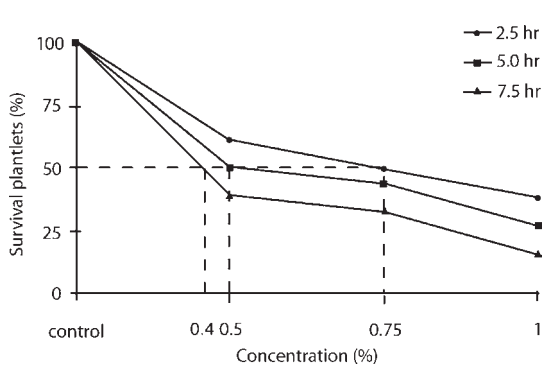

Figure 1 Survival percentages after one month colchicine treatment.

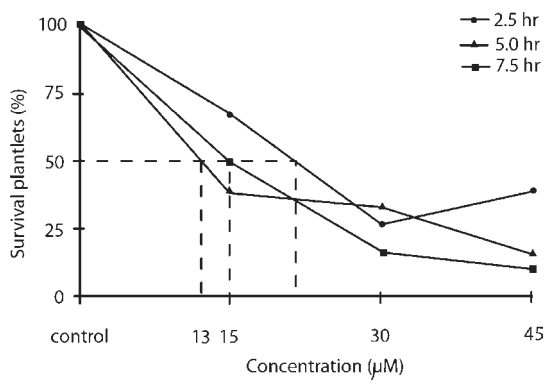

Figure 2 Survival percentages after one month oryzalin treatment.

The selected plants were subcultured with the number of shoots produced shown in Table 4. Numbers of shoot of mutants were found to be significantly different from those of the control. For Z1 or oryzalin treated plants, they were noticed to produce significantly more shoots than the colchicine treated plants in the 5th week (Table 4).

After 5 weeks in MS with 5 ppm of BA, the plantlets were rooted in MS without hormone. 1 point was given to 0-1 root, 5 points for the ones with more than 6 roots. The score above 3 points was good for planting. The oryzalin treated plants obtained the highest score of 4.6 points, which was significantly different to the control and colchicine treated plants (Table 5). Leaves of oryzalin treated plants were also revealed to be bigger than those of the other treatments, resulting in stronger roots produced. The results were quite similar to those of Phoengchan (1995).

One hundred rooted plantlets of each treatment (control, Z1-6,C3-5,C3-6) were transplanted to nursery with 98% found to survive.

Table 2 Average numbers of shoot produced from MV_1-MV_3.

Chemical	Symbol	Concentration	Time(hrs)	MV_1 1/	MV_2	MV_3
Control				2.1^{e}	1.4	1.9
Colchicine	A1	0.5%	2.5	0.8^{a-d}	1.4	1.3
	A2		5.0	0.9^{bcd}	1.1	1.1
	A3		7.5	1.0^{bcd}	1.3	1.3
	B1	0.75%	2.5	1.1^{cd}	0.9	1.0
	B2		5.0	0.9^{bcd}	0.9	1.3
	B3		7.5	1.1^{cd}	1.1	1.6
	C1	1.0%	2.5	1.2^{d}	1.3	1.3
	C2		5.0	1.0^{bcd}	0.4	1.0
	C3		7.5	0.8^{a-d}	1.4	1.0
Oryzalin	X1	15mM	2.5	0.8^{a-d}	1.6	1.1
	X2		5.0	0.7^{a-d}	0.7	0.9
	X3		7.5	0.7^{a-d}	0.9	1.1
	Y1	30mM	2.5	0.5^{abc}	1.0	1.1
	Y2		5.0	0.4^{ab}	1.3	1.1
	Y3		7.5	0.4^{ab}	1.0	1.0
	Z1	45mM	2.5	0.5^{abc}	1.4	1.1
	Z2		5.0	0.5^{abc}	1.1	0.7
	Z3		7.5	0.2^{a}	0.0	-
F-test				**	ns	ns
CV(%)				74.9	91.4	64.8

ns = non significance

** = highly significance

1/ = Means within columns in similar letters were not significantly different at the 5% level by Duncan's multiple range test.

Table 3 The selected plants with stomata longer than 28 mm.

Chemical	Symbol	Concentration	Time(hrs)	Total plantlet	Selected plantlet (no.)
Control			25	25	-
Colchicine	A1	0.5%	2.5	22	5
	A2		5.0	14	4
	A3		7.5	13	1
	B1	0.75%	2.5	13	1
	B2		5.0	21	2
	B3		7.5	15	1
	C1	1.0%	2.5	30	5
	C2		5.0	8	2
	C3		7.5	9	2
Oryzalin	X1	15mM	2.5	18	1
	X2		5.0	5	1
	X3		7.5	17	5
	Y1	30mM	2.5	7	1
	Y2		5.0	16	3
	Y3		7.5	7	0
	Z1	45mM	2.5	27	6
	Z2		5.0	14	2
	Z3		7.5	-	-

Table 4 Numbers of shoot of mutant in tissue culture compared to those of the control.

Treatment	Number of shoot[1/]				
	1st week	2nd week	3rd week	4th week	5th week
Control	1.0	2.1[b]	3.0[b]	4.1[b]	4.9[c]
Z1-6	0.8	1.3[a]	2.2[a]	2.5[a]	3.3[b]
C3-5	0.8	1.1[a]	1.5[a]	2.1[a]	2.2[a]
C3-6	0.9	1.1[a]	1.5[a]	2.2[a]	2.5[a]
F-test	ns	**	**	**	**
CV(%)	70.7	54.8	43.6	44.0	37.7

ns = non significance

** = highly significance

1/ = Means within columns in similar letters were not significantly different at the 5% level by Duncan's multiple range test.

Table 5 Score of healthy root of mutant plantlets compared to those of the control *in vitro*.

Treatment	Score[1/]
Control	3.8[a]
Z1-6	4.6[b]
C3-5	3.7[a]
C3-6	3.8[a]
F-test	**
CV(%)	25.6

** = highly significance

1/ = Means within columns in similar letters were not significantly different at the 5% level by Duncan's multiple range test.

Table 6 The height of mutant plants compared to the control in nursery.

Treatment	Height (cm)	
	1st month [1/]	2nd month [1/]
Control	6.12[b]	8.11[b]
Z1-6	3.63[a]	4.13[a]
C3-5	6.98[c]	7.53[b]
C3-6	6.47[bc]	8.10[b]
F-test	**	**
CV(%)	19.4	19.9

** = highly significance

1/ = Means within columns in similar letters were not significantly different at the 5% level by Duncan's multiple range test.

After 2 months in nursery, the heights of those were measured (Figure 7 and Table 6). Oryzalin could inhibit growth of treated plants to nearly 4.13 cm. high which were significantly shorter than the other treatments. This is due to the fact that oryzalin is herbicide which can retard the growth of plants as indicated by Hassawi and Ling (1991).

In the field

After 3 months in nursery, the plants were transplanted to be cultivated in the field at Kasetsart University, Kamphangsaen campus. The survival plants were recorded after 1 month. Only 43.8% oryzalin treated plants were found to survive whereas the control and colchicine treated ones survived 100, 87.5 and 87.5 % respectively.

Table 7 Height of mutants compared to the control in the field.

Treatment	Height (cm.)[1/]					
	1st month	2nd month	3rd month	4th month	5th month	6th month
Control	8.43[b]	15.23[b]	25.58[b]	36.88[b]	57.20[b]	81.90[b]
Z1-6	3.40[a]	4.95[a]	7.57[a]	10.05[a]	14.30[a]	19.20[a]
C3-5	7.78[b]	14.48[b]	25.58[b]	38.50[b]	61.13[b]	79.53[b]
C3-6	8.15[b]	14.55[b]	27.35[b]	39.45[b]	62.33[b]	85.05[b]
F-test	**	**	**	**	**	**
CV(%)	14.3	16.3	15.9	14.1	14.7	13.8

** = highly significance

1/ = Means within columns in similar letters were not significantly different at the 5% level by Duncan's multiple range test.

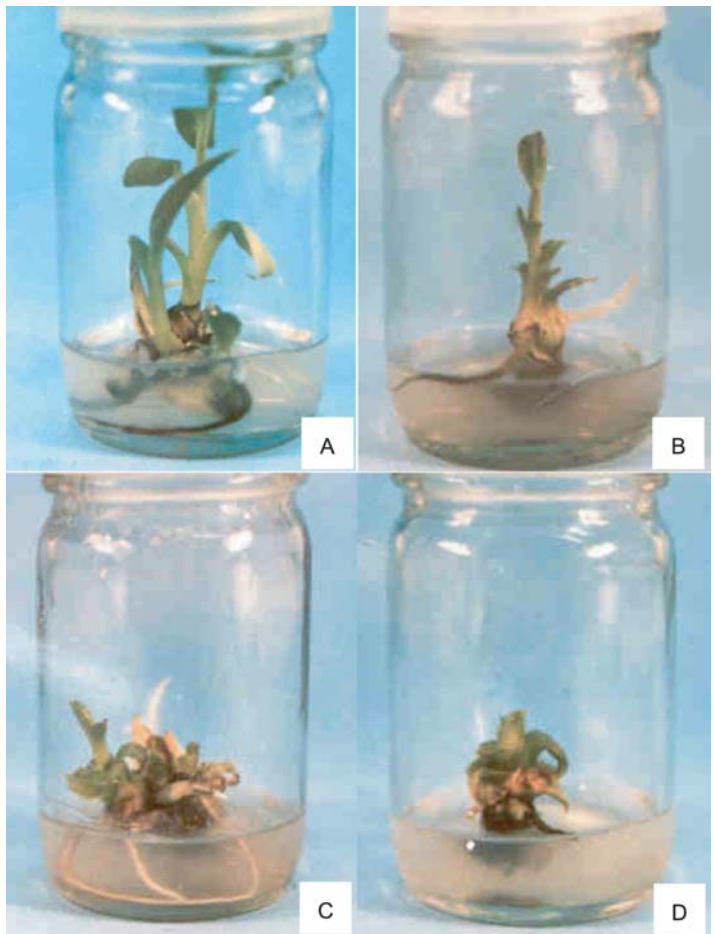

Figure 3 Plantlets in tissue culture.
A. normal B-D. abnormal

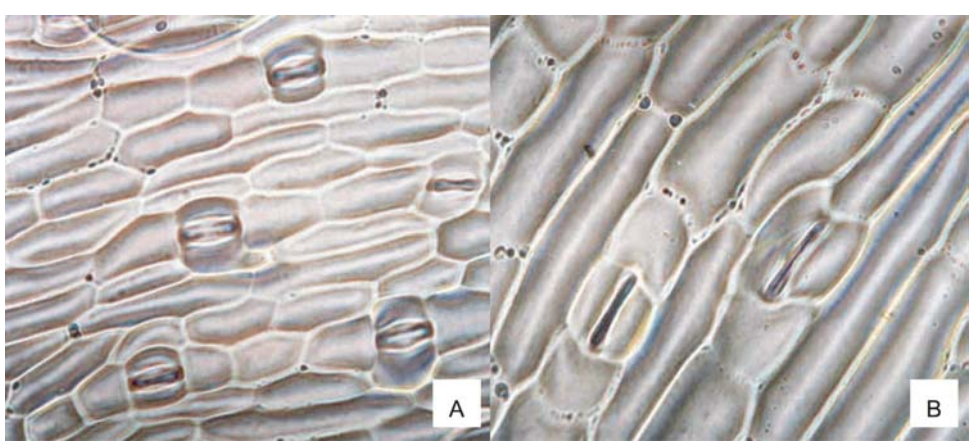

Figure 4 Stomata size (400X).
A. normal plant B. abnormal plant

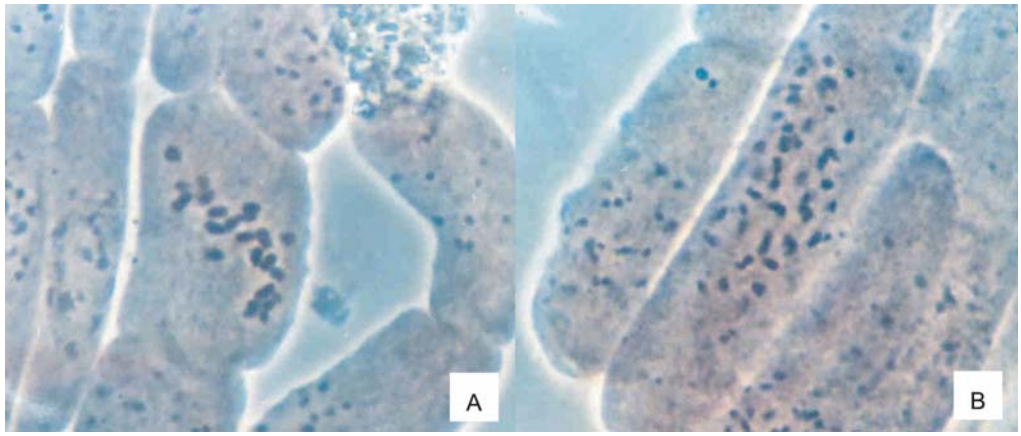

Figure 5 Chromosome number (1850X).
A. 2n=22 B. 2n=44

Figure 6 Control and mutant plantlets in tissue culture.

Figure 7 Control and mutant plants after 2 months in nursery.

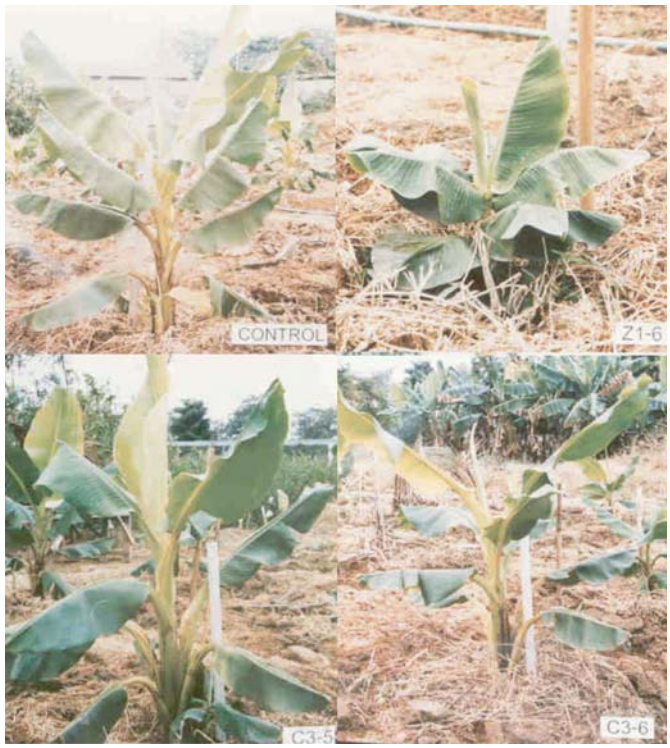

Figure 8 Control and mutant plants in the field.

Figure 9 Bunch of 4X Kluai Khai.

Figure 10 Kluai BEP in 4 inches pot (1 year old).

The heights of plant were measured every month. The oryzalin treated plants were noticed to be at the shortest in every month and were highly significantly different from the other treatments, the same as in nursery. This result was due to the effect of herbicide that retarded growth of broadleaf weeds. The growth of oryzalin treated plants were 3.40, 4.95, 7.57, 10.05, 14.30 and 19.20 cm. at the 1-6th month respectively. On the 6th month, the control was 81.90 cm. while the colchicine treated plants were 79.53 and 85.05 cm. which were not significantly different from one another (Figure 8 and Table 7). The results were similar to the work of Vakili (1967) who studied the growth of 2X and 4X of *Musa balbisiana*.

When the plants were 6 months old in the field, number of leaf, number of sucker and circumference of pseudostem at 10 cm. above the ground were recorded. The circumferences of the oryzalin treated plants were found to be significantly smaller than those of the other treatments (Table 8). Numbers of leaf and sucker were also noticed not to be different.

At six months old, the control was revealed to flower whereas the treated plants had not yet. That could mean the shooting time of treated plants took longer time than the normal ones, which was not good characteristic of banana. It should therefore be discarded.

Following this experiment, the C3-5, C3-6 and Z1-6 were grown for further observing and found that, both of C3 flowered after 1 year. The bunches and fruits were smaller than those of the normal K.Khai as shown in Figure 9. The discard of those plants should obviously be done as suggested. For Z1-6 plant, the growth were very low. Studying on the characteristic of psuedostem, the leaves were also found to be absolutely changed from those of the normal K.Khai. This was due to the fact that oryzalin was a growth inhibitor and antimitotic agent which could retard the growth of plant (Verhoeven *et al.*, 1990; Hassawi and Liang, 1991; Ramula *et al.*, 1991). Z1-6 was found to be very short which was good as ornamental plant (Figure 10). It was then named BEP and the plant was registered at Ministry of Agriculture, Thailand on the year 2000.

Table 8 Circumferences at 10 cm height, numbers of leave and numbers of sucker at 6 months old of mutants compared to the control.

Treatment	Circumference (cm.) 1/	Number of leave	Number of sucker
Control	23.95^{b}	17.58	2.12
Z1-6	15.33^{a}	14.08	1.25
C3-5	23.88^{b}	15.20	1.17
C3-6	24.45^{b}	14.95	1.21
F-test	**	ns	ns
CV(%)	11.4	15.6	56.1

ns = non significance

** = highly significance

1/ = Means within columns in similar letters were not significantly different at the 5% level by Duncan's multiple range test.

CONCLUSION

1. LD_{50} at 2.5, 5.0 and 7.5 hrs of colchicine concentration on Kluai Khai are 0.4, 0.5,0.75 per cent and of oryzalin are 13, 15, 20 mM at 2.5, 5 and 7.5 hrs. of soaking respectively.

2. 1% colchicine at 7.5 hrs. and 45 mM oryzalin at 7.5 hrs. could change the chromosome number of Kluai Khai from 2n = 22 to 2n = 44.

3. The growth of colchicine treated plants were similar to the control but fruiting period was too long and the fruits were quite small.

4. The growth of oryzalin treated plants was very slow, both in the culture media and in the field. Morphology of leaf and psuedostem changed from the normal Kluai Khai. The result was good to be furtherly produced as ornamental plant. It was later registered by the name of BEP.

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Development and Maintenance of Gynoecious Lines of Cucumber (*Cucumis sativus* L.)

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ABSTRACT

The study aimed to develop gynoecious lines of cucumber (*Cucumis sativus* L.) by isolating, selfing and evaluating of selfed progenies from original populations and to maintain these lines by using chemicals to induce staminate flower for genetically selfing. Two F_1 cucumber cultivars of long type (Seminis-1 and Seminis-2) and three short type (Siminis-3, Micro-c and Bingo) from Thailand and three OP cultivars of long type (Long Green, Kusle and Bhakatpur Local) from Nepal were evaluated for gynoecious sex expression. Among the F_1 populations, only Bingo expressed gynoecious type for 5% and the rest were predominantly gynoecious sex type. Open-pollinated populations only expressed monoecious sex type. During the process of gynoecious line development through inbreeding and plant-to-row selection it took three consecutive selfing generation (S_3) for complete gynoecious development of SE1-G (long) and SE3-G (short) lines which were isolated from the original population of Seminis-1 and Seminis-3 respectively. Among the used chemical, silver nitrate ($AgNo_3$) was found statistically significantly superior over gibberellic acid (GA_3) and silver thiosulfate ($Ag(S_2O_2)_2$) for effective staminate flower induction for the maintenance of gynoecious lines. The highest sex ratio (M/F) 0.80:1 in SE1-G line and 0.89:1 in SE3-G line was observed by first lateral chemical application from the chemical silver nitrate 400 and 300 ppm applied twice respectively which confirm the highest possibility of flower synchronization.

Key words: sex expression, gynoecious, development, maintenance and cucumber

INTRODUCTION

Hybrid varieties of cucumber are predominantly used in the production system of many developed and developing countries. The proportion of hybrid varieties is continuously increasing and thus, gynoecious lines of cucumber are important for hybrid seed production.

Sex inheritance plays an important role in cucumber hybrid breeding. Several researchers have worked on sex expression of cucumbers and reported that it was genetically determined but could be modified by growth substance application and also environmental factors (Krishnamoorthy, 1975; Lower and Edwards, 1986; Kalloo, 1988). Considering the above factors many combinations of hybrid seed production have been proposed and recommended using gynoecious parents. Despite of all efforts, sex expression variation of commercial hybrids is still a problem in cucumber cultivation (Lower and Edwards, 1986).

At present, interesting in stabilizing the gynoecious character and development of stable gynoecious inbred parents have been intensified

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and become a common goal of numerous hybrid breeding programs. Therefore this study was conducted with the following objectives.

1. To study on the consisting of gynoecious plants in promising population.
2. To isolate gynoecious lines from the promising population.
3. To find out and appropriate chemical and concentration for staminate flower induction on gynoecious lines for the purpose of line maintenance.
4. To search an effective method in line maintenance through selfing pollination.

MATERIALS AND METHODS

All the experiments under this study were conducted at Horticulture experiment field of Kasetsart University during December 1999 to February 2001.

1. Sex expression in original population and line isolation

Five cultivars of long cucumber namely Seminis-1 (SE1) and Seminis-2 (SE2) and Long Green (LG), Kusle (K) and Bhakatpur Local (BL) together with three short cultivars such as Seminis-3 (SE3), Micro-c (MC) and Bingo (BG) were evaluated the sex expression. Cucumber plants were grown in 15 cm plastic pots for a preliminary study. Bingo and Seminis-2 were sown on December 15, 1999 Bhakatpur Local, Kusle and Long Green were sown on January 8,2000. Seminis-1, Seminis-3 and Micro-c were sown on February 2, 2000

Plants were examined pistillate expression at first five nodes and node order (Lower and Edwards, 1986) and individual plant was classified and recorded gynoecious, predominantly gynoecious and monoecious sex type percentage (Staub and Kipper, 1985; Staub et al. 1986 and Steele and Torrie, 1969).

The outstanding populations of long and short cucumber were considered to isolate S_1 lines. The isolation of gynoecious and predominantly gynoecious lines were treated with 200-400 ppm silver nitrate ($AgNo_3$) to induce staminate flowers to facilitate genetically selfing. Selected lines were isolated by plant-to-row selection up to S_2 and S_3 selfing generations and statistically analyzed by Chi-square contingency tables (Steele and Torrie, 1969), in order to obtain 100% gynoecious plants in the population and were used for succeeding experiments.

2. Chemical induction of staminate flowers for the maintenance of gynoecious lines

Thirteen treatments including control treatment were evaluated under randomized block design in four replications. Two concentrations of cach chemical i.e. GA_3 (gibberellic acid) 500 and 1000 ppm, SN (silver nitrate) 200 and 400 ppm and STS (silver thiosulfate) 6 and 9 mM were applied once or twice and only water was the control treatment. The first chemical application started at 23 days after sowing and subsequent application at 7-day interval. All chemical solutions were prepared with deionized water and applied about 5 ml./plant.

Two plants for each replication were grown in each 15cm plastic pot. Seeds of these plants were sown on November 22, 2000. Number of induced staminate flowers per plant, the position of the first staminate flowers, days to flowering and phytotoxicity caused by chemical applications were recorded for analysis. Phytotoxic rating was respectively scored as <1. 1-2, 2-3 and > 3-4 for less toxic, mild toxic, moderately toxic and severely toxic. Mean separation of each observation was statistically analyzed by Duncan's new multipte-range test.

3. Chemical application on first lateral of gynoecious lines for flower synchronisation

Seven treatments including control with SE1-G and SE3-G lines of cucumber were evaluated under this experiment. The SN 400 and 300 ppm were applied once and twice at 7-day interval and

SN 200 ppm was applied twice and four times at subsequent 3-day intervals on first lateral axes of the plants. The experiment was laid out as a randomized compete block design with four replications.

Two plants in each replication were grown in 15 cm plastic pot. Secds were sown on Dec 7, 2000 and plants were decapitated at three weeks after sowing in order to promote branching. The first application was done on Jan 2, 2000. The male and female flowers bloomed per day on treated and untreated axes were separately recorded and sex ratio was calculated for maximum flower synchronization.

RESULTS

1. Studies of original population and line isolation

1.1 Population comprising gynoecious plants

Among long cucumber cultivars, the most deserving population was Seminis-1 which expressed 35% predominantly gynoecious sex type and the remaining cultivars only expressed 100% monoecious sex type (Table 1). All short cucumber cultivars comprised predominantly gynoecious sex type and only Bingo expressed 5% gynoecious sex type (Table 1).

1.2 The increasing percentage of gynoecious plants over generations

Significantly increasing of gynoecious plant percentage S_2 generation was observed in all the cultivars (Table 2). The two highest pencentages were 85% in SE1-S_2 (long cucumber) and 95% in SE3-S_2 (short cucumber) from original populations 35 and 30% respectively (Table 2).

The 100% gynoecious populations in S_3 generation were obtained as SE1-G and SE3-G lines (Figure 1). Gynoecious plants of these lines were maintained by selfing and the bulked seeds of each S_3 line was used for the experiment on gynoecious line maintenance.

1. Mean separation in column by "chi-square contingency" test indicates differences between generations in gynoecious plants at 95% confidence.

2. Percentage of gynoecious plants in S2 selfing generation was found significant increased at 95% confidence

3. Within the bracket first figure is % of gynoecious plants and second is predominantly gynoecious plants. Both sex types formed into one gynoecious sex type for the analysis.

Table 1 Percentage of sex types in the original population of cucumber.

Source	Status	Type	Sowing date	Total no. of plants	Sex types		
					Gynoecious (%)	Predominantly gynoecious (%)	Monoecious (%)
Seminis-1 (SE1)	F_1	Long	21.02.00	20	0	35	65
Seminis-2 (SE 2)	F_1	Long	15.12.99	20	0	0	100
Long Green (LG)	O_p	Long	8.01.00	20	0	0	100
Kusle (K)	O_p	Long	8.01.00	20	0	0	100
Bhakatpur Local (BL)	O_p	Long	8.01.00	20	0	0	100
Seminis-3 (SE 3)	F_1	Short	21.02.00	20	0	0	100
Bingo (BG)	F_1	Short	15.12.99	20	5	10	85
Micro-c (MC)	F_1	Short	21.02.00	20	0	25	75

Table 2 Increasing percentage of gynoecious plants over selfing generations in four cultivars of cucumber.

Selfing generations	Long cucumber % of gynoecious plants		Short cucumber % of gynoecious plants	
	Seminis-1 (F1)	Long Green (OP)	Seminis-3 (F1)	Micro-c (F1)
S_0	35[a]	0[a]	30	25
	(0+35)		(0+30)	(0+25)
S_1	45[a]	0[a]	35	30
	(10+35)		(15+20)	(0+30)
S_2	85[b]	8[b]	95	50
	(75+10)		(85+10)	(20+30)

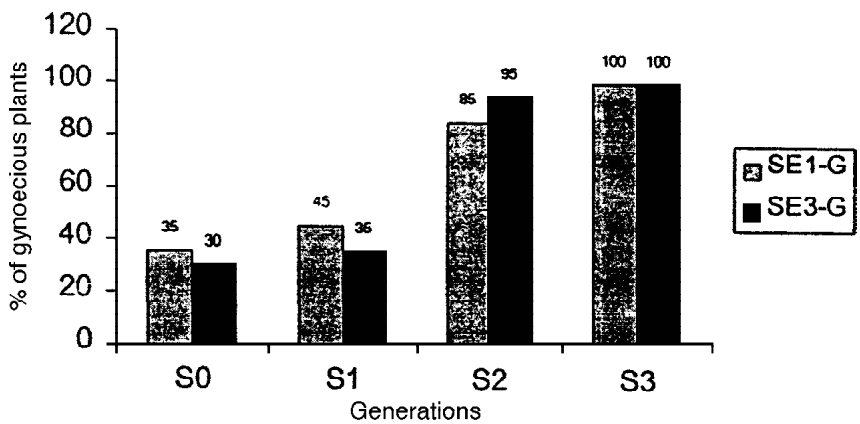

Figure 1 Gynoecious plant percentage of selfing generations in the SE1-G and SE3-G lines of cucumber.

2. Chemical induction of staminate flower in two gynoecious lines

2.1 The case of long cucumber (SE1-G line)

2.1.1 Total staminate flower induction.

Significant difference among the treatments was observed. All the cucumber plants treated with silver nitrate induced more staminate flowers than treatments treated with silver thiosulfate and gibberellic acid (Table 3). Among the silver nitrate treatment 400 ppm applied once induced highest number of staminate flower per plant (34.87) and found no significant difference with 200 ppm applied twice but significantly superior over the rest of the treatments (Table 4).

2.1.2 Days to flowering

Earlier days to male flowering (30.75) of the main axis was recorded in plants treated with SN 400 ppm applied once and found significantly superior over all the treatments except SN 200 and 400 ppm applied twice. Significant difference was not observed among the treatments for days to female flowering (Table 3)

2.1.3 Node number of first male flowering

Plants treated with STS 9 mM applied twice formed staminate flowers at lowest node (1.37) of the main axis and found no significant difference with treatments SN 400 ppm applied once and twice and STS 9 mM applied once but superior over the rest of the treatments (Table 3).

2.1.4 Phytotoxic rating

All the treatments under STS caused more phytotoxic reaction than other treatments. Treatments under GA3 caused less phytotoxic reactions, and SN had the moderate phytotoxic reaction in general (Table 3).

2.2 The case of short cucumber (SE3-G line)

2.1.1 Total staminate flower induction

Results showed the highest significant difference among the treatments for total staminate induction per plant. All the treatments of silver nitrate induced higher staminate flower than others. Among the treatment under silver nitrate, 200 ppm applied twice induced highest (42.37) staminate flowers per plant and found superior over all the treatments (Table 4).

2.1.2 Days to flowering

Earlier days to male flowering (30.0) was observed in plants treated with SN 400 ppm applied once and was not statistically different with treatment SN 200 ppm applied twice but was superior over all the rest treatments (Table 4). No significant difference was recorded among the treatments for days to female flowering (Table 4).

2.2.3 Node number of first male flowering

Treatment STS 9 mM formed the staminate flowers at lowest nodes (3.37) and found no statistical difference with treatments STS 9 mM applied once and SN 400 ppm applied twice but superior over others (Table 4).

2.2.4 Phytotoxic rating

All the treatments under STS caused severely phytotoxic reaction. Treatments under GA_3 had less phytotoxic reactions and SN had moderately (Table 4).

Table 3 Staminate flowers per plant, days to flowering, node number at which first male flower appeared and phytotoxic rating in SE1-G line.

Chemical	Total staminate flowers per plant	Days to flowering in the main axis		Node of first male flowering in main axis	Phytotoxic rating
		Male	Female		
GA 500 ppm once	2.12^{f}	36.12^{d}	27.25^{a}	14.50^{c}	0^{a}
GA 500 ppm twice	2.25^{f}	35.37^{d}	27.75^{a}	12.12^{d}	0.25^{ab}
GA 1000 ppm once	2.37^{f}	35.12^{d}	27.37^{a}	14.50^{c}	0.37^{ab}
GA 1000 ppm twice	4.50^{f}	33.25^{c}	27.38^{a}	11.25^{d}	0.62^{b}
SN 200 ppm once	30.50^{bc}	32.75^{bc}	27.75^{a}	3.62^{bc}	0.75^{bc}
SN 200 ppm twice	32.87^{ab}	31.12^{ab}	27.50^{a}	4.75^{c}	0.87^{c}
SN 400 ppm once	34.87^{a}	30.75^{a}	27.25^{a}	2.62^{ab}	1.12^{c}
SN 400 ppm twice	31.50^{b}	32.25^{abc}	28.0^{a}	1.75^{a}	2.0^{b}
STS 6 mM once	26.0^{dc}	35.25^{d}	28.62^{a}	5.0^{c}	2.75^{c}
STS 6 mM twice	26.62^{dc}	36.25^{d}	27.75^{a}	3.12^{b}	3.25^{cf}
STS 9 mM once	28.12^{cd}	36.87^{d}	27.25^{a}	2.25^{ab}	3.87^{fg}
STS 9 mM twice	23.75^{c}	36.62^{d}	27.12^{a}	1.37^{a}	4.0^{g}
Control	0^{g}	0^{c}	27.12^{a}	0^{f}	0^{a}
CV	10.46	3.3	13.5	23.3	19.4

The same letter in a column are not significant difference at 5% by DMRT.

3. Chemical application on the first lateral of two gynoecious lines for flower synchronisation

3.1 The case of SE1-G (long cucumber)

Male flower induction

Treatment SN 400 ppm applied twice induced highest number of male flower per plant (21.62) on the treated axis and found statistically different and outstanding over all the treatments (Table 5). Significant difference was also observed among the treatments for total male flower induction and SN 400 ppm applied twice had the highest (25.0) male flower per plant and found significantly superior over others (Table 5). Control treatment did not produce staminate flowers.

Female flowers production

No significant difference was observed among the treatments for female flower production on the untreated axes. The lowest female flower on the treated axis (4.25) was obtained from the treatment SN 400 ppm applied twice and the highest (26.50) from the control treatment. The control treatment also produced highest number of total female flowers per plant (54.75) and lowest number (30.87) was obtained by SN 400 ppm applied twice (Table 5).

Sex ratio (M/F)

The maximum sex ratio (M/F) (0.80:1) was obtained from SN 400 ppm applied twice and found significantly difference over all the treatments assigned (Table 5). So there was highest possibility for synchronization of staminate and pistillate flowering.

3.2 The case of SE3-G (Short cucumber)

Male flower induction

The highest number of male flowers per plant (21.12) on the treated axis was induced by

Table 4 Staminate flowers per plant, days to flowering, node number at which first male flower appeared and phytotoxic rating in SE_3-G line.

Chemical	Total staminate flowers per plant	Days to flowering in main axis		Node of first male flowering in main axis	Phytotoxic rating
		Male	Female		
GA 500 ppm once	0.50^{h}	36.25^{c}	25.87^{a}	14.87^{d}	0.25^{ab}
GA 500 ppm twice	3.50^{g}	32.75^{cd}	25.75^{a}	10.87^{c}	0.50^{abc}
GA 1000 ppm once	4.62^{fg}	33.0^{cd}	26.50^{a}	11.0^{c}	0.75^{bc}
GA 1000 ppm twice	6.12^{fg}	31.75^{bc}	26.25^{a}	10.15^{c}	1.12^{cd}
SN 200 ppm once	33.37^{c}	31.62^{ab}	27.12^{a}	5.25^{b}	1.25^{cd}
SN 200 ppm twice	42.37^{a}	30.62^{bc}	27.0^{a}	5.37^{b}	1.75^{dc}
SN 400 ppm once	38.75^{b}	30.0^{a}	26.62^{a}	5.0^{b}	2.25^{cb}
SN 400 ppm twice	33.50^{c}	33.0^{cd}	25.75^{a}	4.25^{ab}	3.0^{bg}
STS 6 mM once	7.0^{cf}	33.75^{d}	26.62^{a}	5.37^{b}	3.37^{gh}
STS 6 mM twice	4.25^{fg}	35.25^{c}	26.12^{a}	5.12^{b}	3.75^{gh}
STS 9 mM once	13.37^{d}	35.25^{c}	25.75^{a}	4.66^{ab}	4.0^{h}
STS 9 mM twice	9.37^{c}	35.50^{c}	26.37^{a}	3.37^{a}	4.0^{h}
Control	0^{h}	0^{f}	26.12^{a}	0^{c}	0^{a}
CV	12.42	3.5	3.18	15.6	21.5

The same letter in a column are not significant difference at 5% by DMRT.

treatment SN 300 ppm applied twice and found statistically superior over all the treatments (Table 6). Total male flower per plant was also observed highest (23.87) by above treatment and found at par with the treatment SN 400 ppm applied one but superior over others. (Table 6).

Female flowers production

The lowest number of female flower on the treatment axis (2.88) was obtained from the treatment SN 300 ppm applied twice and the highest (24.5) from the control treatment. Control treatment also produced highest number of female flower per

Table 5 Sex expression and sex ratio as expressed by number of staminate and pistillate flowers on treated and untreated axes of long cucumber (SE1-G line).

Treatment	Treated axes		Untreated axes		Total		Sex ratio
	Male flowers (No.)	Female flowers (No.)	Male flowers (No.)	Female flowers (No.)	Male flowers (No.)	Female flowers (No.)	M/F
SN 400 ppm once	14.25^{b}	10.62^{b}	3.75^{a}	28.87^{a}	18.0^{b}	39.49^{bc}	0.45:1^{bc}
SN 400 ppm twice	21.62^{a}	4.25^{a}	3.37^{a}	26.62^{a}	25.0^{a}	30.87^{c}	0.80:1^{a}
SN 300 ppm once	13.50^{b}	10.25^{b}	3.75^{a}	27.25^{a}	17.25^{b}	37.50^{cd}	0.46:1^{bc}
SN 300 ppm twice	16.37^{b}	9.12^{b}	3.25^{a}	25.50^{a}	19.64^{b}	34.62^{dc}	0.57:1^{b}
SN 200 ppm twice	5.37^{d}	17.87^{c}	2.0^{a}	24.87^{a}	7.37^{d}	42.14^{b}	0.17:1^{a}
SN 200 ppm 4 times	9.87^{c}	12.87^{b}	2.37^{a}	27.25^{a}	12.24^{c}	40.12^{bc}	0.30:1^{cd}
Control (H2O)	0^{c}	26.50^{d}	0^{b}	28.25^{a}	0^{c}	54.75^{a}	0:1^{c}
CV	12.5	22.6	29.5	14.1	15.12	12.7	19.6

The same letter in a column is not significant difference at 5% by DMRT.

Table 6 Sex expression and sex ratio as expressed by number of staminate and pistillate flowers on treated and untreated axes of short cucumber (SE3-G line).

Treatment	Treated axes		Untreated axes		Total		Sex ratio
	Male flowers (No.)	Female flowers (No.)	Male flowers (No.)	Female flowers (No.)	Male flowers (No.)	Female flowers (No.)	M/F
SN 400 ppm once	164.75^{b}	8.75^{b}	3.75^{a}	22.0^{a}Ÿ	20.5^{ab}	30.75^{c}	0.67:1^{ab}
SN 400 ppm twice	14.50^{bc}	10.37^{b}	3.50^{a}	25.37^{a}Ÿ	18.0^{bc}	35.75^{b}	0.5:1^{bc}
SN 300 ppm once	12.50^{cd}	8.75^{b}	2.0^{a}	23.25^{a}Ÿ	14.50^{d}	32.0^{c}	0.45:1^{c}
SN 300 ppm twice	21.12^{a}	2.88^{a}	2.75^{a}	23.87^{a}Ÿ	23.87^{a}	26.75^{d}	0.89:1^{a}
SN 200 ppm twice	10.60^{d}	10.62^{b}	2.37^{a}	22.87^{a}Ÿ	12.97^{d}	33.49^{bc}	0.38:^{c}
SN 200 ppm 4 times	14.25^{bc}	10.75^{b}	2.50^{a}	25.75^{a}Ÿ	16.75^{cd}	36.50^{b}	0.46:1^{c}
Control (H_2O)	0^{c}	24.5^{c}	0^{b}	26.0^{a}Ÿ	0^{c}	50.50^{a}	0:1^{d}
CV	14.3	15.9	18.8	17.9Ÿ	15.0	15.3	26.5

The same letter in a column is not significant difference at 5% by DMRT.

plant (50.50) and the treatment SN 300 ppm applied twice had the lowest number of female flowers per plant (26.75) (Table 6).

Sex ratio (M/F)

The maximum sex ratio (M/F) (0.89:1) was received from treatment SN 300 ppm applied twice and found no statistical difference with treatment SN 400 ppm applied once but superior over others (Table 6). So, there was highest possibility for synchronization of staminate and pistillate flowering.

DISCUSSION

High female population consisting higher percentage of predominantly gynoecious and the gynoecious plants were observed in the F_1 population as compared to the OP population. It is because of F_1 are the developed population from gynoecious parents.

Selfed progenies of predominantly gynoecious also that of gynoecious plants when followed by plant-to-row selection system had increased percentage of gynoecious plants in SE1 and SE3 populations. It is obvious that by inbreeding and plant-to-row selection the traits become fixed and the progeny or line approach uniformity (Agrawal, 1998)

The most effective staminate flower induction was observed on gynoecious lines treated with silver nitrate rather than silver thiosulfate and gibbereillc acid (GA_3). As silver ion is a potent anti-ethylene agent in cucumber and tomato (Elmo, 1976). It inhibits synthesis of ethylene and thus induce staminate flowers (Krishnamoorthy, 1975). Though GA_3 also inhibit the endogenous ethylene level through auxin, the induction may be more in silver ion because silver nitrate is a chemical and gibberellic acid is a kind of growth regulator (Tolla and Peterson, 1979). Lower *et al.* (1978) and Nijs and Wisser (1979) also reported for less effectiveness of GA_3 than silver nitrate.

Despite the staminate flower formed in the lowest nodes, the days to flowering was observed later in plants treated with silver thiosulfate compared with silver nitrate and gibberellic acid. This is mainly associated with the severe phytotoxic reaction causing plants regain growth quite late and thus affected days to flowering.

Chemical application on the first lateral axis of the plant gave high male and female sex ratio in both treated lines. Results clearly indicated that it is only due to the treated axis induced more staminate flowers while the untreated carried all female flowers.

CONCLUSION

1. Higher percentage of predominantly gynoecious plants under this study was observed in F_1 population as compared to OP populations.
2. Homozygous gynoecious line could be isolated by consecutively selfing with plant-to-row selection. The gynoecious SE1-G and SE3-G lines were successfully developed under this study.
3. Silver nitrate ($AgNo_3$) was an appropriate chemical for staminate flower induction on gynoecious cucumber but the response of it's concentration depends upon cucumber genotypes and environmental condition.
4. First lateral axis chemical application was an effective method for flower synchronization in gynoecious line maintenance through selfing pollination. The SN concentration of 400 and 300 ppm which were two times applied to SE1-G long cucumber and SE3-G short cucumber respectively were found effective concentrations in the line maintenance.

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Hybrid Improvement of Chinese Radish

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ABSTRACT

Nine inbred lines of Chinese radish were investigated for their self-incompatibility levels using fluorescent microscope technique and seed set analysis. The results showed that most of the inbred lines were self-incompatible. They were crossed in all combinations to be selected for the best hybrid variety. From varietal trial and ability to set seeds, it was found that hybrid 27 x 18 and some of hybrid 77 x 18 had good horticultural characteristics such as uniformity, dark green leaf without hair, cylindrical shape, smooth skin, firm texture of root and root weight of about 360-400 g. These characteristics are market acceptable. Root yield of these hybrids in the previous experiments was a lot higher than marketed varieties. The hybrid varieties should be further tested in farmer's field.

Key words: self-incompatibility, cross in all combinations and horticultural characteristic.

INTRODUCTION

Chinese radish *Raphanus sativus* var. *Longipinnatus* L., is one of the popular vegetables in Thailand. It can be used for making varieties of food, both fresh and pickle. F1 hybrid seed of Chinese radish is more popular than open-pollinated seed, but F1 hybrid seed is more expensive than the open-pollinated one. However, F1 hybrid varieties give higher yield and better quality than open-pollinated varieties. All F1 hybrid seeds were imported. If male and female lines for the F1 hybrid seeds could be produced in the country, the price of F1 hybrid seeds would be decreased.

Chinese radish is a cross-pollinated crop and it has a sporophytical reaction for self-incompatibility. The self-incompatibility is controlled by S gene which has many alleles (Haruta, 1962). It was used in F1 hybrid seed production by developing male and female parental lines which were self-incompatible lines. There are two ways of checking self-incompatibility ; seed set analysis (Shinohara, 1981) and fluorescent microscope technique (Kho and Baer, 1968). The first method is a field test by selfing unopened flowers and opened flowers within the same inflorescence. The method is a time consuming, but yielding reliable results. The second method, fluorescent microscope technique, is a laboratory test by observing pollen tubes in styles of female flowers after selfed-or crossed-pollination. The later method is fast but the results are not as reliable as the former.

Varieties of Chinese radish used in this studies were supported by the International Development Research Centre (IDRC) through the Faculty of Agriculture, Chiang Mai University. Originally, they were imported from Japan, Taiwan

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and Korea, and also found as local varieties in the country. They were tested at a few locations for their adaptability and performances. Good varieties were selected from these experimental trials (Wivutvongvana, 1987). The selected varieties were self-pollinated for a few generations using a bud pollination technique (Wivutvongvana, 1987). Inbred lines obtained were tested for their self-incompatibility levels using seed set analysis and fluorescent microscope techniques. The inbred lines which had high levels of self-incompatibility were lines No. 27, 30, 54, 59, 62, 75 and 84 (Nikornpun and Tan-Kim-Yong, 1990-91). These lines were used in the experiments as parental lines.

MATERIALS AND METHODS

Inbred lines of Chinese radish which were developed at Chiang Mai University were used in the experiments. They had high level of self-incompatibility as mentioned above. However, they were tested again for their self-incompatibility levels. Two methods of testing were used : seed set analysis and fluorescent microscope technique. The former method is a common method for testing self-incompatibility. Three to four inflorescences per plant were bagged before flower opened. Three to five days later, the bags were taken off. Unopened and opened flowers were marked with a thread. Emasculation was needed for all flowers tested. They were pollinated with pollen of the same plant. After pollination, the inflorescences were bagged for two weeks. About 10 plants were used for an inbred line, then the pods were counted, the young flowers should be able to bear seeds while the opened flowers should not be able to bear seeds if they are self-incompatible.

Fluorescent microscope technique was used to test self-incompatibility levels (Kho and Baer, 1968). Unopened and opened flowers were taken from the same plant. They were put on a slide in a petridish containing potassium dichromate solution. The potassium dichromate ($K_2Cr_2O_7$) when boiled and kept in a tight chamber, will bring about the level of humidity to 98% RH. The flowers were emasculated before pollinated with pollen from the same plants. Another opened flower was required on the slide, it was cross-pollinated with pollen from different varieties, and was used as a control flower. The flowers were kept in the petridish for one night. Styles of these flowers were sectioned in sodium hydroxide (NaOH) which was heated to 60°C for one hour. Then the styles were stained with 0.2 % aniline blue in 2% potassium phosphate ($K_3PO_4.3H_2O$) for 24 hours. They were squashed on a slide which had a drop of glycerol. Pollen tubes were counted using a fluorescent microscope.

Inbred lines No. 18, 27, 56, 59, 62, 75 and 77 were vernalized for 15 day at 10°C. They were grown in a controlled room at 25 °C under continuous light and 80% relative humidity. They were cross-pollinated in all combination by hands. F1 hybrid seeds were grown in winter 1996 by one seed company in Chiang Mai. Thirty six commercial varieties of Chinese radish were in a trial as control varieties. These varieties were obtained from local seed companies and seed companies abroad. They were grown in comparison with the F1 hybrid varieties, randomly grown for two replications without experimental design. After 48-55 days, horticultural characteristics of a plant were recorded (e.g. plant growth, plant uniformity, leaf color, leaf margin, days to harvest, yield, root shape, root position in soil, root skin, root size, lateral root and root texture, disease, and over all rating).

RESULTS

Self-incompatibility levels of parental lines as evaluated by seed set analysis and fluorescent microscope techniques showed that all lines were self-incompatible. However, different levels of self-incompatibility were obtained (Table 1). Strong self-incompatible lines showed no pollen tube in female style (Figure 1). While weak self-incompatible lines showed some pollen tubes in the

Table 1 Seed yield and incompatibility levels of Chinese radish by seed set analysis and fluorescent microscope techniques.

Cross	Seed weight (g/pod)	SSA	FM	Cross	Seed weight (g/pod)	SSA	FM
18 x 27	0.34	SixSI	SIxWSI	59 x 62	0.11	SIxSI	SIxSI
18 x 56	0.59	SIxWSI	SIxWSI	59 x 75	0.21	SIxSI	SIxSI
18 x 59	0.13	SIxSI	SIxSI	59 x 77	0.10	SIxSI	SIxWSI
18 x 62	0.32	SIxSI	SIxSI	62 x 18	0.12	SIxSI	SIxSI
18 x 75	1.43	SIxSI	SIxSI	62 x 27	0.07	SIxSI	SIxWSI
18 x 77	0.40	SIxSI	SIxWSI	62 x 56	0.15	SIxWSI	SIxWSI
27 x 18	0.68	SIxSI	WSIxSI	62 x 59	0.09	SIxSI	SIxSI
27 x 56	0.43	SIxWSI	WSIxWSI	62 x 75	0.09	SIxSI	SIxSI
27 x 59	0.59	SIxSI	WSIxSI	62 x 77	0.08	SIxWSI	SIxWSI
27 x 62	0.22	SIxSI	WSIxSI	75 x 18	0.88	SIxSI	SIxSI
27 x 75	0.29	SIxSI	WSIxSI	75 x 27	-	SIxSI	SIxWSI
27 x 77	0.22	SIxSI	WSIxWSI	75 x 56	-	SIxWSI	SIxWSI
56 x 18	0.49	WSIxSI	WSIxSI	75 x 59	-	SIxSI	SIxSI
56 x 27	-	WSIxSI	WSIxWSI	75 x 62	-	SIxSI	SIxSI
56 x 59	-	WSIxSI	WSIxSI	75 x 77	-	SIxSI	SIxWSI
56 x 62	-	WSIxSI	WSIxSI	77 x 18	0.14	SIxSI	WSIxSI
56 x 75	0.51	WSIxSI	WSIxSI	77 x 27	0.16	SIxSI	WSIxWSI
56 x 77	0.14	WSIxSI	WSIxWSI	77 x 56	0.13	SIxWSI	WSIxWSI
59 x 18	0.09	SIxSI	SIxSI	77 x 59	0.34	SIxSI	WSIxSI
59 x 27	0.08	SIxSI	SIxWSI	77 x 62	0.16	SIxSI	WSIxSI
59 x 56	0.25	SIxWSI	SIxWSI	77 x 75	0.14	SIxSI	WSIxSI

SSA : Seed set analysis
FM : Fluorescent microscope
SI : Self-incompatibility
WSI : Weak self-incompatibility.

style (Figure 2).

Several lines had strong self-incompatibility as tested with both methods such as No. 18, 59, 62 and 75. Two varieties or lines were self-incompatible when tested by seed set analysis and they were weak self-incompatible when tested by fluorescent microscope technique such as No. 27 and 77. The line which was weak self-incompatible when tested with both methods was No. 56.

The inbred lines were crossed in all combinations. Most crosses yielded some seed as shown in Table 1. Some of these F1 hybrid seeds were further tested in the field using commercial varieties as control varieties. Horticultural characteristics of these hybrid and control varieties were recorded. Root yield was not recorded. However, it was shown in previous experiments that the hybrid varieties yielded higher than many control varieties (Nikornpun, 1988-92). They showed 1-3 times higher than Everest variety which

was a hybrid variety. They also showed 0.5-3 times higher than Jiatai 1 and 2 which were open-pollinated varieties (Nikornpun, 1995).

Not all crosses were evaluated for their horticultural characteristics (Table 2). Among F1 hybrid varieties tested, only hybrids of 27 x 18 (Figure 3) and some hybrids of 77 x 18 were acceptable. Other hybrid varieties were not acceptable due to poor plant performances, especially, short roots. Good F1 hybrid varieties were 27-1-3 x 18-1, 27-1-3 x 18-2, 27-1-3 x 18-3, 27-1-3 x 18-6, 77-2 x 18-11 and 77-4 x 18-7. These varieties should be tested again in farmer's field.

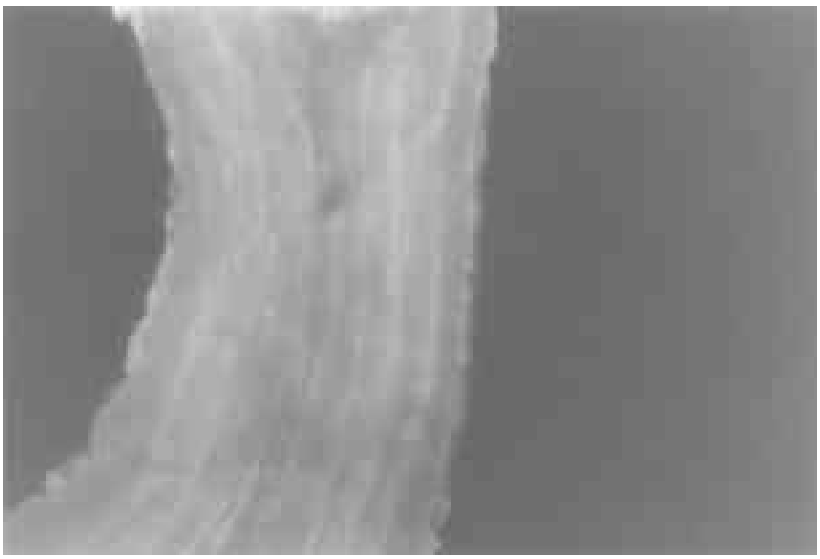

Figure 1 Style of opened flower which was self-incompatible, showed no pollen tube.

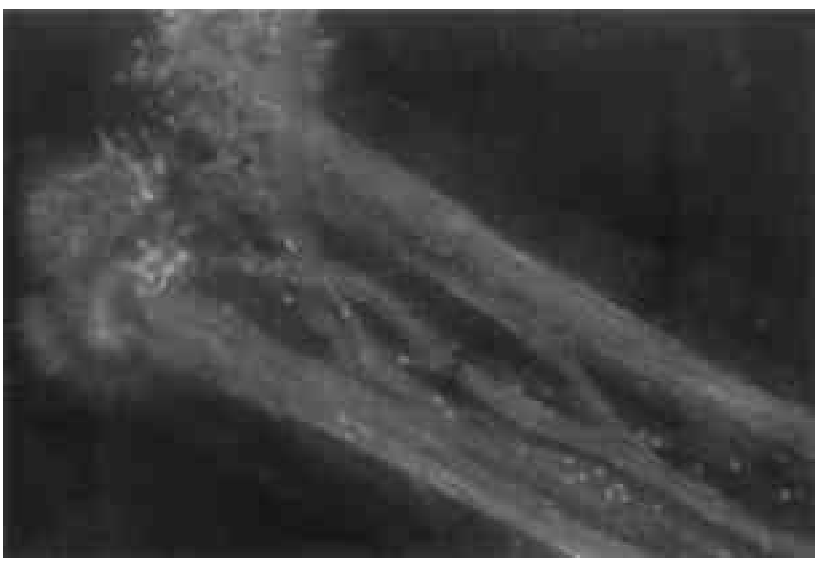

Figure 2 Style of opened flower which was weak self- incompatible, showed some pollen tubes.

Table 2 Horticultural characteristics of F1 hybrid Chinese radish and control varieties.

Cross	Growth	Uniformity	Leaf			Days to Harvest
			Color	Shape	Spine	
Hybrid						
27-1-3x18-1	Extremely vigorous	Uniform	Green	Lyrate	None	40
27-1-3x18-2	Extremely vigorous	Uniform	Dark green	Entire	None	40
27-1-3x18-3	Extremely vigorous	Uniform	Dark green	Lyrate	None	40
27-1-3x18-6	Extremely vigorous	Uniform	Dark green	Lyrate	None	40
27-1-3x18-7	Extremely vigorous	Uniform	Dark green	Lyrate	None	40
59-10x18-1	Extremely vigorous	Variation	Dark green	Entire	Few	45
59-10x18-2	Extremely vigorous	Partly uniform	Green	Entire	Few	40
59-10x18-5	Extremely vigorous	Variation	Green	Entire	Few	40
62-2x18-1	Extremely vigorous	Variation	Dark green	Entire	Few	45
62-2x18-5	Extremely vigorous	Variation	Green	Entire	Few	45
62-2x18-6	Extremely vigorous	Variation	Green	Entire	None	40
77-2x18-1	Extremely vigorous	Variation	Green	Serrate	Few	40
77-2x18-2	Extremely vigorous	Variation	Green	Serrate	Few	40
77-2x18-11	Extremely vigorous	Uniform	Green	Serrate	Few	40
77-4x18-7	Extremely vigorous	Uniform	Green	Serrate	Few	40
Control						
55 Days, CT	Vigorous	Variation	Green	Serrate	-	40
A-1, Fighter car	Vigorous	Variation	Dark green	Entire	None	40
Himalai, Thepwatana	Extreme Vigorous	Uniform	Dark green	Entire	None	40

Table 2 Contnued.

Cross	Root									
	Shape	Position in soil	Skin surface	Weight (g)	Width (cm)	Length (cm)	Scar	Lateral root	Fresh texture	Overal rating
Hybrid										
27-1-3x18-1	Cylindrical	Half buried	Smooth	450	3.7-4.3	30	Narrow	More than half	Firm	Very good
27-1-3x18-2	Cylindrical	Mostly buried	Smooth	400	3.3-4	37	Absent	More than half	Firm	Good
27-1-3x18-3	Cylindrical	Mostly buried	Smooth	450	3.4-4	30	Wide	More than half	Firm	Very good
27-1-3x18-6	Cylindrical	Mostly buried	Smooth	360	3.3-4.2	29	Absent	More than half	Firm	Good
27-1-3x18-7	Taper	Mostly buried	Smooth	350	3.4-3.9	28	Wide	More than half	Firm	Reject
59-10x18-1	Short	Half buried	Smooth	160	2.7-3.6	15	Absent	Few	Firm	Reject
59-10x18-2	Short	Half buried	-	310	2.4-4.2	23	Wide	More than half	Firm	Reject
59-10x18-5	Short	Half buried	-	250	2.6-4.2	22	Wide	More than half	Firm	Reject
62-2x18-1	Short	Half buried	Smooth	280	3.1-4.4	18	Narrow	Few	Firm	Reject
62-2x18-5	Short	Half buried	-	400	3.4-4.7	17	Narrow	Few	Firm	Reject
62-2x18-6	Short	Mostly buried	Smooth	340	2.8-4.5	18	Narrow	Few	Firm	Reject
77-2x18-1	Short	Mostly buried	Smooth	240	3	20	Narrow	More than half	Firm	Reject
77-2x18-2	Short	Mostly buried	Smooth	250	2.9-4	22	Narrow	More than half	Firm	Reject
77-2x18-11	Cylindrical	Mostly buried	Smooth	430	3.4-4.7	26	Narrow	More than half	Firm	Good
77-4x18-7	Cylindrical	Mostly buried	Smooth	390	3.4-5.3	23	Narrow	Few	Firm	Good
Control										
55 Days, CT	Cylindrical & Taper	Mostly buried	Smooth	300	3.3-4.2	22	Narrow	More than half	Firm	Reject
A-1, Fighter car	Cylindrical	Mostly buried	Smooth	350	3.8-5.2	23	Absent	Morr than half	Firm	Good
Himalia, Thepwatana	Cylindrical	Mostly buried	Smooth	280	4.0	22	Absent	Lower	Firm	Sale

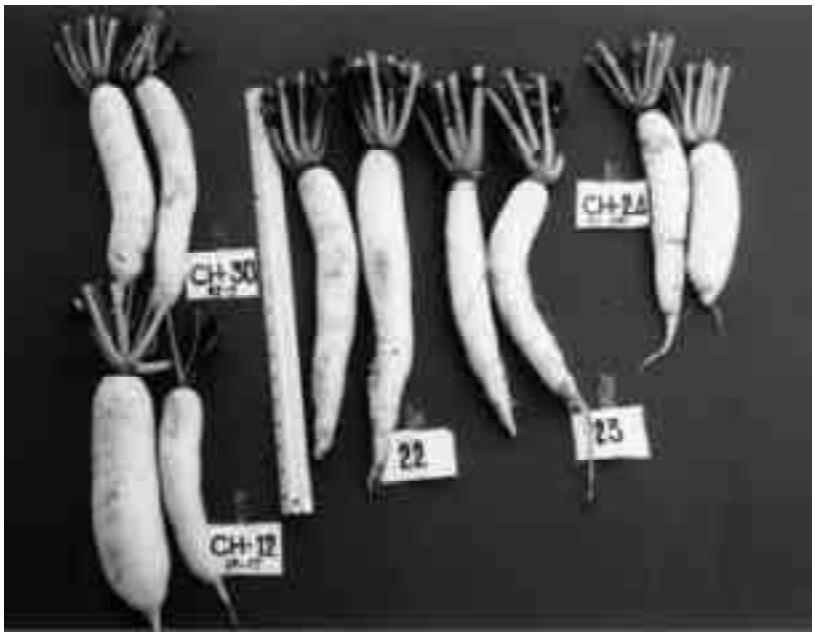

Figure 3 Good F1 hybrid varieties No. 22 (27-1-3 x 18-1) , No. 23 (27-1-3 x 18-2), No. 24 (27-1-3 x 18-3) and No. 25 (27-1-3 x 18-6) compared with control varieties : No. 12 (55 Days CT OP #2) and No. 30 (RF-3).

CONCLUSION AND DISCUSSION

It was shown from seed set analysis and fluorescent microscope techniques that most of the inbred lines of Chinese radish were self-incompatible and weak self-incompatible. They could be further used for F1 hybrid production. When these inbred lines were crossed in all combinations, most of them gave some hybrid seeds. Some crosses were tested in the field by the private company, characteristics, only crosses 27 x 18 and 77 x 18 were acceptable. They had long cylindrical shape of root with smooth skin surface, no scar or narrow scar, firm texture and good weight. Yield was not tested in this experiment, but we had shown that these hybrid varieties had much higher yield than open-pollinated and commercial F1 hybrid varieties (Nikornpun, 1988-92).

F1 hybrid Chinese radish always give higher root yield than open-pollinated varieties. Local selection for male and female lines of the hybrid would give good F1 hybrid varieties because they are adapted to our local climatic conditions. Therefore, our F1 hybrid varieties always give higher root yield than imported F1 hybrid varieties.

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Some Insecticidal Plant Extracts for Controlling Maize Weevil, *Sitophilus zeamais* Motschulsky (Coleoptera : Curculionidae)

Somnuk Wongtong[1] and Sangkhae Nawanich[2]

ABSTRACT

The toxicity of crude extracts from three selected plants namely Waan-nam (*Acorus calamus* L.), Noi-naa (*Annona squamosa* L.) and Lian (*Melia azedarach* L.) was investigated under laboratory conditions of 27±3°C and 80±20% RH. By the use of dry film method, for the toxicity test, the result revealed that crude extract from Waan-nam was more toxic to maize weevil than crude extract from Noi-naa seed. Due to low toxicity of Lian to maize weevil, thus, it was dropped out from further investigation. The efficacy and long-term residual effect of crude extracts from Waan-nam root and Noi-naa seed were also studied. Crude extracts of both plants were treated to maize seeds and the number of dead and alive maize weevils were counted. The results indicated that crude extract from Waan-nam root at the concentration of 10% (w/v) effectively control the number of maize weevil and its F_1 for 120 days after treated (DAT). Additionally, the same results on the control of maize weevil were obtained from crude extract of Noi-naa seed at the concentrations of 5 and 10% (w/v) whereas the concentration of 2.5% (w/v) could control the number of maize weevil for 90 DAT. Moreover, all concentrations of crude extract from Noi-naa seed could effectively control the number of F_1 maize weevil for 120 DAT. The effects of crude extracts from Waan-nam root and Noi-naa seed, on maized seed germination were also evaluated. The between-paper method was employed for germination test. It was found that maize seeds treated with crude extract from Waan-nam root almost completely lost their germination percentage while seed germination was high up to 90 DAT when treated with crude extract from Noi-naa seed at 2.5% (w/v) concentration.

Key words : insecticidal plant extract, maize weevil control, Waan-nam, Noi-naa, Lian

INTRODUCTION

Maize Weevil : *Sitophilus zeamais* Motschulsky (Coleoptera : Curculionidae) is one of the most serious, internal feeding pests of maize seed and grain. *S. zeamais* are found in all warm and tropical parts of the world (Dobie *et al.*, 1984). Maize insect pests are moved and distributed around the world by means of the stored product transportation. Moreover, the differences of stored condition, and regional environments can bring about survival of maize weevil.

Maize weevil and rice weevil (*Sitophilus oryzae* L.) can infest many kinds of agricultural stored products such as maize, sorghum, wheat, barley, rice and paddy (rough rice) (Chankaewmanee, 1997) by internal feeding. Seed stored for six months was infested 22 percent

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(Sukprakarn *et al.,* 1996) resulted in light weight seed, lower food quality and lower germination percentage. Control of maize weevil can be done in several ways such as management and clean storage, reduce seed humidity, hot/cool temperature control, vacuum storage, seed treated by some materials, insecticides and fumigant treated. In order to reduce post harvest loss and food quality loss, organically alternative controlling methods are needed. Lately, several reports indicated the insecticidal actions of certain plant species. Additionally, in Thailand, the existence of tropical medicinal flora is abundance and awaits investigation. Therefore, it may be very useful for farmers if some of such plant extracts with insecticidal effect against insects such as the maize weevil are found. Moreover, these extracts should not react as pollutants and/or should not be the compounds that bring about insect resistance. Expectation, if the extracts control maize weevils, is that importation of insecticides will decrease. Plant extracts from Waan-nam (*Acorus calamus* L.), Noi-naa (*Annona squamosa* L.) and Lian (*Melia azedarach* L.) have been found to have insecticidal action and can be used for controlling maize weevil.

The objectives of this study were to determine the toxicity and the efficacy of the crude extracts of some medicinal plants in controlling maize weevil. The effect of the crude extracts of some medicinal plants on maize seed germination was also studied.

MATERIALS AND METHODS

Culture of maize weevil, *Sitophilus zeamais* Motsch.

Stock cultures of maize weevil were obtained by collecting adults maize weevil from the storage room at the National Corn and Sorghum Research Center, Pakchong, Nakorn Ratchasima. The cultures were kept in a plastic box (17.5 × 25 × 9.5 cm) and wide mouth jars covered with filter papers. The filter paper was sealed with glue at the top of the jar to prevent the infestation of stored grain mites. The food media on which the test insects were cultured were seeds corn var. Pacific 328 that had 10.59% moisture content.

In order to obtain adults of known age from insect cultures, 100 unsexed adults were added to 200 g fresh culture media in a glass jar. After 3 days of egg-laying period, the adults were removed by means of sifting from the culture media and a new generation was obtained from the hatched eggs in approximately 30 to 45 days. The insect culture was reared at 27±3°C and 80±20% RH in the laboratory of the Entomology Research Building, Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok.

1. The toxicity of some medicinal plant extracts to maize weevil

1.1Criteria for selecting plant species

Plants without damage from insect and plants known to have insecticidal effects both by the local people and researchers were the criteria for selecting plant species. The plant samples were, then, air-dried under normal room temperature until extraction. From the above criteria, 3 plant species were selected for this investigation.

1.2 Extraction of medicinal plants

Each plant sample was extracted using of the same extraction procedures modified from

Table 1 List of plant species, common names and Thai names including plant parts.

Plant species	Common name (Thai name)	Plant parts
Acorus calamus L.	Sweet flag (Waan-nam)	Rhizomes
Annona squamosa L.	Sugar apple (Noi-naa)	Seeds
Melia azedarach L.	Bastard cedar, Persian lilac (Lian)	Leaves

several authors work (Butterworth and Morgan, 1971; Roongsook, 1992 and Palaharn, 1996). This involved chopping of the selected plant samples and weighed 500 g. After weighing, the chopped sample was completely soaked in 1,000 ml of 95% ethanol for 3 days and then filtered. The sample was extracted twice. After that ethanol was evaporated with evaporator. The ethanolic crude extract was kept at 4°C in a refrigerator for further studies.

1.3 Screening for insecticidal action and toxicity tests of some medicinal plant extracts to maize weevil

4 concentrations, i.e. 25, 50, 75 and 100 percent (w/v). The ethanolic crude extracts were used in the screening test. The extract of each sample was dissolved in the solvent (95% ethanol). Then the solution was ready for screening test using the residual exposure method (contact method). One ml of solution was dropped onto the filter paper No. 1, 70 mm diameter. The treated filter paper was air-dried under the laboratory conditions, thereafter, transferred into petridish. Then the glass ring (5.0 cm diameter, 2.5 cm height) was placed on the treated filter paper as the confining cage for the test insects. Twenty adult maize weevils, *S. zeamais* at the age of 7-10 days were introduced onto each treated filter paper inside the cage. Then the cage was covered with the petridish cover of which 6-8 holes were made to allow air circulation. Observation was made at 24 hours after treating. The number of dead and alive insects were recorded

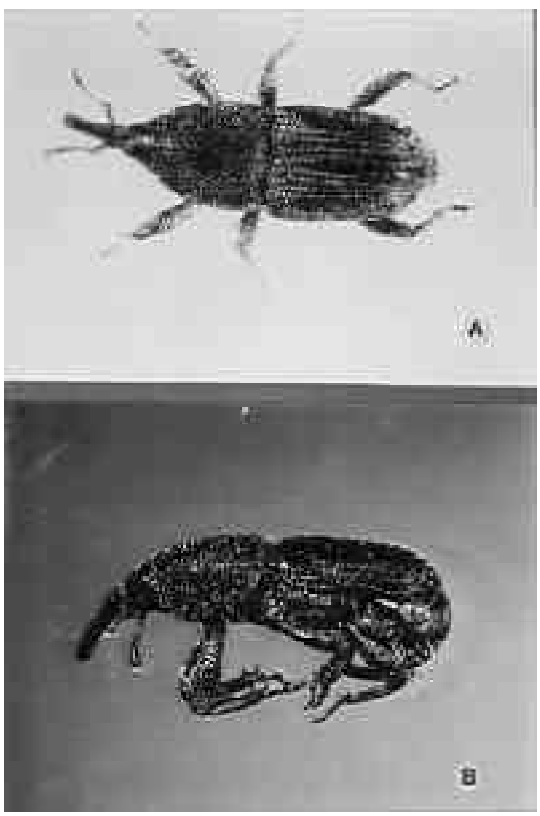

Figure 1 Adult of maize weevil, *Sitophilus zeamais* Motsch.
A. Dorsal view
B. Lateral view

Figure 2 Waan – nam , *Acorus calamus L.*
A. Leaves
B. Rhizomes

and occasional control death was corrected by Abbott's formula (Abbott, 1925).

The percentage of mortality is corrected by Abbott's formula :

$$\% \text{ Corrected mortality} = \frac{\% \text{ test mortality} - \% \text{ control mortality}}{100 - \% \text{ control mortality}} \times 100$$

Therefore, the extracts that showed insecticidal action were used in toxicity study and the method used in toxicity study was similar to the screening test except the concentrations which serial dilution technique was employed. This experiment was a completely randomized design with five replications and four levels of concentration as treatments. Observation of each treatment was made at 24 hours after treating. Data on insecticidal action testing were recorded and calculated using the probit analysis (Finney, 1971) to obtained LC_{50} values. In this toxicity test, ethanolic crude extracts of plant part of each species were used, i.e. rhizome of Waan-nam and seed of Noi-naa.

2. Efficacy of crude extracts of some medicinal plants to control maize weevil

2.1 Efficacy test

Two ethanolic crude extracts were used for maize seed treatment employing a completely randomized design with four replications. Maize seeds were treated with the 50 ml of the ethanolic crude extracts at the rates of 2.5, 5 and 10% w/v per 1 kg of seeds. The treated seeds were left to dry under laboratory conditions. Thereafter, 100 g of seeds were transfered into each four wide-mouth

Figure 3 Noi-naa, *Annona squamosa* L.
A. Fruit
B. Seeds

Figure 4 Lian, *Melia azedarach* L.
A. Leaves
B. Leaves and branch

glass jars. Then about 20 adult maize weevils at the age of 7-10 days were released into the jar and the top was glued to prevent infestation of the store grain mite . The jars were kept in the laboratory at 27°C and 80% RH. Ten days later, dead and alive maize weevils were counted and were taken out of the jars. Fifty-five days after, new maize weevils were counted and maize seeds were checked for damage and undamage.

2.2 Residual test

The methods used in this study were similar to the efficacy test except the periods to release the maize weevils into the treated seeds. The seeds were treated with crude extract at the rates of 2.5, 5 and 10% w/v per 1 kg of seed. One hundred gram of treated seeds were stored in the jar for 30, 60, 90 and 120 days before introducing twenty adult maize weevils into the jar. The observation was made for each storage period, on the number of dead and alive maize weevils.

3. The effect of crude extracts of some medicinal plants on maize seed germination

3.1Germination test

The germination test was performed by the use of between-paper method (rolled method). One hundred seeds of maize were sampled from each treatment of the efficacy test and residual test. The double pieced paper towel (25 cm × 40 cm) was soaked in water and was stretched on the table. Then maize seeds were laid onto the paper towel and covered with another damp paper towel. After that, paper towels with maize seeds rolled and kept in the plastic box under laboratory conditions of 27±3°C and 80±20% RH. Four days later, the paper towels were opened and normal seedlings were counted (Duangpatra, 1986). Data were recorded and calculated for the germination percentage.

RESULTS AND DISCUSSION

1. The toxicity of some medicinal plant extracts to maize weevil

1.1 Selection of plant samples

Three species of plants were collected in Bangkok and Nakorn Ratchasima province during the investigation, namely: rhizomes of Waan-nam (*Acorus calamus* L.), seeds of Noi-naa (*Annona squamosa* L.) and leaves of Lian (*Melia azedarach* L.).

1.2 Extraction of medicinal plants

Samples of the three test plants were extracted for bioactive principles, and screened for insecticidal activities against the maize weevil. The results of plant extract are shown in Table 2.

1.3 Screening and toxicity test of some medicinal plants extracts to maize weevil

1.3.1 Screening test

The crude extracts of three plant parts were screened of insecticidal activities against the maize weevil, *S. zeamais* at the age of 7-10 days. The results revealed that only crude extracts at the concentration of 25% w/v from Waan-nam and

Table 2 The weight of crude extract of three plant samples extracted with 95% ethyl alcohol.

Plant common name	Plant part	Weight of sample (g)	Weight of crude extract (g)
Waan-nam	Rhizome	500	28.08
Noi-naa	Seed	500	24.35
Lian	Leaves	500	11.31

Noi-naa had the insecticidal activities against maize weevils, causing mortality at 24 hours of 82.50% and 35.00%, respectively. The crude extract of Lian did not kill the maize weevil although high concentration of 100% w/v was used (Table 3).

1.3.2 Toxicity testing (LC_{50}) of plant crude extracts of Waan-nam and Noi-naa on maize weevil

Plant crude extract of Waan-nam was tested against maize weevils at 5 concentrations; 0 (ethyl alcohol 95%), 5, 10, 20 and 40% (w/v). The results in Table 4 and 6 shows that the crude extract from rhizome of Waan-nam was toxic against maize weevil, *S. zeamais* with an LC_{50} value of 11.7341% (w/v). The regression lines are shown in Figure 5. The results were more or less agreed with Roongsook (1992) who reported that the LC_{50} value for toxicity of crude extract from rhizomes of Waan-nam to diamondback moth, *Plutella xylostella* was 6.5% (w/v).

Plant crude extract of Noi-naa was tested against maize weevils at 5 concentrations; 0 (ethyl alcohol 95%), 25, 30, 35 and 40% (w/v). Crude extract from seed of Noi-naa was found to show the toxicity against maize weevil with an LC_{50} value of 32.3434% (w/v). The results are shown in Table 5, 6 and the regression lines in Figure 6. Roongsook (1992) also reported the same result for toxicity of crude extract from seed of Noi-naa to diamondback moth with an LC_{50} value of 0.50% w/v.

The summarized results in Table 6 and Figure 7 show that crude extract of Waan-nam was more

Table 3 Screening activities of crude extracts from tested plants against the maize weevil adults, 7-10 days old, under laboratory conditions, 27±3°C and 80±20% RH.

Plant	Plant part	Corrected mortality at 24 hours (mean, %)[1/]
Waan-nam	Rhizome	82.50
Noi-naa	Seed	35.00
Lian	Leaf	0

1/ Means at 24 hours after treated with crude extract at 25% (weight/volume) and calculated by the use of Abbott's formula (Abbott, 1925).

Table 4 Mortality percentage of maize weevil, *S. zeamais* as affected by crude extract of Waan-nam, *A. calamus* L. 24 hr. after treated under the laboratory conditions.

Dose (% w/v)	Log dose (x)	Total treated	No. dead adult/treatment	% corrected mortality	Empirical probit (y)
Treated check[1/]	0.0000	100	0	0	0.0000
5	0.6990	100	23	23	4.2619
10	1.0000	100	50	50	5.0008
20	1.3010	100	62	62	5.3023
40	1.6021	100	85	85	6.0356

1/ treated check = 95% ethyl alcohol

Table 5 Mortality percentage of maize weevil, *S. zeamais* as affected by crude extract of Noi-naa, *A. squamosa* L. 24 hr. after treated under laboratory conditions.

Dose (% w/v)	Log dose (x)	Total treated	No. dead adult/treatment	% corrected mortality	Empirical probit (y)
Treated check1/	0.0000	100	1		
25	1.3979	100	21	20.20	4.182
30	1.4771	100	37	36.36	4.6529
35	1.5441	100	51	50.50	4.9873
40	1.6021	100	91	90.90	6.2787

1/ treated check = 95% ethyl alcohol

effective on maize weevil than that of Noi-naa. However, lower slope of 1.84 was found for Waan-nam extract whereas Noi-naa extract had higher slope, 8.7559 (Table 6). It seemed that maize weevil was more sensitive to Noi-naa extract than Waan-nam extract. According to Roongsook (1992) the crude extract from leaves of Lian, *M. azedarach* L. was used only for screening test because of the toxicity of this plant to diamondback moth was quite low. Palaharn (1996) also confirmed that three crude extracts were effective to beet armyworm, *Spodoptera exigua* Hubner. Percentage corrected mortality of three plants at 72 hours were ranked in decreasing orders as 82.14% for ethanolic crude extract from seed of Noi-naa, 79.31% for leaves of lian and 62.06% for rhizome of Waan-nam. However, in this experiment crude extract of the Lian leaves was harmless to maize weevils.

2. The efficacy of crude extracts of some medicinal plants to control maize weevil

2.1Efficacy test

Table 7 shows the efficacy test of medicinal plant extracts on maize weevil when treating maize seeds for 10 days. The results showed that 5 and

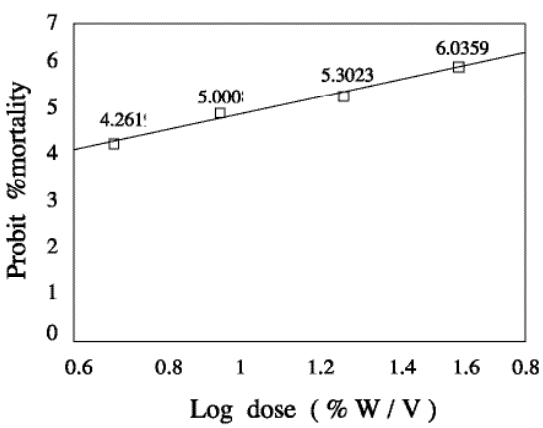

Figure 5 Probit mortality line of maize weevil, *S. zeamais* as affected by crude extract of Waan – nam, *Acorus calamus* L.

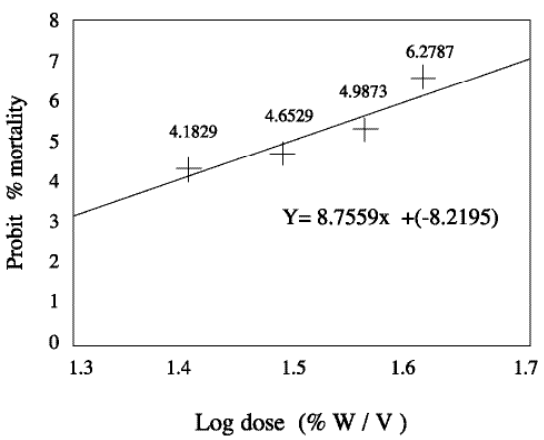

Figure 6 Probit mortality line of maize weevil, *S. zeamais* as affected by crude extract of Noi – naa, *Annona squamosa* L.

Table 6 Toxicity of two ethanolic crude extracts against maize weevil, *S. zeamais* (Coleoptera : Curculionidae)

Plants	LC_{50} (% w/v) (95% CI.)	Slope ± SE
Waan-nam	11.7341(9.7959-13.8455)	1.8400±0.0373
Noi-naa	32.3434(31.2103-33.5909)	8.7559±0.0079

10% w/v concentrations of Waan-nam rhizome extracts, 2.5, 5 and 10% w/v concentrations of Noi-naa seed extracts and the standard check (pirimiphos methyl) killed maize weevil 100%, whereas, 2.5% w/v concentrations of Waan-nam rhizome extract killed maize weevil at 96.25%.

The data from Table 8 shows the number of adults of F_1 maize weevil developed in treated seeds for 55 day. The results showed that all treatments except treated check (ethyl alcohol 95%) completely controlled the number of adult of F_1 of maize weevil ranging from 0-0.25.

2.2Residual test

Residual effects of medicinal plant extracts on maize weevil when treating seed for 30, 60, 90 and 120 days are shown in Table 9. It was found that 10% w/v concentration of Waan-nam rhizome extract, 5 and 10% w/v concentrations of Noi-naa seed extracts and standard check gave residual effect for 120 days long. The percentage of dead maize weevil ranged from 86.25-100% whereas, 2.5% w/v concentration of Noi-naa seed extract had residual effect for 90 days long killing maize weevil up to 93.75% w/v after which its residual activity declined. In addition, 5% w/v concentration of Waan-nam rhizome extract could completely control maize weevil only for 60 days (Table 9).

Considering concentrations, Waan-nam at 10% w/v concentration gave the best result in controlling maize weevils (120 days). The same was true for Noi-naa crude extracts at 5 and 10% w/v concentrations. To control maize weevil, Noi-naa crude extract at 5% w/v concentration is recommended because of cost competitiveness. Neem extract consisted of azadirachtin 0.002% at the concentrations of 5, 10 and 20 ml/kg gave good protection of seed for 120 day (Nilpanit *et al*, 1992). The result of this experiment was in line with Nilpanit *et al.* (1992).

According to this investigation, the seeds treated with crude extracts of Waan-nam and Noi-naa at 2.5, 5 and 10% (w/v) concentrations and standard check for 30 and 60 days (Table 10) completely controlled the number of adults of F_1 maize weevil, allowing only up to 1.25 adults to emerge. However, for the treated check for 30 and 60 days, number of adults of F_1 maize weevil

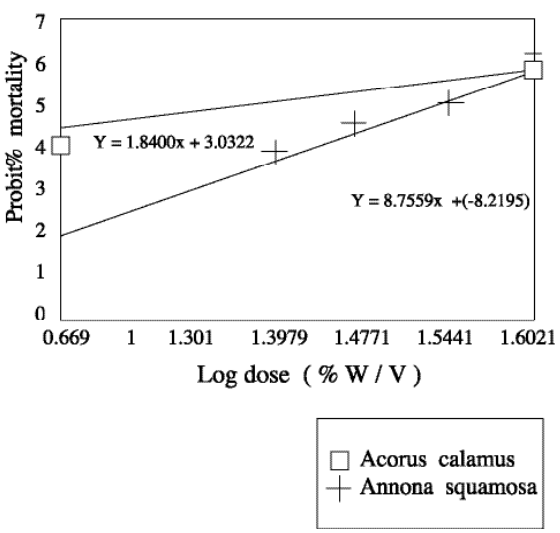

Figure 7 Probit mortality line of maize weevil, *S. zeamais* as affected by Crude extract of Waan – nam, *Acorus calamus L.* and Noi – naa, *Annona squamosa* L.

Table 7 Percentage of dead maize weevils after introducing into maize seeds treated with crude extracts for 10 days.

Treatments	Percentage of dead maize weevil (%)
Waan-nam 2.5% w/v	96.25 b
Waan-nam 5.0% w/v	100 a
Waan-nam 10% w/v	100 a
Noi-naa 2.5% w/v	100 a
Noi-naa 5.0% w/v	100 a
Noi-naa 10% w/v	100 a
Standard check[3/]	100 a
Treated check[2/]	2.50 c
CV (%)	2.26

[1/] Means followed by a common letter in the same column are not significantly different at 5% level of DMRT
[2/] Treated check = 95% ethyl alcohol
[3/] Standard check = pirimiphos methyl at the rate of 2 ml of pirimiphos methyl/300 ml of water/100 kg of seed

Table 8 Number of emerging adults of F_1 maize weevil in maize seeds treated with crude extracts for 55 days.

Treatments	Number of emerged maize weevil[1/]
Waan-nam 2.5% w/v	0.00 b
Waan-nam 5.0% w/v	0.00 b
Waan-nam 10% w/v	0.00 b
Noi-naa 2.5% w/v	0.25 b
Noi-naa 5.0% w/v	0.00 b
Noi-naa 10% w/v	0.00 b
Standard check[3/]	0.00 b
Treated check[2/]	38.50 a
CV (%)	134.71

[1/] Means followed by a common letter in the same column are not significantly different at 5% level of DMRT
[2/] Treated check = 95% ethyl alcohol
[3/] Standard check = pirimiphos methyl at the rate of 2 ml of pirimiphos methyl/300 ml of water/100 kg of seed

Table 9 Percentage of dead maize weevils after introducing into maize seeds treated with crude extracts for 30, 60, 90 and 120 days.

Treatments	Percentage of dead maize weevil (%)[1/]			
	30 days	60 days	90 days	120 days
Waan-nam 2.5% w/v	73.75 b	70.00 b	16.25 c	18.75 d
Waan-nam 5.0% w/v	98.75 a	100 a	52.50 b	75.00 bc
Waan-nam 10% w/v	100 a	100 a	98.75 a	100 a
Noi-naa 2.5% w/v	100 a	100 a	93.75 a	65.00 c
Noi-naa 5.0% w/v	100 a	100 a	88.75 a	86.25 ab
Noi-naa 10% w/v	100 a	100 a	97.50 a	98.75 a
Standard check[3/]	100 a	100 a	100 a	100 a
Treated check[2/]	5.00 c	1.25 c	0.00 c	2.50 e

[1/] Means followed by a common letter in the same column are not significantly different at 5% level of DMRT
[2/] Treated check = 95% ethyl alcohol
[3/] Standard check = pirimiphos methyl at the rate of 2 ml of pirimipho smethyl/300 ml of water/100 kg of seed

emerged were 37.75 and 56.00, respectively. It seemed that treated check was the least toxic to F_1 maize weevil. The result from the seed treated for about 90 days indicated that the seed treated with Waan-nam at 5, 10% w/v, Noi-naa at 2.5, 5.0, 10% w/v concentrations and standard check controlled number of adult of F_1 maize weevil allowing up to 4.75 adult emerged, while Waan-nam at 2.5% w/v concentration gave different result, up to 14.75 adults emerged. Additionally, treated check allowed 72.75 adults of F_1 maize weevil to emerge. When seed were treated for 120 days, all treatments were equally effective in controlling maize weevil but in the treated check number of adults F_1 maize weevil was as great as 56.0.

It was also found that Waan-nam at 10% w/v and Noi-naa at 2.5, 5, 10% w/v concentrations and standard check gave the longest residual effects (120 days) while Waan-nam at 2.5 and 5.0% w/v concentrations gave the shorter residual effect (60 days) (Table 10). However, among Noi-naa group, Noi-naa crude extract at the concentration of 2.5% w/v was recommended so as to save the budget.

3. Study of the effect of crude extracts of some medicinal plants on maize seed germination

3.1The germination test

The germination percentages of seed treated with crude extract of Waan-nam at different concentrations and for different periods were significantly different from treated check and standard check. They were ranging from 10.00-16.25%. It showed that all concentrations of crude extracts of Waan-nam group affected seed germination right after seeds were treated (Table 11).

The germination percentage of seed treated with crude extracts of different concentrations of Waan-nam was low and not recommend for maize seed treatment. Pingsutiwong and Wattanakij (1996) suggested that seed germination percentage of field corn (*Zea mays* Linn.) must not be lower than 75%. The results of this experiment clearly showed that Waan-nam crude extracts reduced maize seed germination percentage.

Acceptable germination percentage of maize

Table 10 Number of adults of F_1 maize weevil emerging in maize seeds treated with crude extracts for 30, 60, 90 and 120 days.

	Percentage of dead maize weevil (%)[1/]			
	Days	Days	Days	Days
Waan-nam 2.5% w/v	1.25 b	1.25 b	14.75 b	13.00 b
Waan-nam 5.0% w/v	0.00 b	0.00 b	4.75 c	2.50 b
Waan-nam 10% w/v	0.25 b	0.00 b	0.00 c	0.25 b
Noi-naa 2.5% w/v	0.25 b	0.50 b	1.25 c	5.50 b
Noi-naa 5.0% w/v	0.75 b	0.00 b	3.25 c	2.25 b
Noi-naa 10% w/v	0.25 b	0.00 b	0.75 c	0.50 b
Standard check[3/]	0.00 b	0.00 b	0.00 c	0.00 b
Treated check[2/]	37.75 a	56.00 a	72.75 a	56.00 a

[1/] Means followed by a common letter in the same column are not significantly different at 5% level of DMRT

[2/] Treated check = 95% ethyl alcohol

[3/] Standard check = pirimiphos methyl at the rate of 2 ml of pirimiphos methyl/300 ml of water/100 kg of seed

seed (>80%) was obtained from crude extracts of Noi-naa at 2.5, 5, 10% w/v concentrations, standard check and treated check up to 120 days after seed treatment. However, if 90% germination was required, the standard check gave the best result of up to 120 days whereas a better result was achieved when seeds were treated with crude extract of Noi-naa at the concentration of 2.5% w/v and from treated check, up to 90 days. In addition, maize seeds treated with crude extracts of Noi-naa at 5 and 10% w/v concentrations gave germination percentages of approximately 90% within the period of 60 days. It was obvious from the standard check gave greater germination percentage than treated check and Noi-naa crude exracts (Table 11). The assumption was that the use 95% ethyl alcohol as solvent for extraction of both medicinal plants led to the decrease of the germination percentage of maize seed.

However the result in Table 11 showed that seed treated with crude extract of Noi-naa at the concentration of 2.5% w/v gave the longest residual effect for 90 days as similar to those of the standard check and the germination percentage of seed was not decreased. Thus, treated with crude extract of Noi-naa at the concentration of 2.5% w/v is to be recommended.

CONCLUSION

The laboratory studies of medicinal plant extracted from 3 plant species with 95% ethyl alcohol for insecticidal activities against maize weevil can be concluded as follows :

1. Two species of medicinal plants were found to contain the insecticidal principles against maize weevil, i.e. Waan-nam rhizomes, *Acorus calamus* L. and Noi-naa seeds, *Annona squamosa* L. Concerning the toxicity of Waan-nam rhizome and Noi-naa seeds extract to maize weevil, Waan-nam rhizome extract was found to be toxic to maize weevil with an LC_{50} value of 11.73% w/v and Noi-naa seeds extract was toxic with an LC_{50} value of 32.3434% w/v, but maize weevil was more sensitive to Noi-naa extract than Waan-nam extract.

2. Among Waan-nam crude extracts, maize

Table 11 The germination percentage of maize seeds after treated seeds with crude extracts and introduced 20 adults of maize weevil for 10 days.

Treatments	Germination percentage of maize seeds after treated (%)[1/]				
	0 days	30 days	60 days	90 days	120 days
Waan-nam 2.5% w/v	16.00 c	16.25 b	14.00 d	15.00 c	14.75 c
Waan-nam 5.0% w/v	11.75 d	11.25 c	14.50 d	12.00 c	13.75 c
Waan-nam 10% w/v	10.00 d	13.50 b	13.25 d	14.25 c	14.25 c
Noi-naa 2.5% w/v	96.75 ab	95.25 a	91.75 bc	91.50 b	82.50 b
Noi-naa 5.0% w/v	95.50 b	95.00 a	90.00 c	87.00 b	83.50 b
Noi-naa 10% w/v	95.75 ab	94.75 a	89.50 c	89.50 b	81.25 b
Standard check[3/]	99.25 a	99.50 a	98.25 a	98.50 a	93.25 a
Treated check[2/]	98.50 ab	96.25 a	95.50 ab	90.25 b	83.50 b

[1/] Means followed by a common letter in the same column are not significantly different at 5% level of DMRT

[2/] Treated check = 95% ethyl alcohol

[3/] Standard check = pirimiphos methyl at the rate of 2 ml of pirimiphos methyl/300 ml of water/100 kg of seed

seed treated with 10% w/v concentration of Waan-nam rhizome extract could kill and control F_1 maize weevil for 120 days long. This residual effect was longer than that of the 5.0% w/v of Waan-nam rhizome extract which killed and controlled F_1 maize weevil for 60 days.

Among Noi-naa crude extracts, maize seed treated with 5.0 and 10% w/v concentrations of Noi-naa crude extract killed maize weevil for 120 days long. This residual effect was longer than that of the 2.5% w/v concentration of Noi-naa seed extract which killed maize weevil for 90 days long. Additionally, all treatments of Noi-naa seed extract could kill F_1 maize weevil for 120 days long.

3. Considering seed germination percentage, maize seed treated with Waan-nam crude extracts had very low germination percentage. These treatments were not suitable for seed maize. Among seed treated with Noi-naa crude extracts, only Noi-naa crude extract at the concentration of 2.5% w/v did not decrease germination percentage until 90 days after treatment. However, the use of 95% ethyl alcohol as solvent for extraction of both medicinal plants led to the decrease in germination percentage of the maize seed.

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Yield Losses Assessment Due to Pests on Cotton in Lao PDR

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ABSTRACT

From 1985 to 1991 research on cotton protection was carried out in Lao PDR to determine the incidence of the major pests and the efficiency of the recommended protection program. Results showed that without any pest control, the development of the plants was very low and yield losses reached almost 70% of the potential production. The recommended program, with seven insecticide sprayings, reduced the incidence of the pests but was not enough to ensure the required production. Moreover, it increased the cost of production and was not profitable. Only the intensive program with a weekly insecticide application could protect the crop satisfactorily. To cope with this situation, an IPM strategy, aiming at reducing the cost of pest control, must be implemented.

Key words: cotton, pests, yield losses, Integrated Pest Management, Lao

INTRODUCTION

In Lao PDR, cotton is traditionally grown throughout the country in small family plots without inputs (Trébuil *et al.*, 1994). The average of the planted area per farmer is about 1,500 m^2 (Thirasack, 1994). Five main types of cotton plant are cultivated. They belong to three botanical species *Gossypium hirsutum* L. (Fai Niai), *G. arboreum* L. (Fai Noi, Fai Moui, Fai Mok) and *G. bardadense* L. (Fai Djan) (Tr?buil *et al*., 1994). These cultivars produce a short to medium size lint of 18 to 27 mm in length. The ginning out turn is about 30 %. The yields are low and the national average is around 500 kg/ha of seed-cotton (Castella *et al*., 1993).

Among the problems that must face the farmers are the attacks of several insect pests that reduce yield (Angladette, 1948). Over the crop season two main kinds of pest are recorded. The first are sucking insects represented essentially by the leafhopper *Amrasca biguttula* Ishida (Homoptera: Cicadellidae), and the cotton aphid *Aphis gossypii* Glover (Homoptera: Aphididae). Secondly there is the bollworm *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) which larvae, according to the period of infestation, can damage either the squares, the flowers or the green bolls (Matthews and Tunstall, 1994). Moreover, *A. gossypii* transmits a viral disease called blue disease or leaf roll (Cauquil and Follin, 1983). Pests of minor importance are also recorded. The larvae of the semi-looper *Anomis flava* (F.) (Lepidoptera: Noctuidae) and of the leaf roller *Syllepte derogata* (F.) (Lepidoptera: Pyralidae) damage the foliage of the plants. Nymphs and adults of the whitefly *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) and of the cotton stainer *Dysdercus cingulatus* (F.) (Hemiptera: Pyrrhocoridae) are sucking insects. The former feeds on the leaves but can also contaminate the lint with honeydew and associated fungi. The latter feeds on immature as well as ripe cotton seeds. Its punctures can either

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cause the shedding of the young green bolls or the rotting of the developed bolls which produce seeds and lint of poor quality. Finally, the spiny bollworm *Earias vittella* (F.) (Lepidoptera: Noctuidae) and the pink bollworm *Pectinophora gosssypiella* (Saunders) (Lepidoptera: Gelechiidae) damage the flowers and the green bolls. Several other secondary pests are also present in the fields, but no data is available concerning these species.

Until the creation in 1987 of the Cotton Lao Factory in Vientiane (Kousol, 1994) cotton was usually grown for self-consumption (Trébuil *et al.*, 1994). Between 60 to 80 kg of seed-cotton, ginned by hand, are needed to meet a family's fibre requirement (Mahdavi, 1995; Peutot, 1996). Nowadays the development of the textile industry, particularly in Thailand, opened new outlets (Fichet, 1995). But the exportation to these countries depends, among other things, on the fibre quality which must be adapted to the industrial spinning technology (Mahdavi, 1995). Consequently new varieties with high level of production and good fibre quality must be proposed to the farmers. In the 1960's, new cultivars were tested with more intensive cultural practices; hence seed-cotton yield increased initially. But after a few years, the production started to decrease due to their susceptibility to *A. biguttula* and *H. armigera*, and to the augmentation of the cost of production generated by the numerous insecticide applications that the farmers must do to control these pests. This situation, which has continued until now (Castella *et al.*, 1993), demonstrates that the development of cotton production in Lao PDR must be accompanied by the implementation of technical itineraries which allow the growth of cotton without increasing the use of chemicals. This is possible with the setting up of an Integrated Pest Management strategy adapted to the local conditions of production which are specific to the country with regard to ecological and human environments, varieties, outlets and organisation of the cotton industry (Follin and Crozat, 1993). But the elaboration of such a strategy necessitates a thorough knowledge of the factors influencing the production, particularly the pests.

The objective of this study is to quantify yield losses caused by insects, and the cost-effectiveness of the recommended management strategy.

MATERIALS AND METHODS

The experiment was conducted over a period of seven years, from 1985 to 1991, in farmer fields, except at Napok where it was carried out at the National Agricultural Research Station. These locations were in the following provinces: Luang Prabang, Sayabury, Vientiane with two places Napok and Ban Hai, Bolikhamxay and Savannakhet (Figure 1). This allowed researchers to collect results in various ecological conditions (Table 1). Cotton was grown during the rainy season from the end of

Table 1 Altitude and annual rainfall registered at the different experiment localities.

Localities	Altitude (m above sea level)	Annual rainfall (mm)
Bolikhamxay	180	2200-3000
Savannakhet	170	1400-1900
Vientiane	170	1400-1900
Sayabury	350	1400-1900
Luang Prabang	450	less than 1400

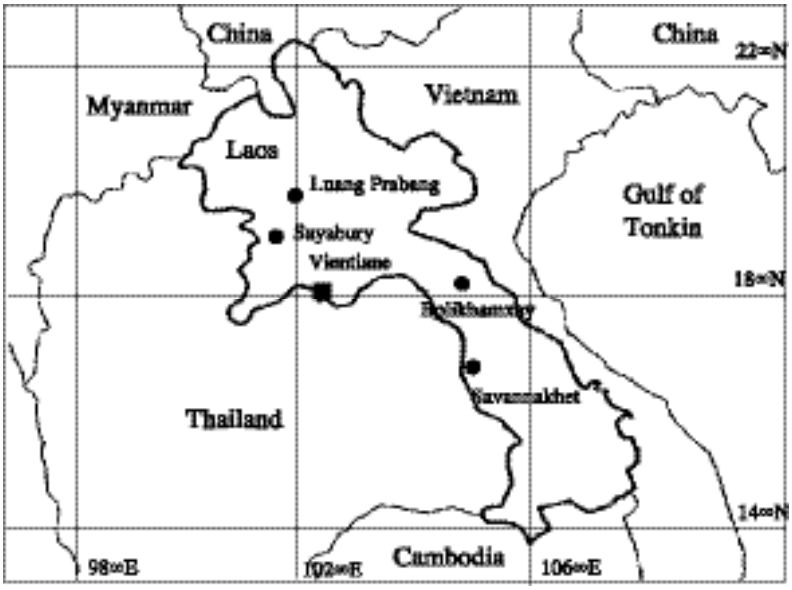

Figure 1 Map of Lao PDR with location of the experiments.

June to November. At Luang Prabang in 1985, Napok Research Station in 1985 and Ban Hai 1991, the sowing dates were on June 29th, July 1st and June 1st respectively.

According to the cropping season and the locality, several traditional and bred varieties were planted (Table 2).

No seed treatment either with insecticide or fungicide was used. Throughout the season three insect pest protection levels were compared as follows:

- No Treatment (NT): no insecticide application.

- Standard Protection (SP): 7 insecticide applications at 15 day intervals, starting from 21 days after sowing.

- Intensive Protection (IP): weekly application of insecticide starting from 16 days after sowing.

All insecticide applications were carried out by knapsack sprayer equipped with a single lance. Pesticides were used as follows: two applications for SP and four for IP with dimethoate were implemented at the rate of 375 to 560 g ai/ha depending on plants growth, followed by the applications of a mixture of dimethoate (as previous) and deltametrin at the rate of 7 to 10 g ai/ha. Dimethoate was applied to control early and late sucking pests whereas deltametrin was used to control bollworm.

For each experiment, during the whole cropping season, data were recorded using 40 plants in each plot. For aphids, this meant the number of plants with at least one individual. The populations of leafhoppers were estimated by counting together the number of nymphs and adults observed on five top leaves of each scouted plant. Finally the number of bollworm larvae encountered on the whole plant was recorded.

Two replications were implemented. Aiming to reduce environmental effects, the untreated plots were located at both extremities of the planted area whereas the plots with the intensive protection were situated in the middle of the experimental parcel. The standard program was applied between the two previous levels of protection (Figure 2). Elementary

Table 2 Yield and relative yield losses due to all pests in experiments carried out at different locations, from 1985 to 1991, and average per locality.

Localities	Years	Varieties	NT yield (kg/ha)	SP yield (kg/ha)	IP yield (kg/ha)	Losses in NT (% of IP)	Losses in SP (% of IP)
Napok	1985	DI.5	12	507	2623	99	81
Station	1986	DI.5	8	816	1160	99	30
(Vientiane)	1989	SR 2	742	2125	2700	73	21
		Fai Niai	175	236	436	60	46
	1990	SR 2	66	281	862	92	67
		KK 1	650	752	1377	53	45
	average		276	786	1526	80	49
Ban Hai	1987	Fai Niai	1035	1570	1530	32	0
(Vientiane)	1991	KK 1	846	1847	2445	65	25
	average		941	1709	1988	53	14
Bolikhamxay	1991	KK 1	355	1503	2247	84	33
		Fai Niai	569	1091	1474	61	26
	average		452	1297	1861	76	30
Sayabury	1991	KK 1	298	1466	2180	86	33
		Fai Niai	558	1040	1400	60	26
	average		428	1253	1790	76	30
Savannakhet	1991	KK1	437	1491	2095	79	29
		Fai Niai	760	966	1542	51	37
	average		599	1229	1819	67	32
Luang Prabang	1985	DI.5	339	1665	1685	80	1
	Average of all the trials		457	1157	1717	73	33

NT	SP	IP	IP	SP	NT
No Treatment	Standard Program	Intensive Protection	Intensive Protection	Standard Program	No Treatment

Figure 2 Experimental design used to compare the three levels of protection.

plots consisted of 16 rows of 25 m long of 1 m apart (400 m^2).

Correlation analysis was performed by Statitcf Version 5 (Institut Technique des Céréales et des Fourrages) and the curves were plotted using Excel 97 software (Microsoft).

RESULTS AND DISCUSSION

The incidence of the pests varied from an experiment to experiment. Presented below are the main results, obtained when pest populations were important enough to reveal differences between

Table 3 Yield obtained with the three levels of protection, and losses in NT and SP programs according to the potential production (IP yield) and the varieties (average over locations and years).

Varieties	NT kg/ha	SP kg/ha	IP kg/ha	Losses in NT (% of IP)	Losses in SP (% of IP)
DI.5	120	996	1823	93	45
SR 2	269	802	1187	77	32
KK 1	517	1412	2069	75	32
Fai Niai	619	981	1364	55	28

treatments or trials.

a) Plant growth

According to the incidence of pests the development of the plants showed important differences.

At Luang Prabang in 1985, early pest incidence was low and no difference in plant growth was noted. Plant development was gradual and similar in the three treatments (Figure 3). At the end of the season, the plants reached 140 cm.

At Napok station in 1985, significant difference was observed between NT and SP as well as SP and IP (Figure 4). With IP program up to 90 days after sowing, the plant growth was normal and the result obtained was rather similar to the one of Luang Prabang. After this date the rains stopped early affecting the plant growth. With the standard protection, the plant development slowed down after the beginning of the season and the maximum height was only 50 cm. Finally, when no insecticide was applied the plants remained small and did not exceed 20 cm in height.

Hence, at Napok station, plant growth was directly affected by insect pest incidence and varied according to insecticide control level. The difference of plant growth was essentially due to leafhopper damage because populations of other early pests remained low. Important populations of these homopterous insects could affect the plant

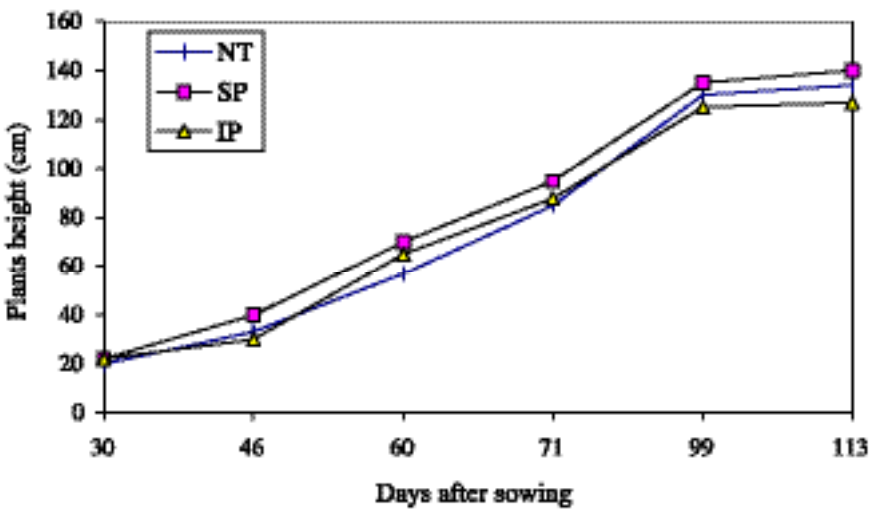

Figure 3 Plant growth at Luang Prabang in 1985 (sowing date June 29).

development considerably. Apart from leaves drying, the punctures of leafhoppers induce a shortening of the inter nodes. Moreover, only the IP program yielded a satisfactory control of the pest. The standard program, with spraying rate of 15 day intervals could reduce the incidence of pests but was not enough to allow a normal plant development.

b) Pest control

Control of aphids

At Napok station the number of plants infested by aphids increased gradually throughout the growing season with noticeable fluctuations (Figure 5). In NT and SP plots, the development of the colonies was almost the same. Two main peaks

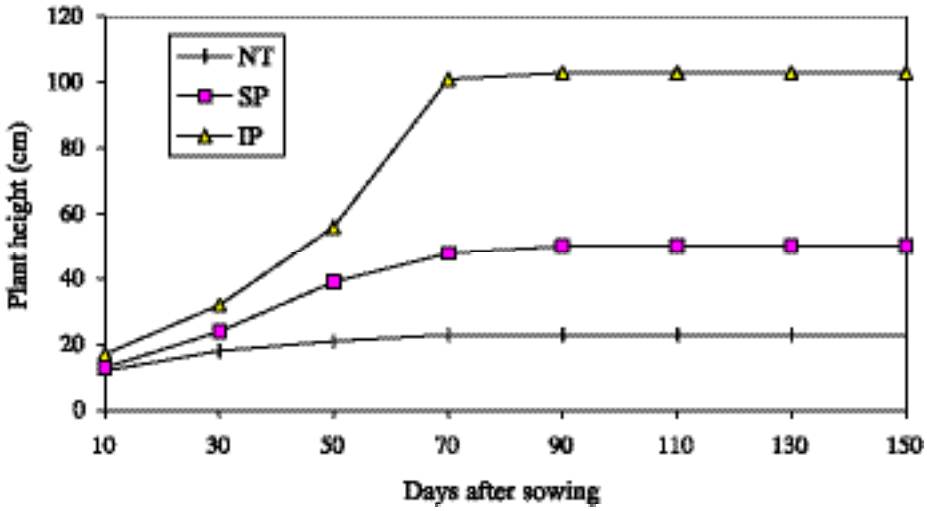

Figure 4 Plant growth at Napok in 1985 (sowing date July 4).

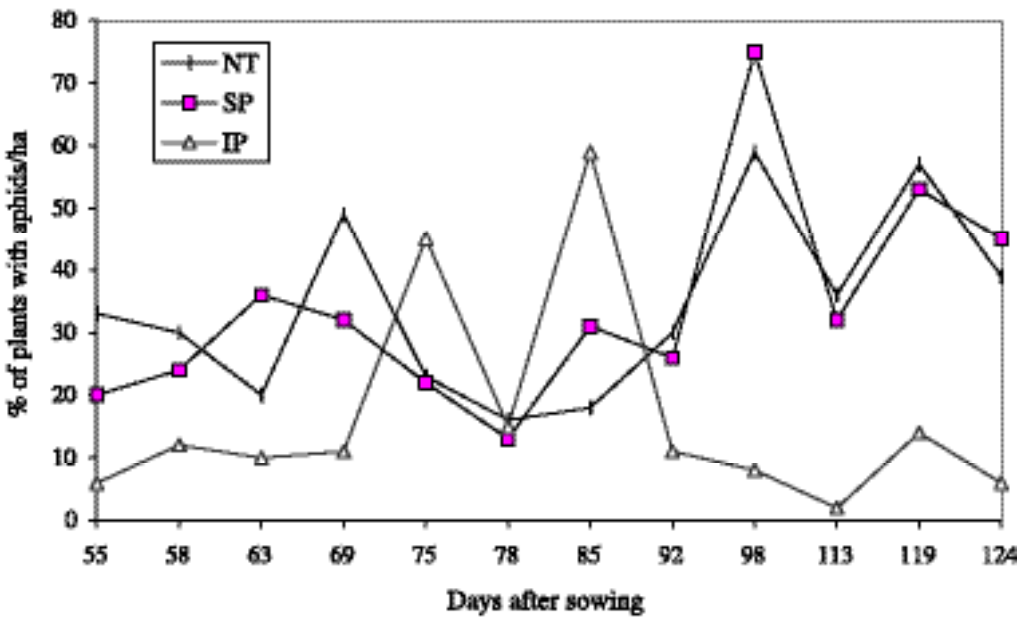

Figure 5 Plant infestation by aphids at Napok in 1985 (sowing date July 4).

of infestation were recorded at the middle of September (almost 69 days after sowing) and the beginning of October (98 days after sowing) with almost 50 % and 75 % of plants attacked, respectively. With IP program, except at 75 and 85 days after sowing, the rate of infested plants remained lower than in NT and SP. This was particularly true during the month of October (from 92 days after sowing) where the infestation decreased quickly with IP program, and affected in most of cases less than 10 % of the plants.

At Ban Hai in 1991 the infestation of aphids was important at the beginning of the growing season, before the application of insecticide, particularly in IP and NT plots (Figure 6). Therefore, the number of infested plants decreased quickly, including in NT plot where no insecticide was applied. Nevertheless, the results showed differences between the treatments. During the whole season the highest level of infestation was recorded in NT plot and the lowest in IP plot.

The data collected at Napok in 1991 indicated that in case of serious infestation of aphids, the standard program of protection was not sufficient to control the development of the aphid colonies.

Control of leafhoppers

Leafhoppers appeared very early, from the second week after the emergence of seedling and the infestation continued until harvesting time. At Napok station in 1985, the populations recorded on NT and SP increased gradually and remained high during the entire crop season (Figure 7). Three peaks of infestation could be observed, at the beginning of July (35 days after sowing) and August (59 days after sowing), and at the end of October (87 days after sowing) with a population of more than 1,200,000 leafhoppers per ha in SP. Only with the intensive protection the density of leafhoppers was lowered to reach a maximum of almost 200,000 leafhoppers per ha in July (43 days after sowing).

At Ban Hai in 1991, the results were not similar to those of Napok (Figure 8). The most important damaging populations were recorded in NT plots. The standard protection, in spite of good efficiency at the beginning and the end of the crop season, could not control leafhopper infestation from 45 to 60 days after sowing.

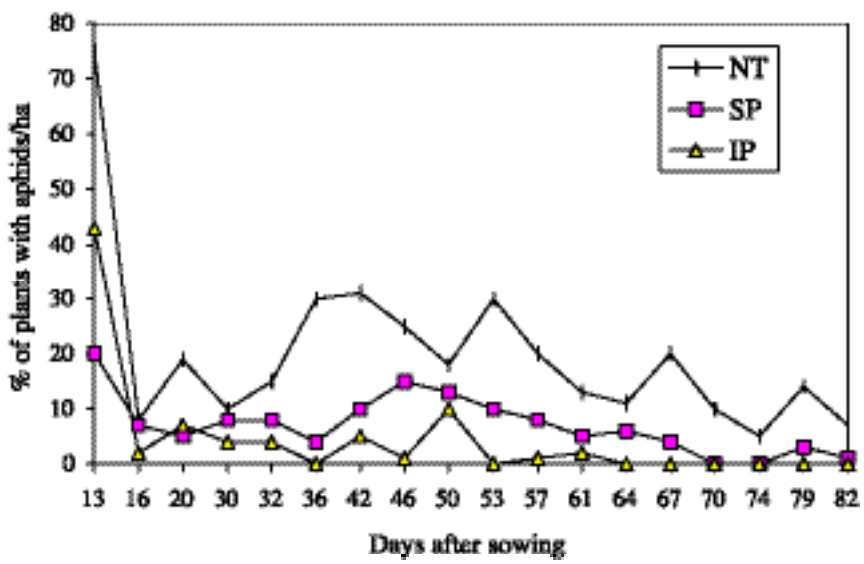

Figure 6 Plant infestation by aphids at Ban Hai in 1991 (sowing date June 1).

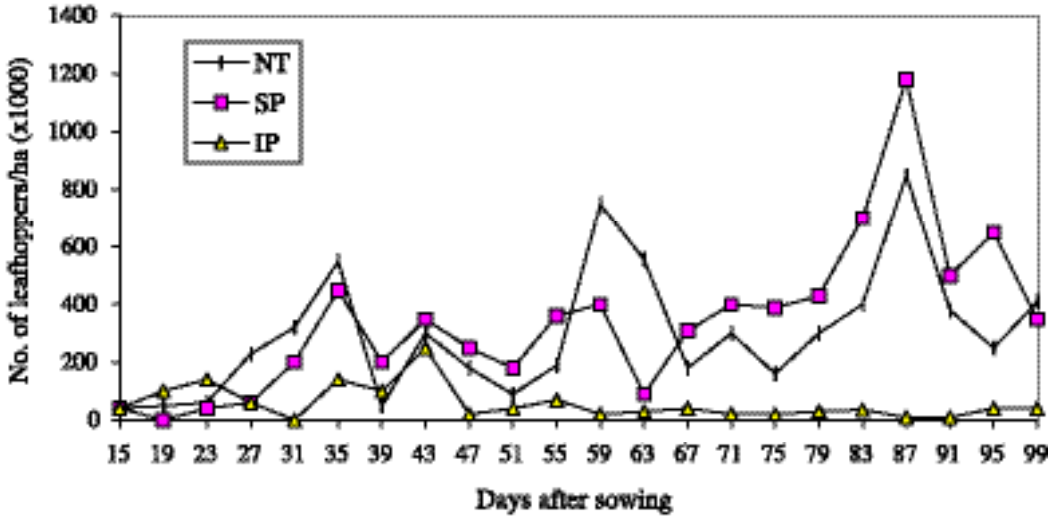

Figure 7 Plant infestation by leafhoppers at Napok in 1985 (sowing date July 4).

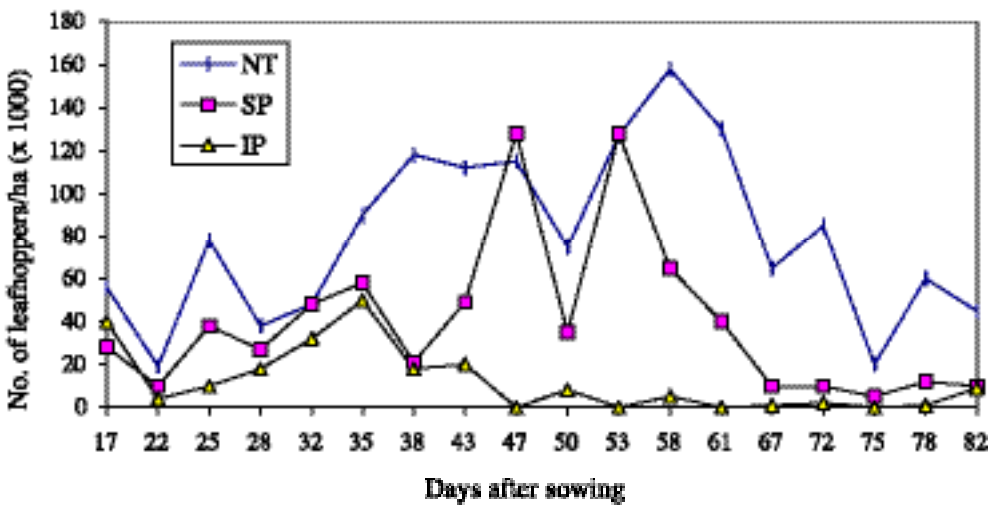

Figure 8 Plant infestation by leafhoppers at Ban Hai in 1991 (sowing date June 1).

Control of bollworm

Bollworm infestation was recorded only in Ban Hai in 1991 (Figure 9). The first larvae were observed at the beginning of the flowering period. The data collected showed that with no insecticide application the population of larvae increased quickly starting from 60 days after sowing and could exceed 10,000 larvae/ha. In SP plots the dynamic of population was almost the same as in NT plot, but with lower density of larvae which did not exceed 3,000 larvae per ha. In IP plots very few larvae were recorded. The population increased at the end of the growing season, around 80 days after sowing, in the three programs of protection. But the level of population depended on the level of protection.

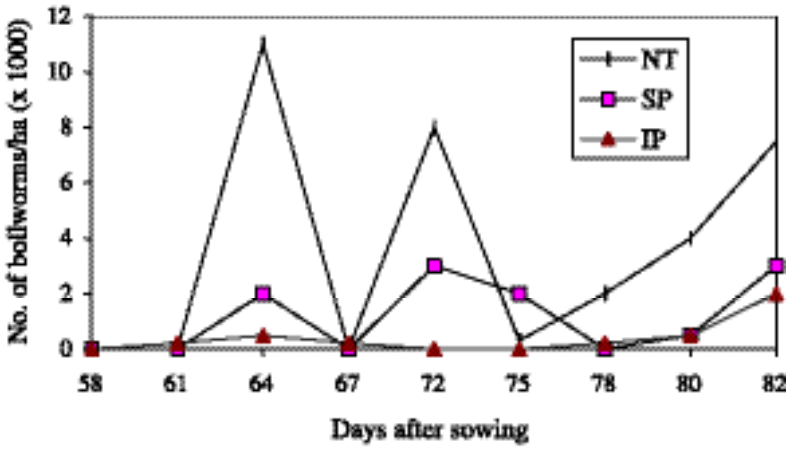

Figure 9 Dynamic of population of bollworm at Ban Hai in 1991 (sowing date June 1).

b) Seed-cotton yield

The production of seed cotton depended on several factors such as climate, soil, variety and pest incidence. Therefore, to estimate the losses caused by insect pests it was necessary to compare the production obtained in NT plot with the one in IP plot which is not significantly correlated with pests incidence ($P>>0.05$; $R^2 = 0.0596$). Similarly, the efficiency of the standard program was estimated comparing the productions obtained in SP and IP plots.

First of all it was noted that the yield varied accordingly with the year and the locality (Table 2). For instance, at Napok station in NT plot, with the same variety SR 2, the production was 742 kg/ha and 66 kg/ha in 1989 and 1990, respectively. This difference was due, in part, to the level of pests incidence, particularly leafhopper. In NT plot in1985 with the variety DI.5 the production was 12 kg/ha at Napok and 339 kg/ha at Luang Prabang. The same variation was observed in 1991 with the variety KK1 planted in four locations, Ban Hai, Savannakhet, Bolikhamxay and Sayabury. In this case the pest incidence was the highest at Sayabury, with 86 % of losses in NT comparing with IP, and was the lowest at Ban Hai with 65 % of losses. In spite of these important variations, the results showed that in Lao PDR, if no insecticide protection is administered, the average losses due to pests could reach about 70 % of the potential production of the crop (Table 2). This estimation is almost identical to the one obtained in Thailand, where the average loss of production due to all the pests fluctuating between 70 % and 80 % of the potential production (Genay, 1994). With the standard program, the average loss decreased to around 30 % in comparison with IP program (Table 2). It indicated that, even if the yield obtained with SP program was positively correlated with the yield obtained with IP ($P< 0.01$) (Figure 10), with seven sprays of insecticide it was not possible to reach the potential production. Moreover, the average loss of seed cotton between NT and SP is 700 kg. Actually the price of dimethoate 400 g ai/l and deltametrin 12.5 g ai/l is US $ 9/l and US $ 22/l respectively, whereas the kg of seed cotton bought to the farmers is almost 22 cents. That means that a program of 7 insecticide applications, 2 with dimethoate followed by 5 with dimethoate mixed with deltametrin, costs approximately the equivalent of 800 kg of seed-cotton and is not profitable. If all the results are taken into account, the efficiency of SP program was not significantly influenced by pests incidence ($P>0.05$) (Figure 11). Nevertheless, the tendency

showed by the curve (Figure 11) is confirmed if the data recorded at Luang Prabang in 1985 is not taken into account (P< 0.05; R? = 0.3276). In this case, the more important the pests incidence, the less efficient the standard protection.

The comparison of seed-cotton yield among varieties showed that without pest control the variety mostly affected was DI.5, with 93 % yield losses, and the least damaged was Fai Niai, with only 55 % yield losses (Table 2). Even though these results

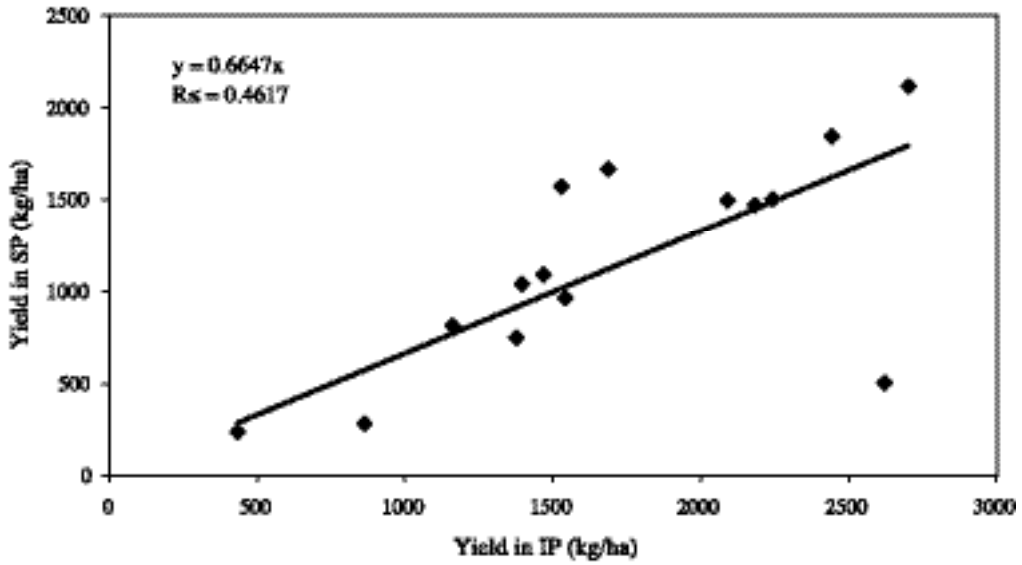

Figure 10 Corellation between the yields obtained with IP and SP programs.

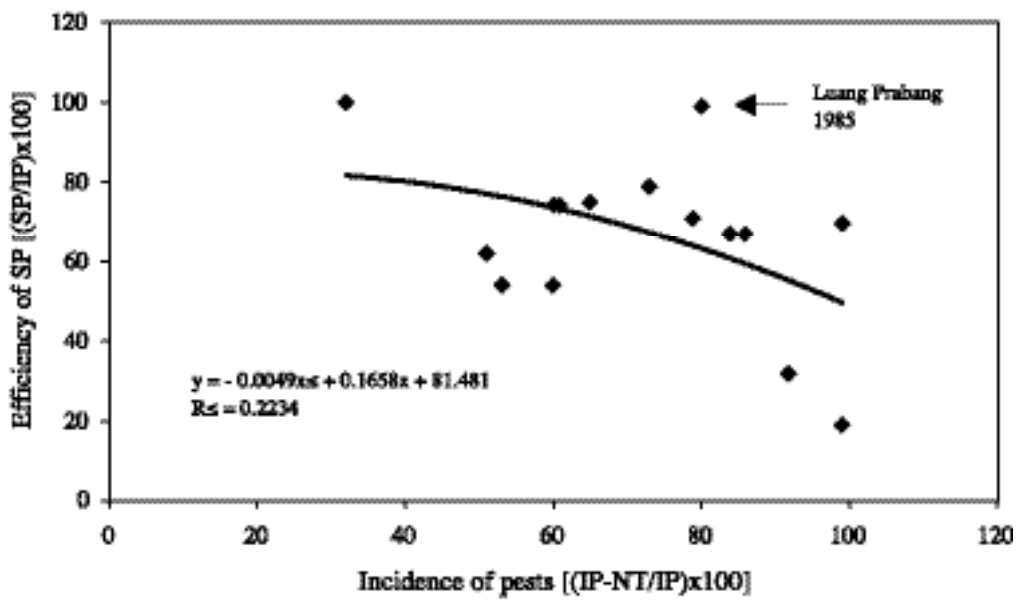

Figure 11 Relationship between the incidence of pests and the efficiency of SP program.

must be confirmed by more experiments, it is interesting to note that the two varieties, DI.5 and SR 2, are almost glabrous whereas KK 1 and over all Fai Niai are hairy. Consequently, this variation of productivity can be explained, at least in part, by the degree of sensibility to leafhoppers. As demonstrated by Parnell *et. al.* (1949) the pubescent varieties are resistant to this pest whereas the glabrous ones are highly affected. More recently, studies carried out in Thailand pointed out the implication of hairiness in the control of *A. bigittula* and the importance of this variety character for the improvement of Integrated Pest Management Strategies in South-east Asia (Castella, 1995; Renou, 1999).

CONCLUSION

The most important pests of cotton recorded from Lao PDR were the leafhopper *A. biguttula* and the bollworm *H. armigera*. But the damages caused by these insects were widely variable among locations and from one year to another. Nevertheless, the trials carried out for seven years in different regions of the country showed that the losses of production could reach an average of 70 % of the potential production when no insecticide was applied. The standard program of seven insecticide applications recommended to the farmers could help reduce the losses to 30 % on average, but its efficiency depends on the rising level of pest incidence and on the capacity of the variety to resist to leafhoppers attack. Moreover it increases the cost of production too much, and it is not profitable. Consequently, the chemical solution to control insect pests, appears to be very expensive and unsuitable according to the conditions of production encountered in Lao PDR.

To face this situation, it is important to develop in Lao PDR an Integrated Pest Management strategy aiming at reducing the pest incidence but maintaining a low production cost. As shown by the experiments, the first step to reach such a goal is to plant hairy cotton varieties with high resistance to leafhopper. This alternative should eliminate the insecticide applications to control this homoptera. Nevertheless, as it was demonstrated in Thailand whatever the cultivar, cotton leaf is more suitable for leafhopper egg deposition during the two first weeks of plant development, before the appearance of hairs. Moreover, in Lao PDR the field infestation by early pests like leafhoppers and aphids can take place during this period. From these observations it is possible to recommend to treat the seeds with an insecticide to control the early pests. This technique avoids insecticide sprayings at the beginning of the season and consequently leads to a better preservation of the beneficial species. As mentioned above, the other main pest encountered in Lao PDR was the bollworm *H. armigera*. No variety character seems to be efficient enough to avoid attack of *Helicoverpa*. Neither morphological character like atrophied or absent bracts (frego bracts) which can hinder oviposition (Angelini *et al*., 1965; Matthews, 1989), high gossypol which have an antibiotic effect on bollworm (Vaissayre *et al*., 1997), nor the combination of both (Khalifa, 1979) result in a satisfactory level of protection. Consequently, the control of this pest is until now mainly based on insecticide applications. But, the creation of genetically modified cotton varieties able to produce toxin of the bacteria *Bacillus thuringiensis* to control bollworm offers new possibilities. This has already been applied on a large scale in some countries, principally the U.S.A. (more than 70% of cotton acreage in 2000) and China (more than 10% of cotton acreage in 2000) (Giband *et al.*, 2001). This alternative to chemical control could be experimented in Lao PDR within the framework of an integrated pest management strategy.

Finally, some additional measures, like planting date or intercropping could be of interest for Lao production, and should be tested.

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Toxicity of 4,11-Selinnadien-3-one from Nutsedge (*Cyperus rotundus* L.) Tuber Extracts to Diamondback Moth Larvae *(Plutella xylostella* L.), Detoxification Mechanisms and Toxicity to Non Target Species

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ABSTRACT

Tubers of nutgrass (*Cyperus rotundus* L.) were collected from various locations of Thailand during the summer and rainy seasons. Toxicity against the diamondback moth (*Plutella xylostella* L.) was observed using different concentrations of the active compound, 4,11-selinnadien-3-one. The toxic effects were also determined on mice (*Mus musculus*), fish (*Poecillia reticulata*) and bee larvae (*Apis florea)*.

It was found that the active principle of nut grass was higher in summer than that in rainy season by ca. 2 folds. This active principle varied according to geographical vareas with Chanthaburi and Chaing Mai producing the highest amounts of 4,11-selinnadien-3-one (0.13-0.16% ai. yield) compared with the other. The LC_{50} against 2^{nd}–3^{rd} instar larvae of diamondback moth were 7-12 ppm. Detoxification enzyme activities as well as synergistic effects revealed that monooxygenase, esterases and some degrees of glutathione-S-transferase played a role in detoxification. Furthermore, synergists, PB and TPP, could raise the effectiveness of the active principle up to ca. 2-6 fold. At 2,000 ppm of 4,11-selinnadien-3-one, exposed mice showed no sign of acute dermal, acute oral or eye irritation effects. However, the active principle was toxic to other non target organisms with LC_{50} of 28.01 ppm and 10.8 ppm to 1-month old guppies and bee larvae, respectively.

Key words: nut grass, *Cyperus rotundus*, diamondback moth, *Plutella xylostella*, detoxification enzymes

INTRODUCTION

Thailand faces several pesticide problems, in terms of residues in food crops, insect resistance to pesticides, toxicity to humans and non target species as well as the high cost of pesticides used in agricultural production (Valleyaluck, 1983).

Currently, Thailand has imported some 20,000 tons of pesticides was imported into the country costing thousands of millions of Thai baht (Katanyukul, 2002). Although pesticide application method of controlling pests, this method is experiencing numerous problems especially in the developing world where knowledge of pesticide toxicity has been ignored (Visetson, 2001).

Apart from toxic residues in food, environmental pollution and the high cost of pest control, pesticide resistance has also become pronounced in terms of cross-resistance and has been a major problem for vegetable producing farmers. The diamondback moth is one pest that has shown a quick development of cross resistance

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and is one of the most important pests in the Central Plain of Thailand.

Botanical pesticides derived from neem, lemon grass, galanga, and Siam weed have been proven to be appropriate insecticide alternatives for control of insect pests (Schmutterer, 1990; Singh *et al*. 1989; Visetson and Milne, 2001). At least 5 commercial products from these plants have been introduced into the Thai pesticide market (Visetson, 2001), including products recommended for use against diamondback moth.

Ohsawa*et al*. (1996) found that crude extracts of nut grass (*Cyperus rotundus* L.) tubers gave 80% mortality to diamondback moth larvae in 1 hour post treatment. They indicated that the active compound, 4, 11-selinnadien-3-one, was responsible for killing the larvae. Nut grass also called purple nutsedge, belongs to the Family Cyperaceae. This grass is beneficial as a herb for curing stomach complain (Kiritikar and Basu, 1991). Although Thai farmers regard it as a major weed, it is used as an ingredient in herbal medicine to maintain body function (Anonymous, 1986).

In this research we report the efficacy of the active compound, 4,11-selinnadien-3-one, extracted from the tubers of nut grass for controlling diamondback moth larvae as well as identify its detoxification mechanisms in terms of enzyme reactions, namely esterase, glutathione-S-transferase and monooxygenase activity. Toxicity tests were also conducted for non target organisms, namely the mouse (*Mus musculus*), guppy (*Poecillia reticulata*) and bee larvae (*Apis florea)*.

MATERIALS AND METHODS

Insect larvae and plant samples

Diamondback moth larvae were collected from a vegetable producing area in Kanchanaburi province, 150 kms west of Bangkok. The larvae were cultured for two generations under laboratory conditions at 23 ± 2°C following the method of Leckprayun *et al*. (1999). Nut grass tubers were collected from 5 different locations, Chaing Mai, Ubon Ratchathani, Kanchanaburi, Chanthaburi, Songkhla, in Thailand during the rainy and summer seasons.

Plant extraction and efficacy tests

Five kilogrammes of each sample from each location and each season were ground and dried at room temperature. Ethanolic Soxhlet extraction at 70°C was administered for 48 hours. The crude extracts were then evaporated and freeze dried. The yield of extract was measured. The extracts were carried out by the modified method of Ohsawa *et al.* (1996) with a counter-current distribution method using n-hexane and methanol. The product was separated by silica gel column chromatography using acetone and n-hexane as solvents. The first fraction was used to quantify the amount of 4,11-selinnadien-3-one. Highly purified (99.9%) 4,11-selinnadien-3-one, purchased from Merck Ltd. (Thailand), was used as a standard. The second fraction was employed for preparative TLC using acetone and n-hexane at the ratio of 1:9. The final extracted product was kept at -20°C until required for the following experiments.

The extracts were diluted with acetone and tested against 3^{rd}–4^{th} instar larvae of the diamondback moth. Three replicates, comprising 20 larvae each, were used. A 5% leaf surfactant, triton X-100, was mixed with each concentration prior to use. A no-chioce leaf dipping method was used for the experiments. One leaf circle disk of Chinese kale with diameter of 5 cm was placed for each group of larvae. Mortality was checked after 24 hours exposure. A Completely Randomized Design with 3 replicates was used. All experiments were run at 23 ± 2 °C. Abbott's formula (Matsumura, 1976) was employed to correct control mortality. LC_{50}'s were calculated from regression equations.

Detoxification mechanisms and synergist assays

The life larvae from treatments were used in *in vitro* assays to optimize enzyme activity of

esterase, glutathione-S-transferase and monooxygenase following the methods of Visetson and Milne (2001) and modified from Rose (1985) using paranitrophenyl acetate (PNPA), chlorodinitrobenzene (CDNB) and aldrin to determine enzyme activity. The synergists, piperonyle butoxide (PB), triphenyl phosphate (TPP) and diethyl maleate (DEM), were used at 1% to detect the inhibition mechanisms of activity of the enzymes, monooxygenase, esterase and glutathione-S-transferase, respectively.

Toxicity tests on mice, fish and bee

The 2,000 ppm extract was used to test for acute dermal, oral and eye irritation toxicity on 2 month-old mice (*Mus musculus*) following the method of Ecobichon (1992). Hair on the back of the mice was shaved prior to one treatment of the extract containing 2,000 ppm 4, 11-selinnadien-3-one to be applied. Addition of the extract in the food pellets prior to feed the mice were assayed for acute oral test. The extract was applied topically in the mouse eyes for irritation test. The toxicity was determined by the abnormality of the dermal tissues after 14 days exposures. LC_{50}'s were determined for 1 month old guppies (*Poecillia reticulata*) and bee larvae (*Apis florea)*.

DMRT was employed for all mean comparisons using a significance level of probability > 95% following the method of Finney (1964).

RESULTS AND DISCUSSION

Extraction and 4,11-selinnadien-3-one

The ethanolic Soxhlet extraction of nut grass tuber showed 15-25% more yields in summer than in the rainy season (Table 1). In summer, samples collected from Chaing Mai and Chanthaburi gave the highest contents of 4, 11-selinnadien-3-one (0.13-0.16%w/w) on a dried weight basis. Nut grass tuber extracts from these areas showed 15% less active compound in the rainy season than in the summer. Possible reasons for this included hydrolysis of the active compounds, increased metabolism during the season or the effect of higher moisture content in the tuber prior to extraction. These results were similar to the fluctuations of azadirachtin content of neem seed kernels found in

Table 1 Percentages of 4, 11-selinnadien-3-one in nut grass extracts from samples collected during two seasons from different parts of Thailand.

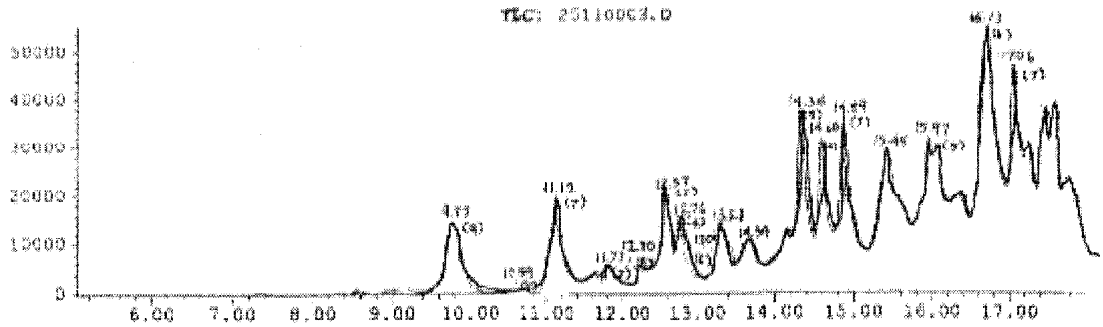
Province	% (w/w) [1,2,3,4]	
	Summer	Rainy
Chaing Mai	0.13 ± 0.07b	0.09 ± 0.02a
Ubon Ratchathani	0.09 ± 0.02b	0.06 ± 0.02b
Kanchanaburi	0.04 ± 0.04a	0.03 ± 0.02a
Chanthaburi	0.16 ± 0.03b	0.09 ± 0.06a
Songkhla	0.03 ± 0.05a	0.03 ± 0.01a
Average	0.09 ± 0.04	0.06 ± 0.03

1 means followed by different letters within the same column are significantly different at $P < 0.05$

2 means ± SD, 5 replicates for each area and season. Larvae from F_2-generation were used for all experiments at the same period of time.

3 all extracts made using ethanolic Soxhlet extraction at 70-80°C, 48 hours

4 The method of 4, 11-selinnadien-3-one identification was per Ohsawa *et al.* 1996 and the high purity standard of the active ingredient was purchased from Merck (Thailand).



Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
1	9.77	10.88	C:\DATABASE\WILEY275.L			
			Cyperene $$ 3H-3a,7-Methanoazulene, 2	89329	002387-78-2	98
			.alpha.-Gurjunene $$ 1H-Cycloprop[e]a	89468	000489-40-7	91
			(-)-.ALPHA.-GURJUNENE	89794	000000-00-0	91
2	10.09	1.43	C:\DATABASE\WILEY275.L			
			Dispiro(cyclopropane-1,2'-bicyclo[2.2	32938	094348-06-8	25
			1,5-diphenylhex-3-ene	122491	103240-60-4	25
			1,5-Diphenylhex-3-ene	122507	000000-00-0	25
3	11.12	11.50	C:\DATABASE\WILEY275.L			
			trans-Pinocarveol $$ Bicyclo[3.1.1]he	38420	000547-61-5	38
			trans-Pinocarveol $$ Bicyclo[3.1.1]he	38422	000547-61-5	35
			Bicyclo[3.1.1]heptan-3-ol, 6,6-dimeth	38425	005947-36-4	35
4	11.77	0.96	C:\DATABASE\WILEY275.L			
			1-.alpha.-Terpineol $$ 3-Cyclohexene-	40197	010482-56-1	53
			3-Cyclohexene-1-methanol, .alpha.,.al	40185	000098-55-5	50
			.DELTA.3-Carene $$ Bicyclo[4.1.0]hept	25254	013466-78-9	47
5	12.10	1.02	C:\DATABASE\WILEY275.L			
			(1,3E,5E)-Undeca-1,3,5-triene	36322	051447-00-6	43
			(tetrahydroxycyclopentadienone)cricar	164566	117896-75-0	38
			(Tetrahydroxycyclopentadienone)cricar	164567	000000-00-0	38
6	12.37	3.90	C:\DATABASE\WILEY275.L			
			Bicyclo[3.1.1]hept-2-ene-2-carboxalde	35961	000564-94-3	50
			3,4-DIMETHYLPYRIDINE	6885	000000-00-0	49
			Benzenemethanol, .alpha.-methyl- (CAS	15789	000098-85-1	49
7	12.76	3.97	C:\DATABASE\WILEY275.L			
			Myrtenol $$ Bicyclo[3.1.1]hept-2-ene-	38412	000515-00-4	90
			MYRTENOL	27977	000515-00-4	90
			Myrtenol $$ Bicyclo[3.1.1]hept-2-ene-	38413	000515-00-4	89
8	13.09	1.68	C:\DATABASE\WILEY275.L			
			(Cyclopropyl)trivinylsilane	35641	000000-00-0	47
			a-Citral $$ 2,6-Octadienal, 3,7-dimet	37677	000106-26-3	38
			Cyclohexanol, 3,3,5-trimethyl- (CAS)	29939	000116-02-9	35
9	13.28	2.15	C:\DATABASE\WILEY275.L			
			1-Phenanthrenol, 2,4,4a,4b,5,6,7,8,8a	133576	057804-14-7	42
			1-Phenanthrenol, 2,4,4a,4b,5,6,7,8,8a	133574	057804-13-6	42
			METHYL 2,5-DIMETHYLTHIO-9-OXOGERANOAT	171438	060774-84-7	28
10	14.34	8.57	C:\DATABASE\WILEY275.L			
			Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-	35974	000080-57-9	86
			Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-	35969	000080-57-9	76
			Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-	35968	018309-32-5	68

Figure 1 GC-mass spectroscopy chromatogram (methods as described in the text) showing the different compounds that constituted the extracts.

various seasons and places in a tropical climate (Schmutterer, 1990).

Chromatograms, from GC-mass spectroscopy following the method of Ohsawa *et al*. (1996), showed that 10 active principles had accumulated in the tubers (Figure 1). The figures also revealed that 4, 11-selinnadien-3-one concentration was higher in our extracts than those of Ohsawa*et al.* (1996). This may indicate location influences, e.g. trace elements which play a role in the accumulation of secondary plant substances in major plants. These results also showed the same trend as rotenone extracted from derris root collected from different locations (Visetson and Milne, 2001).

Efficacy against diamondback moth larvae

The no-choice leaf dipping tests showed significant variation in efficiency against the 3rd-4th instar diamondback moth larvae within the same season (Table 2). On the other hand, differences were pronounced when comparisons were made between two seasons. The extracts showed 1.5-2.0 folds lower LC_{50}'s in the summer than in the rainy season ca. 1.5-2.0 folds. Chantaburi and Ubonratchatani gave the lowest LC_{50} of 7.05 ± 0.03 and 9.06 ± 2.02 ppm in summer but in the rainy season the LC_{50} increased ca. 2 folds. These figures indicated that there were either some substances other than 4,11-selinnadien-3-one in the extracted samples in summer and these substances might have elevated the mortality or some other substances accumulated in tubers in the rainy season might have reduced the mortality. Future experiments should look at other compounds extracted from the tubers that influence the larvae mortality. However, the aims of this experiment were to look at efficacy and detoxification mechanisms in this insect as well as to find the way to use extracts in the field. It is recommended to collect tubers in the summer rather than in the rainy season.

Mechanisms of enzyme activity and synergistic effects

The three synergists, PB, TPP and DEM, increased the efficacy of 4,11-selinnadien-3-one ca. 2-3 folds in samples collected in the summer and increased the efficacy ca. 2-6 folds in samples from the rainy season (Table 3).

Table 2 LC_{50} values for 4,11-selinnadien-3-one in nut grass extracts against 2nd -3rd instar larvae of the diamondback moth (*Plutella xylostella L.*) with a no-choice leaf dipping method for tubers collected during two seasons from different parts of Thailand.

Provinces	ppm [1,2]	
	Summer[3]	Rainy[4]
Chaing Mai	10.05 ±2.03a	17.11 ±2.11a
Ubon Ratchathani	9.06 ±2.02a	19.16±2.12b
Kanchanaburi	12.05± 1.01b	18.12 ±4.07a
Chanthaburi	7.05 ±0.03a	12.11 ±1.03a
Songkla	15.09±1.02b	25.18 ±1.04b

1 means followed by different letters within the same column are significantly different at $P < 0.05$

2 means ± SD, 5 replicates for each area and season. Larvae from F_2 -generation were used for all experiments at the same period of time.

3 all extracts made using ethanolic Soxhlet extraction at 70-80°C, 48hrs. following the method of Ohsawa *et al*. (1996).

4 no-choice leaf disks tests with 20 individuals of 2nd -3rd instar larvae were employed, 24 hours , LD_{50} from regression equation of discriminating doses from 10-90% mortality.

Table 3 LD_{50} and enzyme activity after addition of 1% synergist extracts containing 4,11-selinnadien-3-one extracted from nut grass tubers collected during two seasons from Chanthaburi with synergistic ratio (SR), correction factors {CF} and correlation determinations [r^2] also presented.

Synergist added	LD_{50} and Detoxification enzyme activity [1,2,3,4]					
	Summer (SR)[r^2]	Enzyme activity[5]	{CF}	Rainy (SR)[r^2]	Enzyme activity	{CF}
None	7.05 ±0.03b	Est	12.14 ±2.23	12.11 ±1.03c	Est	16.23 ±5.83
		GSH	32.13 ±2.46		GSH	42.45 ±6.48
		Mo	4,320 ±126		Mo	5,550 ±422
PB	2.41 ±1.2a	Mo	2,111 ± 233	2.32±2.05a	Mo	3,232 ±333
	(2.92) [0.8]		{2.04}	(5.22)[0.9]		{1.71}
TPP	2.11 ±1.13a	Est	8.14 ±2.11	2.14±3.02a	Est	9.12 ±3.13
	(3.34)[0.7]		{1.49}	(5.66) [0.8]		{1.78}
DEM	5.13±1.23b	GSH	21.22 ±2.76	5.12±1.10b	GSH	29.15 ±2.24
	(1.37)[0.6]		{1.51}	(2.37)[0.6]		{1.46}

1 means followed by different letters within the same column are significantly different at P = 0.05

2 means ± SD, 5 replicates for each area and seasons. Larvae from F_2 -generation were used for all experiments at the same period of time.

3 all figures for extracts made using ethanolic Soxhlet extraction at 70-80°C, 48 hrs. following the method of Ohsawa et al. (1996)

4 no-choice leaf dipping tests with 20 individuals of 3^{rd} –4^{th} instar larvae were employed. 24 hr checked.

5 Est, GSH and Mo stand for esterase, glutathione-S-transferase and monooxygenase with units of nM paranitrophenol produced/min/mg protein, nM DCNB conjugated product/min/mg protein and picM aldrin epoxidation/min/mg protein, respectively. CF was a correction factor derived from the division of enzyme activity with no synergist by synergistic enzyme activity. The synergistic ratio, SR, was derived from division of the LD_{50} with no synergist by the synergistic LD_{50}. r^2 was a correlation determination between LD_{50} and enzyme activity. "None" means no synergist was added to the active compound.

Moreover, correlations ($r^2 > 0.8$) were found between efficacy and enzyme activity, thus indicating that monooxygenase and some general esterases play a role in detoxification of this compound. Synergistic ratios (SR) and correction factors (CF) for the synergists, PB and TPP, showed that addition of these synergists resulted in a high inhibition of monooxygenase and esterase, respectively, in diamondback moth larvae. These results confirmed those of numbers of work in the area of detoxification mechanisms (Rose, 1985; Mackness *et al*., 1983; Yu and Hsu, 1985 and Visetson and Milne, 2001). The results showed that esterase, glutathione-S-transferase and monooxygenase would be inhibited 2-folds after addition of PB, TPP and DEM. These mechanisms were different from those involved with the detoxification of rotenone extracted from derris and of azadiractin from neem which revealed that monooxygenase was the only enzyme system that played a major role (Visetson and Milne, 2001). On the other hand, esterase had a major role in the detoxification of galanga and Siam weed extracts (Visetson *et al*. 2001). In another case, cholinesterase played a role in detoxifying citronella extracted from lemon grass in the dog tick (Visetson and Chuchouy, 1999). It can be concluded that because more than two mechanisms are involved in the detoxification of the 4,11-selinnadien-3-one in this insect larvae, nutgrass tuber extract should be a better insecticide alternative to control this insect pest. Any pesticide in which more than one

mechanism is involved in its detoxification results in a longer time taken for the pest to produce resistant genes (Yang *et al*., 2001; Yu, 1983; Yu, 1984).

Toxicity to non target species

The concentration of 2,000 ppm of 4,11-selinnadien-3-one in the extract from nut grass tubers gave no signs of growth effects (Figure 3). In addition, no acute dermal, acute oral as well as eye irritation were observed after 14 days of exposure. These results indicated no toxic effects based on the criteria of Ecobichon (1992).

One-month old guppies (*Poecillia reticulata*) were cultured in aquaria containing 10, 20, 30, 40 and 50 ppm of active ingredient. The mortality after 24 hours exposure showed 13.12 ±1.16, 38.28±2.12, 58.11±1.45, 88.35±2.34 and 96.12±1.23%, respectively, and gave an LC_{50} of 28.01 ppm (Y = -6.02 + 2X) (Table 5).

Nut grass tuber extracts at 1, 3, 5, 10 and 20 ppm of 4,11-selinnadien-3-one were applied to 1 week-old bee larvae (*Apis florea)* using a topical mist method and showed 8.12 ±3.36, 10.23±3.56, 34.39±2.51, 67.12±10.44 and 89.32±12.14% mortality, respectively, after 24 hours exposure indicating a LC_{50} of 10.8 ppm (Y = - 6.8 + 4X) (Table 6). Because this $LC_{50}$was more or less similar to the LC_{50} for diamondback moth larvae, then care must be taken with using nut grass extracts in areas of large bee populations. However, toxic levels on adult bee should be done in the future to evaluate the safety level in the bee population.

Table 5 Effects of 4,11-selinnadien-3-one extracted from nutgrass tubers on juvenile guppies (*Poecillia reticulata*).

Concentration (ppm)[1,2]	% mortality
10	13.12 ±1.16
20	38.28±2.12
30	58.11±1.45
40	88.35±2.34
50	96.12±1.23
	(LC_{50} = 28.01ppm, Y = -6.03 + 2X)

[1] means ± SD, n = 20, 0ne-month old juvenile fish.

[2] the active compound dissolved in acetone and then poured into water to make each concentration. Jars containing only acetone in water served as untreated controls, 24 hr check

CONCLUSION

Nut grass tubers in summer showed higher contents of 4,11-selinnadien-3-one compared to tubers collected in the rainy season. Chiengmai and Chantaburi tuber extracts had the highest 4,11-selinnadien-3-one contents. The LC_{50} against 3rd-4th instar larvae of diamondback moth was 7 – 12 ppm. The addition of PB, TPP and DEM to the extracts increased their efficacy. The reduced amounts of esterase, monooxygenase and glutathione-S-transferase after synergists were added indicated that more than one mechanism were involved in the detoxification of this compound. These results show that the insect may

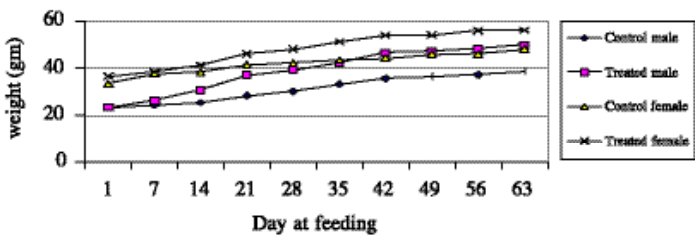


Figure 2 Growth rates of mice after feeding on nut grass tuber extract at 2,000 ppm 4,11-selinnadien-3-one.

Table 6 Effects of 4,11-selinnadien-3-one from nutgrass extracts on bee larvae (*Apis florea).*

Concentration (ppm)	% mortality[1,2]
1	8.12 ±3.36
3	10.23±3.56
5	34.39±2.51
10	67.12±10.44
20	89.32±12.14
	(LC_{50} = 10.8 ppm, Y = -6.8 + 4X)

[1] means ± SD, n = 20 individuals of 1-week old bee larvae.
[2] the active compound dissolved in acetone to a certain concentration then applied to the larvae. Acetone served as untreated controls. 24 hr check

take more time to produce resistance to this substance compared with other plant products. Although the substance did not show acute dermal, acute oral and eye irritation to the mouse, the LC_{50} values for diamondback moth larvae and bee larvae were similar. Therefore care must be taken in the use of this compound where bees are important to an area. This compound expressed a LC_{50} ca. 28.01 ppm for the guppy so the use of this extract near water was quite safe for fish. However, purification of both the enzyme systems and the active compound are crucial for the future in order to determine the exact synergistic relationships. This will lead to improvement of alternative plant substances for production on a commercial scale in the future.

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Effect of Seasonal Variations on Production of Australian Friesian Sahiwal (AFS_3) Cows in Thailand

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ABSTRACT

Data from Tabkwang Research and Breeding Centre, Department of Livestock Development, Ministry of Agriculture and Cooperatives, during 1993–1999, were used to determine the effect of climatic conditions on production performance of Australian Friesian Sahiwal Appendix-3 (AFS_3) cows .

The results revealed that summer had the highest THI ($P<0.05$) while winter had the lowest THI ($P<0.05$). The AFS_3 cows had the lowest productive performance during summer in Thailand.

Key words: AFS_3 cows, production performance, seasons, tropical conditions

INTRODUCTION

The effect of heat stress on animal production has been investigated and documented for a number of years (Brody *et al.*, 1949; Johnson, 1985). In pioneering research works at the Climatology Laboratory in Missouri, USA, the relationship were established between high ambient temperature and increased rectal temperature of dairy cows associated with the subsequent impact on milk yield (Kibler and Brody, 1950). Elevated body temperature was due largely to a reduction in the temperature gradient between skin surface and the environment. High relative humidity reduces cutaneous evaporation and thus makes more difficult body heat dissipation as the environmental temperature is approaching the cow's body temperature (Gerbermedhin, 1985). The interaction of the environment and body temperature would affect body heat dissipation and dairy production. Then the challenge in managing dairy cattle under tropical conditions is to minimize the need for compensatory reactions for thermal balance that compromise dairy productivity (Johnson, 1985).

The Australian-Friesian-Sahiwal Appendix 3 cattle (AFS_3) were imported from Australia and kept at the Tabkwang Livestock Research and Breeding Centre (TRBC), Saraburi Province, Thailand. The AFS_3 was the third generation of the Australian-Friesian-Sahiwal cattle consisting of 75% Friesian and 25% Sahiwal types. Their production under the Australian conditions were 3,000 kg/lactation with milk protein and fat percentage of 3.4% and 4%, respectively (Anon, 1989). In Thailand, Bunyanuwat *et al*. (1996) reported that production of the AFS_3 cattle during

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their second lactation (November 1995–October 1996) in terms of daily milk yield, daily 4% fat-corrected milk (FCM) yield, milk fat content, milk fat percentage and conception rate at ambient temperature of 28.9°C and relative humidity (RH) of 74.1% were 10.5 kg, 10.2 kg, 0.39 kg/d, 3.9% and 32.5%, respectively.

The objective of this investigation is to assess the effect of seasonal variations on milk production and reproductive performance of the AFS_3 cows under the area of Khang-khoi district, Saraburi Province.

MATERIALS AND METHODS

Data from TRBC, Department of Livestock Development, Ministry of Agriculture and Cooperative, during 1993-1999, were used. The TRBC is located approximately 123 km north of Bangkok and at Latitude 14° 13′ N, Longitude 100° 54′ E and altitude of 70 meters above sea level (Tabkwang Livestock Research and Development Centre, 1996).

Data collection and analysis

Records from a total 1,117 AFS_3 cows during 1993-1999 were used to determine the effect of climatic conditions on milk production and reproductive performance of AFS_3 cows.

The cows were fed with fresh forage and hay supplemented with meal concentrate to meet the requirement according to the NRC standard (NRC, 1989). They were twice daily (05:00 am and 3:30 pm) milked in the Herring Bone milking parlour. The udders were routinely cleaned and tested for mastitis before milking and teat dipping with iodine solution was done to prevent mastitis after milking. The cows were pregnancy checked by rectal palpation at day 60 post artificial insemination (AI).

Meteorological data from the Muak-Lek Weather Station, Saraburi Province between 1993-1999 were also used in the analysis. The data consisted of daily maximum-minimum temperature, wet-dry bulb temperatures and RH. The temperature–humidity index (THI) was also calculated using the method of Johnson*et al.* (1963) as followed:

THI = td - (0.55 - 0.55 RH) (t_d - 58)

where: t_d = dry bulb temperature (°C)

RH = relative humidity (%)

Months of the years 1993-1999 were also categorized into seasons according to general chronological classification of the Meteorological Department (1998), Ministry of Transports and Communication;

- Rainy season (May 1 to September 30)
- Winter season (October 1 to January 31)
- Summer season (February 1 - April 30)

Mean maximum-minimum temperatures (°C), RH (%) and THI, and milk yield (kg/lactation), 305-day standardised milk yield (kg), average milk yield (kg/d), days in milk (d), number of service (time), calving interval (d) and pregnancy period (d) were collected and compared according to seasons using the least square analysis of variance (Harvey, 1975) and SAS (1985) for the calculation. The statistical model was:

$$Y_{ijk} = \mu + A_i + B_j + AB_{ij} + E_{ijk}$$

Where

Y_{ijk} = observed value at k, lactation i and season j.

μ = mean of total observed values.

A_i = effects of lactation (i = 1, 2, 3, 4)

B_j = seasonal effects (j = 1, 2, 3, 4)

AB_{ij} = co-relation between lactation and season.

E_{ijk} = random sampling effects $E_{ijk} \sim$ NID (O, δ^2

RESULT AND DISCUSSION

Seasonal climatic parameters in Table 1 were statistically different ($P<0.05$) among seasons. Maximum summer temperature was higher ($P<0.05$) than those from both rainy and winter seasons, while maximum temperature of winter was at the

lowest value (P<0.05).

Since THI was higher than 72 throughout the year, the environmental conditions at the station would not be suitable for milk production, especially from Friesian cows (Johnson, 1987).

Milk yield (kg/lactation), 305-day standardised milk yield and the average milk yield in winter were not significantly different when compared to those from the rainy season, as shown in Table 2. However, the mentioned parameters in both winter and rainy seasons were statistically higher (P<0.05) than those in summer possibly due to high THI in summer (Table 1). Heat stressed cows would increase their maintenance energy (Yousef, 1985) and would reduce their feed intake (Stermer *et al*., 1986) for their thermal balance resulting in milk production.

Furthermore, number of services and calving interval in summer (Table 2) were higher (P<0.05) than those in both rainy and winter seasons. There was no significant difference between seasons for pregnancy period, presumably due to the fact that higher body temperature of heat stressed cows would affect their reproductive system via hypothalamo-pituitary-gonad axis causing lower secretion of oestrogen (Abilay *et al*., 1975), lutenising hormone (Madan and Johnson, 1973) and lower progesterone (Wise *et al*., 1988).

Table 1 Seasonal meteorological values between 1993-1999 at TRBC.

Parameter	Season		
	Rainy	Winter	Summer
Maximum temp. (°C)	32.3±0.41b	31.6±1.03c	34.9±0.99a
Minimum temp. (°C)	22.7±0.58b	18.0±0.84c	24.4±0.51a
Mean temp. (°C)	27.5±0.39b	25.3±0.60c	29.6±0.65a
Relative humidity (%)	84.0±2.78a	76.7±3.92c	79.7±5.33b
THI	78.7±7.14b	75.4±1.73c	84.1±2.51a

Means within row with different superscripts are statistically different (P<0.05).

Table 2 Seasonal production performance of AFS_3 cows during 1993 – 1999 at TRBC.

Milk performance	Season		
	Rainy	Winter	Summer
Number of cows	382	374	361
Lactation yield (kg/m)	1,978.2±72.64a	2,019.1±136.38a	1,837.5±188.64b
305-day standardized milk yield (kg)	2,025.2±114.28a	2,073.6±97.63a	1,962.3±104.21b
Daily milk yield (kg/day)	6.6±0.58a	6.8±0.64a	6.5±0.62b
Days in milk	298.1±13.50a	296.0±69.27a	283.6±13.52b
Service	1.82±0.24b	1.64±0.16b	2.24±0.26a
Calving interval (d)	438.5±57.89b	437.6±76.21b	483.5±76.48a
Pregnancy period (d)	277.7±5.74	278.3±6.32	276.9±6.26

Means within row with different superscripts are statistically different (P<0.05).

Table 3 Seasonal milk composition of AFS_3 cows during 1993-1999.

Milk composition	Season		
	Rainy	Winter	Summer
Milk fat (%)	3.8±0.04	3.8±0.03	3.7±0.03
Protein (%)	3.4±0.01	3.5±0.01	3.4±0.01
Lactose (%)	5.6±0.23	5.7±0.27	5.5±0.25
Solid not fat (%)	9.9±0.16	9.9±0.14	9.4±0.17
Total solid (%)	13.7±0.17a	13.8±0.15a	12.9±0.17b

Means within row with different superscripts are statistically different (P<0.05).

Calving interval in summer was longer (P<0.05) than that in both rainy and winter seasons (Table 2), presumably due to nutritional effect during the summer calving period. High environmental temperature would make a reduction in feed intake and would have a severe nutritional effect on both quantity and quality of roughage consumed (Tudsri and Sawasdipanit, 1993). Haematological (Hafez, 1968) and endocrinological (Yousef, 1985; Wise *et al*., 1988) responses from heat stress in summer might also cause a longer calving interval in summer (Ingraham *et al.*, 1974; Gwazdauska *et al*., 1979).

Milk composition in terms of milk fat, protein, lactose and solid not fat percentage in rainy, winter and summer were not statistical different. However, the total solid of milk during rainy and winter were higher (P<0.05) than those during summer. Milk composition from the current study was similar to those reported by Bunyanuwat *et al.* (1996) and Boonprong (1999), possibly due to the AFS breed characteristics (Anon, 1989). In fact, milk composition presented in Table 3 are at higher levels than thate recorded by many works such as Prasanpanich *et al.* (2002) and Tudsri *et al.* (2002). However, trends in milk composition were actually in contrast to low milk yield (Table 2), presumably indicated that most the tested animals were in late lactation (Rook and Campling, 1965; Abd El-Razek *et al*., 1982).

In general, heat stress would cause a change in milk composition (Johnson and Givens, 1961; Yousef, 1985). Maust *et al.* (1972) found that milk fat percentage was influenced by climatic effects of ambient temperature, humidity, wind velocity, rainfall and daylength. Brown *et al*. (1961) concluded that high ambient temperature did have a significant effect on lower milk fat production. However, there was no significant difference (P>0.05) in milk fat percentage which was in harmony with the finding of Bunyanuwat *et al*. (1996) who found no difference in fat percentage among the AFS cows kept at below 27°C, between 27-30°C and above 30°C.

CONCLUSION

The milk quality of the AFS_3 cows was not affected by heat stress and the cows could perform satisfactorily during rainy and winter seasons under tropical conditions in Thailand. However, under a high THI condition during summer both milk production and reproduction of the AFS_3 cows were affected by heat stress.

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Net Energy of Sweet Corn Husk and Cob Silage Calculated from Digestibility in Cows

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ABSTRACT

Sweet corn husk and cob (SC) which is a residue of sweet corn cannery contained 19.75% dry matter (DM). The nutrient composition on DM basis was 6.86% CP, 3.21% EE, 3.97% ash, 70.89% NDF, 35.61% ADF and 15.07% NFC. The silage (SCRB) was made by mixing SC with rice bran (RB) on fresh weight 86:14 and adding 4.38 g of formalin/kg fresh weight then filling it in 2 layered plastic bags. After filling, the inner bag was vacuumed and tied while the outer was sewed. Each bag contained 30 kg. They were kept for 40 days before determining digestibility in 4 cross bred Holstein Friesian cows at nonpregnant and nonlactating stage.

After a 14 day adaptation period, the cows were fed with the silage *ad libitum* for another 14 days followed by a 6 day collection period. Feed intake and faeces output were recorded and sampled for chemical analysis. Digestibility of nutrients and TDN were calculated while DE was also directly determined. ME and NE were calculated from TDN and DE using NRC equations (1988).

The result revealed that SCRB was a good quality silage with 28.34% DM. The content of other nutrients on DM basis was 10.91% CP, 11.71% EE, 57.95% NDF, 28.87% ADF, and 12.28% NFC. The digestibility of these nutrients was 58.50, 56.26, 84.68, 58.97, 51.30 and 70.68% respectively. Nonpregnant dry cows consumed the silage on dry matter basis 0.91% BW or 41.81 g/kg$W^{0.75}$. The silage had 71.31% TDN, 3.10 DE, 2.68 ME and 1.60 NEL (Mcal/kgDM) respectively.

Key words : silage, sweet corn husk and cob, digestibility, net energy, dairy cow

INTRODUCTION

Sweet corn husk and cob is a residue of sweet corn cannery plants. The proportion of this residue is around 65.8% of the whole ear (Thiraporn and Setabandhu, 1994). The estimated amount of this residue in Thailand is around 173,727 tons/year or 476 tons/day. Although the residue is an attractive feed for ruminants, in the wet season when plenty of green feed is available the residue may be left over and causes pollution problem. It should be preserved as a silage for using in the dry season. Unfortunately its moisture content is appreciably high (79-82%) therefore it should be mixed with some absorbent such as cassava chip, ground corn or rice bran. Cheva-Isarakul (1990) reported that baby corn husk which contained 86.4% moisture could be well preserved as a silage by mixing with either 20-25% of rice bran or ground corn. Performance of native cattle being fed with this kind of silage as a roughage

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was superior than the group fed with Napier grass.

Pumisutapool *et al.* (2000) reported that SC ensiled with 14% rice bran either with or without formalin was a good quality silage. However its nutritive value has not been investigated. Therefore the objective of this experiment was to determine the digestibility and energy value of sweet corn husk and cob silage (SCRB).

MATERIAL AND METHOD

Ensiling process

Sweet corn husk and cob from a cannery plant in Chiang Mai, Thailand was chopped into 1-2 cm length. It was mixed with rice bran at the ratio of 86:14 (fresh weight basis). Then 4.38 g formalin/kg fresh weight was added, mixed thoroughly and filled in 2 layered vacuumed plastic bags. The inner bag was tied while the outer bag was sewed and kept for 40 days. Each bag contained 30 kg of the material (SCRB).

Digestibility trial

Four heads of dry nonpregnant crossbred Holstein Friesian cows with average body weight 455.9±42.2 kg were kept individually in a metabolism stall. They were weighed for 3 consecutive days at the beginning and the end of the experiment which lasted 39 days. The animals were fed twice daily at 8.30 and 16.00 hrs. Water was freely accessed. Mineral mix was added on top of the feed at 100 g/day. The first 14 days was a transition period during which feed was gradually changed to SCRB silage. For the next 14 days, SCRB silage was given as a sole diet *ad libitum* in order to determine voluntary feed intake (VFI). The following 5 days feed was restricted to 90% of VFI in order to avoid feed ort. The last 6 days were a collection period during which feed intake, feed ort, faeces and urine were recorded and sampled. The faeces was separated from the urine by a harness with a specially designed funnel which covered the vulva of the cows. Urine was collected in a bag in which 100 ml of 18 N H_2SO_4 was added as a preservative. All samples were kept in a freezer (-20°c) before being subjected to chemical analysis (AOAC, 1984 and Goering and Van Soest, 1970)

Apparent nutrient digestibility and total digestible nutrient (TDN) was calculated as follows:-

$$\text{Apparent digestibility (\%)} = \frac{\text{Nutrient intake - Nutrient in faeces}}{\text{Nutrient intake}} \times 100$$

$$\text{TDN (\%)} = \text{DCP} + \text{DNDF} + \text{DNFC} + (\text{DEE} \times 2.25)$$

where DCP, DNDF, DNFC and DEE were digestible crude protein, neutral detergent fiber, non fiber carbohydrate and ether extract respectively (g/100 g DM of feed)

Digestible energy (DE), metabolizable energy (ME) and net energy for lactation (NEL) were calculated from total digestible nutrient (TDN) using NRC (1988) equations as follows:-

$$\text{DE (Mcal/kg DM)} = 0.04409 \times \text{TDN (\%)}$$

$$\text{ME* (Mcal/kg DM)} = -0.45 + 0.0445309 \times \text{TDN (\%)}$$

$$\text{NEL (Mcal/kg DM)} = 0.0245 \times \text{TDN (\%)} - 0.12$$

In addition ME and NEL were also calculated from DE, which was determined directly, as follows:-

$$\text{ME (Mcal/kg DM)} = -0.45 + 1.01\ \text{DE}$$

$$\text{NEL* (Mcal/kg DM)} = 0.5557 \times \text{DE} - 0.12$$

NB.* adapted from NRC (1988)

RESULT AND DISCUSSION

The chemical composition of sweet corn residue and its silage either without or with additives is shown in Table 1.

Sweet corn cob had higher dry matter (DM), organic matter (OM), crude protein (CP) and ether extract (EE) but lower neutral detergent fiber (NDF) and acid detergent fiber (ADF) than the husk. It might be due to the partial attachment of the kernel which had higher nutritive value than the husk.

Sweet corn husk and cob had higher EE and

Table 1 Chemical composition (% DM basis) of sweet corn residues.

Sweet corn residue	DM	OM	CP	EE	NDF	ADF	NFC	pH
Husk	17.79	96.13	5.41	1.51	77.48	38.73	11.73	-
Cob	24.24	97.52	6.11	4.44	68.50	33.48	18.47	-
Husk and cob	19.75	96.03	6.86	3.21	70.89	35.61	15.07	-
Husk and cob silage[1/]	21.98	96.90	6.27	2.28	77.32	33.90	11.03	4.21
SCRB silage[2/]	28.34	92.85	10.91	11.71	57.95	28.87	12.28	4.98

%ash = 100 - %OM

[1, 2] are from different experiments.

SCRB silage is sweet corn husk and cob ensiled with rice bran and formalin.

NDF but lower CP than baby corn husk (Cheva-Isarakul, 1990). Dry matter content of both residues was similar. SCRB which is the ensiling product of SC with rice bran and formalin had higher DM, CP and EE but lower NDF and ADF than the original sample. It might be due to the higher nutritive value of rice bran, The ratio of RB : SC at 14 : 86 seems to be optimum since DM of the mixture was in proper range for ensiling (25-35%). DM and CP of SCRB silage was similar to baby corn husk ensiled with 25% ground corn (Cheva-Isarakul, 1990). In addition it was also similar to Ruzi grass (Waipanya *et al.*, 1999)

Nonpregnant dry cows consumed SCRB silage of 4.13 kg DM/day which was equal to 0.91% BW or 41.81 g/kg $BW^{0.75}$. This value is lower than Jaster *et al.* (1983) who reported that dry matter intake (DMI) of heifers consuming sweet corn and cob silage was 1.5% BW. The low DMI in this experiment might be due to the high fat and high energy content of the feed, as well as the fact that animals were at a nonpregnant and nonlactating stage and thus only required nutrients for maintenance. In addition the high moisture and acidity of the feed may restrict feed intake. Cheva-Isarakul (1990) reported that DMI of sheep consuming baby corn husk was 2.7% BW or 65.4 g/kg $W^{0.75}$ when the material was in dry form but in a fresh form DMI decreased to 1.6% BW or 32 g/kg $W^{0.75}$ and as silage the animal consumed only 1.2% BW or 27.9 g/kg $W^{0.75}$.

Yammuen-Art *et al.* (2000) and Wongjarearn *et al.* (2001) also found a low DMI for cows which consumed sweet corn stalk silage and whole corn silage at the rate of 0.97 and 1.14% of BW respectively.

Digestibility of nutrient

Nutrient digestibility and energy value of SCRB as well as nitrogen balance of dry cows fed SCRB as a single feed are shown in Table 2.

Dry matter digestibility of silage was 58.50% which was similar to SC silage of Jaster *et al.* (1983, 59.1%) but lower than that of sweet corn stover silage (65.5%; Yammuen-Art *et al.*, 2000) although NDF and ADF of both reports were higher than SCRB in this experiment. This might be due to the inhibitory effect of formalin as observed by Brown and Valentine (1972) in sheep and Valentine and Radcliffe (1975) in *in vitro* digestibility trial.

Since the digestibility of energy of SCRB was 63.09%, therefore its DE content was 3.05 Mcal/kg DM. Average protein intake of the cows was 403.07 g/day which was slightly higher than the requirement (341-364 g/day) of nonpregnant cows at 450-500 kg BW (NRC, 1988). Although DMI of the cows was only 0.91% BW, nitrogen balance was slightly negative (-0.53 g/day).

Table 2 Digestibility of nutrient, energy and N-balance of HCRB in dry cows**.**

Nutrient	% Digestibility	Nutrient	% Digestibility
Dry matter	58.50	Acid detergent fiber	51.30
Organic matter	63.45	Non fiber carbohydrate	70.68
Crude protein	56.26	Total digestible nutrient	71.31
Ether extract	84.68	Digestible energy (Mcal/kgDM)	3.05
Neutral detergent fiber	58.97	N-balance (g/day)	-0.53

Table 3 TDN and DE from direct measurement as well as DE, ME and NEL from calculation.

Energy (Mcal/kgDM)	*In vivo* measurement	Calculated from		Average
		TDN	DE	
TDN (%)	71.31	-	-	-
DE	3.05	3.14	-	3.10
ME	-	2.73	2.63	2.68
NEL	-	1.63	1.57	1.60

Energy content of SCRB

TDN calculated from digestibility, DE from direct measurement and from TDN as well as ME and NE calculated from both values are shown in Table 3. It is noticed that ME and NEL calculated from DE are slightly lower than from TDN. At the same time DE calculated from TDN is slightly higher than the direct measurement (3.14 vs 3.05 Mcal/kg DM). The result is similar to Yammuan-Art (1999) in rice straw and Vasupen (2000) in dry sugarcane stalk. The average values (Mcal/kg DM) of HCRB from both calculations are 3.10 DE, 2.68 ME and 1.60 NEL which are similar to those of sweet corn stover silage (Yammuan-Art, 2000) i.e. 3.09, 2.67 and 1.60 Mcal/kg DM.

CONCLUSION

Sweet corn husk and cob (SC) had 19.75% DM and 6.86% CP (on DM basis). Good quality SCRB silage could be obtained by ensiling SC with rice bran (RB) at 86.14 plus 4.38 g formalin/kg fresh weight and filled tightly in 2 vacuumed layered plastic bags. The silage had 28.34% DM and 10.91% CP.

The digestibility of most nutrients were 51-59% except EE and NFC which were 84.68 and 70.68% respectively. Dry matter intake of SCRB by dry cows was 0.91% BW or 41.81 g/kg $W^{0.75}$. The silage had high TDN (71.31%). The DE, ME and NEL were 3.10, 2.68 and 1.60 Mcal/kg DM respectively.

The ensiling might be done without formalin. In addition the cob should be hammered before ensiling in order to prevent feed selection by animals.

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Lectins Histochemical Studies in Submandibular Salivary Gland of the House Musk Shrew, *Suncus murinus*

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ABSTRACT

Submandibular glands of male house musk shrew, *Suncus murinus*, were examined by light microscopic histochemical methods. The staining procedures employed were horseradish peroxidase conjugated lectins, Alcian blue (AB) pH 1.0, AB pH 2.5, Periodic acid-Schiff (PAS) and AB pH2.5-PAS in combination with enzyme digestion with neuraminidase. The lectins used in the present study were Peanut agglutinin (PNA), *Dolichos biflorus* agglutinin (DBA), Wheat germ agglutinin (WGA), *Limax flavus* agglutinin (LFA), *Ulex europaeus* agglutinin-I (UEA-I), and *Loutus tetragonolobus* agglutinin (LTA)

The submandibular gland of *Suncus murinus* is a branched tubuloacinar gland. Its secretory endpiece contains both serous and mucous cells. Granular ducts, modified striated ducts, were found to be well developed. All the mucous acinar cells were colored deep blue with the AB pH 2.5-PAS procedure and were stained strongly with LFA. Neuraminidase digestion changed the deep blue coloration with the AB pH 2.5-PAS procedure to light red and abolished all staining of mucous cells with LFA. Removal of sialic acid with neuraminidase imparted weak to strong affinity for PNA. Serous cells showed strong red coloration with the AB pH2.5-PAS procedure and were reacted strongly with PNA, DBA and WGA. Granular duct cells exhibited moderate reaction with PAS, LTA and UEA-I.

Key words: lectin, submandibular, salivary gland, house musk shrew

INTRODUCTION

House musk shrew, *Suncus murinus*, a mammalian species belonged to Soricidae in Insectivora is generally regarded as having close evolutionary affinity with fossil primate. These mammalian species has recently been domesticated as a new experimental animal (Oda and Kondo, 1977; Kondo *et al*., 1978). Although a large number of papers have been published on various organs of the house musk shrew (Cooper and Bedford, 1976; Dryden and Anderson, 1977), our knowledge of morphology and histochemistry of the submandibular gland of this animal is still very limited.

The glycoconjugates in secretory epithelium of mammalian submandibular gland have been previously studied (Shackleford and Klapper, 1962; Pinkstaff, 1975; Menghi *et al*., 1983). Furthermore, an information has been obtained regarding the cytochemistry of glycoconjugates in the comparable mandibular gland of the chicken (Suprasert *et al*., 1986). The glycoconjugates are found in intracellular as well as extracellular sites in most, if not all, tissue. The significance of the glycoconjugates is poorly understood but they have been implicated in

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a wide range of important biological activity such as cell adhesion and recognition, fertilization, growth, differentiation, and also in many pathological processes, including malignancy. (Sharon and Lis, 1982).

In view of the circumstance mentioned above, it is the aim of this study to investigate the morphology and the distribution of the glycoconjugates in secretory epithelium of the submandibular of the house musk shrew by means of lectins, a current available light microscopic methods.

MATERIALS AND METHODS

A total of 12 adult male musk shrews were the donors of the submandibular gland examined. After the animals were sacrificed by exsanguination under ether anesthesia their submandibular glands were dissected out. Tissue pieces from these glands were fixed in one of the following fixative 1) 10% formalin containing 2% calcium acetate for 12-24 h. at 4°C. The tissue specimens were then dehydrated in graded ethanol series, cleared with benzene and embedded in paraplast.

Sections were cut at a thickness of 3µm and then subjected to the following histological and histochemical staining procedures.

1. Hematoxylin-eosin (HE) procedure for general structures.

2. AB pH 1.0 procedure (Lev and Spicer, 1964) for sulfated glycoconjugates.

3. AB pH 2.5 procedure (Spicer *et al*., 1967) for acidic glycoconjugates.

4. Periodic acid-Schiff (PAS) (Pearse, 1968) for vicinal diol groups of glycoconjugates.

5. AB pH 2.5-PAS (Spicer *et al*., 1967) for differentiating acidic and neutral glycoconjugates.

Lectin staining procedures

To access the saccharides residues further, the peroxidase conjugated lectin diaminobenzidine procedure was performed to the paraplast sections. Following lectin were employed : *Limax flavus* agglutinin (LFA), *Peanut agglutinin* (PNA), wheat germ agglutinin (WGA), *Dolichos biflorus* agglutinin (DBA)

Furthermore, the following confirmation and control experiments were performed as well.

1) *Enzyme digestion:* Neuraminidase (form *Arthrobacter ureafaciens*). Prior to stain with AB pH 2.5, AB pH 2.5-PAS, LFA, and PNA, sections were incubated in 0.1 M acetate buffer (pH 5.3) containing 1 unit/ml of the enzyme and 0.04 M $CaC1_2$ at 39-41°C for 12-16 h. (Spicer *et al*., 1967)

For the enzyme digestion procedures, two types of control procedures were performed: a) some sections were incubated in respective buffer solutions without enzymes under the identical conditions of temperature and duration, b) The others sections were kept intact without any incubation procedures.

RESULTS

The submandibular gland of *Suncus murinus* is a branched tubuloacinar gland. An adenomere of the submandibular gland has secretory portion composed of glandular cells (secretory endpieces), conducting intercalated ducts, and granular duct cells (modified striated duct cells). Granular duct cells are well developed. At the base of secretory endpieces, myoepithelial cells are present. The secretory endpieces consisted of two layer of cuboidal cells of varying heights. The cuboidal cells are uninucleate and provided with secretory granules between nucleus and free surface. In the space between adenomere, relatively small amounts of connective tissue elements are interposed.

When tissue sections were reacted with AB pH 1.0, the cytoplasm of all secretory cells was found to exhibit negative reaction. The AB pH 2.5, likewise, to divide the secretory endpiece into two types : cells either with a strong alcianophilic cytoplasm at outer layer (mucous cells) or with a negatively AB pH 2.5 reactive cytoplasm at inner

layer (serous cells). When the secretory endpieces were reacted with PAS, the mucous cells at the outer layer were weakly positive. In contrast, the serous cells at inner layer were strongly positive. In the secretory endpieces of the submandibular gland, the dual staining with AB pH 2.5-PAS (Figure 1) resulted in deep blue coloration for the mucous cells at the outer layer and in deep red coloration for the serous cells at the inner layer.

The striated duct cells and granular duct cells were stained negatively with AB pH 1.0, AB pH 2.5. However, they were stained moderately with PAS. The mast cells of connective tissue were found to exhibit strong positive reaction with AB pH 1.0. However, they stained negatively with AB pH 2.5 and PAS.

Digestion with neuraminidase greatly diminished the intensity of AB pH 2.5 reaction the outer layer of the secretory endpieces. Neuraminidase digestion changed the deep blue coloration with the AB pH 2.5-PAS procedure to light red for the mucous cells (Figure 2)

When the secretory endpieces were reacted with lectins, mucous cells were strongly and moderately positive with LFA (Figure 3) and PNA (Figure 4) repectively. In contrast serous cells were negatively stained with LFA (Figure 3) and strongly positive with PNA (Figure 4) and WGA. The serous cells were furthermore found to be negative with UEA-I and LTA but the mucous cells were weakly positive with UEA-I and LTA. The granular duct cells were stained negatively with PNA, LFA and WGA. However they were stained moderately with LTA and UEA-I.

After neuraminidase treatment, the mucous cells stained strongly with PNA. In contrast, LFA did not stain mucous cells after the treatment. In the control tissues for the enzyme digestion experiments, the results of staining reactions were nearly comparable in intensity to those observed in sections kept intact without any incubation procedures.

All the results obtained are also summarized in Table 1.

DISCUSSION

The morphological, histochemical and biochemical characteristics of the mammalian salivary glands have been the objective of numerous researches (Leeson, 1967 ; Shackleford and Klapper, 1962; Bondi *et al*., 1978). A marked diversity of these characteristics have been noted by many investigations, including Shackleford and Klapper (1962) and Leppi and Spicer (1966). Such studies are chiefly abundant in rodents, because of the characteristic features of the gland : 1) the homogenous seromucous or serous cells of the secretory endpieces. 2) the presence of granular convoluted tubule cells, and 3) the sexual dimorphism of the gland. However, no previous publication was found on the morphology and histochemistry of the musk shrew submandibular gland.

In view of the staining specificities of AB pH 2.5 (Spicer *et al*., 1967) and PAS (Pearse, 1968). Two cell types were identified histochemically in the secretory endpieces of the musk shrew submandibular gland : neutral glycoconjugates-containing cells (serous cells) that react strongly with PAS, and acidic glycoconjugates containing cells (mucous cells) that react strongly with AB pH 2.5. The mucous cells were also contained some amount of neutral glycoconjugates as judged from deep blue coloration when they stained with AB pH 2.5-PAS (Spicer *et al*., 1967). In general, the serous cells were found at inner layer of the secretory endpieces and the mucous cells existed at the outer layer. In the light of the substrate specificity of neuraminidase (Spicer *et al*., 1967), Furthermore, the presence effects of digestion of this enzyme upon the AB pH 2.5 and AB pH 2.5-PAS reaction of the mucous cells are taken to indicate the existence of the sialic acid residues.

In view of the positive WGA and DBA reaction of the mucous cells, the glycoconjugates furthermore contained a notable amount of N-acetylglucosamine and N-acetygalactosamine. The

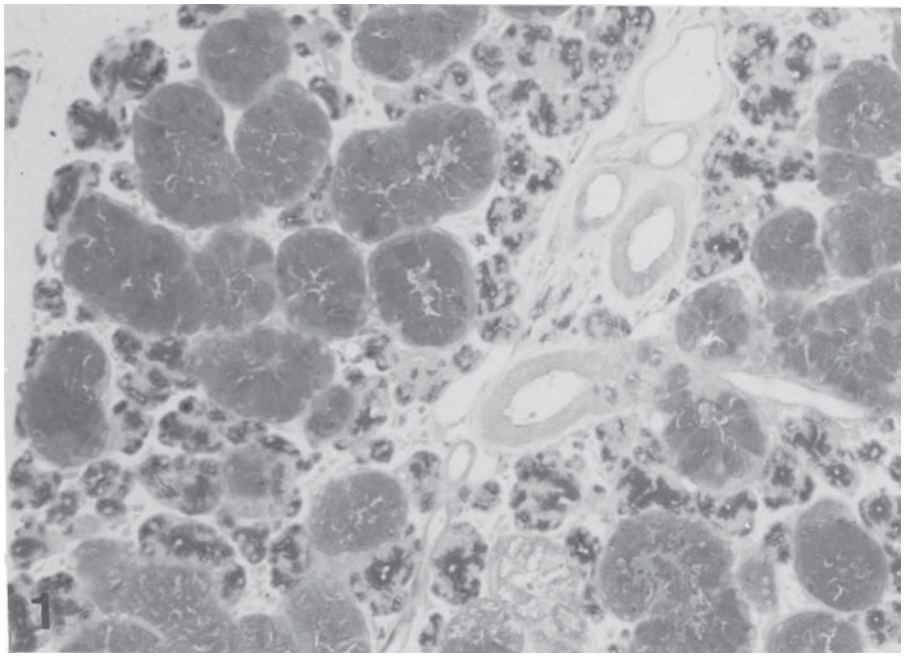

Figure 1 The secretory endpieces of musk shrew submandibular gland consist of serous and mucous cells. The dual staining with AB pH2.5-PAS resulted in deep blue with mucous cells and strong red with serous cells. The granular duct cells are well developed and stained moderately with PAS. X 120.

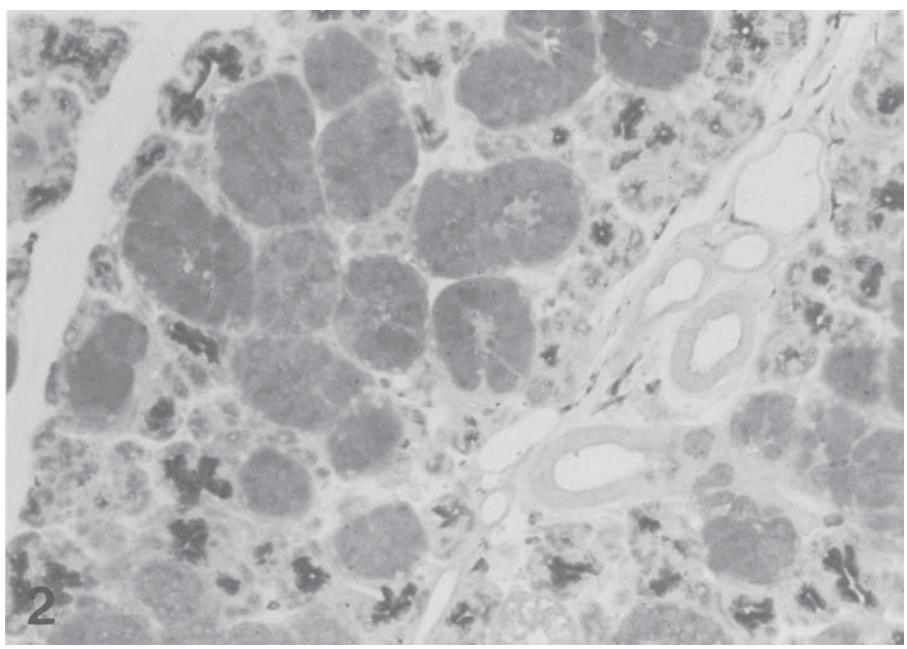

Figure 2 Neuraminidase digestion changed the deep blue coloration with AB pH 2.5-PAS procedure to light red for the mucous cells. X 120.

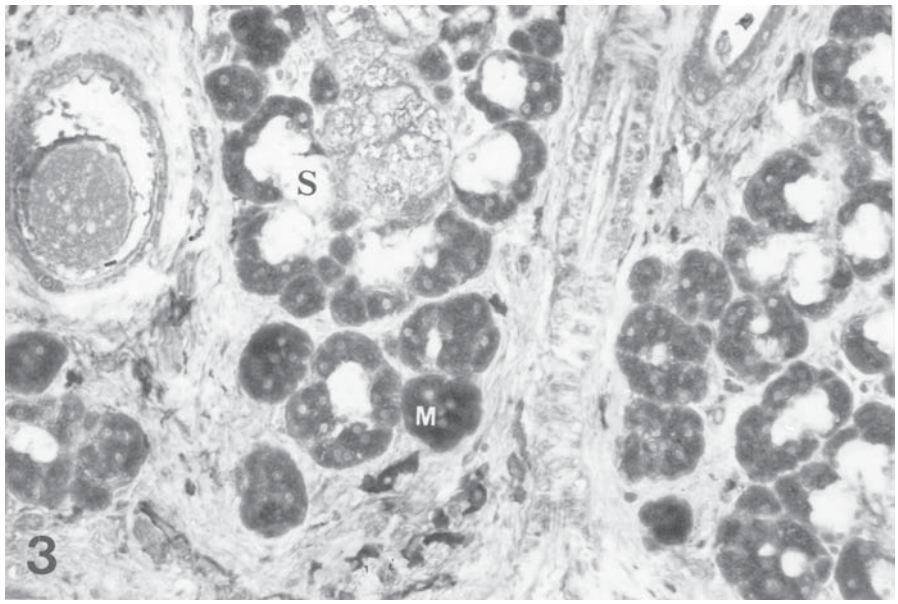

Figure 3 The mucous cells (M) stained strongly with LFA. In contrast, the serous cells (S) stained negatively with LFA. X 264

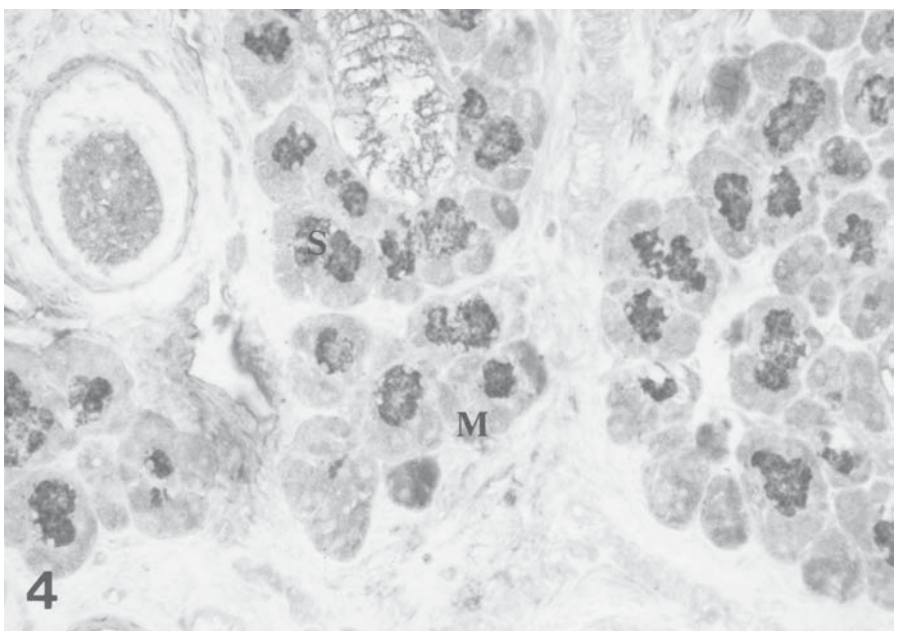

Figure 4 The serous cells (S) exhibit strong reaction with PNA. In contrast, the mucous cells (M) exhibited moderately reaction with PNA. X 264.

Table 1 Histochemical reaction of glycoconjugates in submandibular gland of the house musk shrew.

Staining procedures \ Histologic structures	Serous cells	Mucous cells	Granular duct cells
AB pH 1.0	0	0	0
AB pH 2.5	0	3 B	0
PAS	4 M	1 M	2 M
AB pH 2.5 – PAS	4 M	3 MB	2 M
N.AB pH 2.5	0	0	0
N.AB pH 2.5 – PAS	3-4 M	1-2 M	2M
LFA	0	4 Br	0
PNA	4 Br	1-2 Br	0
WGA	2 Br	1 Br	0
DBA	3 Br	1 Br	0
UEA-I	0	1 Br	2 Br
LTA	0	1 Br	1–2 Br
N.LFA	0	0	0
N.PNA	1 Br	3 Br	0-1 Br

Abbreviation
B = Blue, Br = Brown, M = Magenta, O =Negative reaction
1-n = number indicates intensity of staining reaction
N = neuraminidase

positive staining of PNA at serous cell and LFA at mucous cells suggested that terminal dimer galactose-(1-3) N-acetylgalactosamine occur in secretery granules of serous cells, while terminal sialic acid residues occur in granules of mucous cells. The presences of sialic acid-galactose dimer in mucous cells were more confirmed since an enhanced PNA reaction could be detected following digestion with neuraminidase.

The histochemical study in present investigation permits the identification of differences between elements of the secretory endpieces and those that granular duct cells. As a matter of fact, while the former contains primarily acid component in the mucous cells and neutral component in the serous cells, in the latter involves on neutral glycoproteins.

All the tissue structures of the musk shrew submandibular gland are found to devoid of sulfated groups as presummed from negative reaction with AB pH 1.0 (Lev and Spicer, 1964). This is in contrast to the acini in submandibular gland of various mammalians and avian which contain sulfomucins (Bondi *et al*., 1984, Suprasert *et al*., 1986).

The results of the present study with regard to the morphology and histochemistry have some controversy surrounds the cellular composition of the submandibular gland. In the musk shrew, the secretory endpiece is, of course, serous and mucous cells. In contrast, the submandibular gland of most rodents presents a homogenous secreting components which exhibit morphologically a single serous type but histochemically they display seromucous characteristics (Shackleford and Klapper, 1962). Furthermore, the comparable mandibular glands of the chicken are found to contain exclusively with mucous cells (Suprasert *et*

al., 1986). However, the present of granular duct cells in submandibular gland of musk shrew is similar to those of the rodents but is contrast to those of the chickens.

It is special interesting that at least two types of cells were histochemically differentiated in the secretory endpieces and one cell type in the granular convoluted tubule of the musk shrew submandibular gland examined in the present work. This fact appears to support to the concept that the secretory glycoconjugates are various types in chemical nature and that the physiological activities performed by this carbohydrates are therefore of diversified feature.

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Microstructural Changes in Instant Noodles During Production *via* Triple Staining and Confocal Laser Scanning Microscopy and Scanning Electron Microscopy

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ABSTRACT

Confocal Laser Scanning Microscopy (CLSM) was used to study changes in microstructure throughout the process of instant noodle production. Multiple staining of acridine orange, fluorolink Cy-3 and sulforhodamine was applied in order to differentiate protein, starch and lipid, respectively, in the same image. Scanning Electron Microscopy (SEM) was also applied, complementarily, to contribute stereoscopic images. The microscopic data obtained from triple-labeled samples examined by CLSM were found to correspond to the stereoscopic SEM micrographs. Morphological changes and microstructural arrangement of starch granules and protein matrix, and the presence of lipids were found to respond to different regimes of process: addition of water, inputs of mechanical force and heat and introduction of frying. Thus, triple staining, via CLSM, could be regarded as a potential tool for monitoring microstructural differences resulting from varied processing conditions.

Key words: microstructure, instant noodle, Confocal Laser Scanning Microscopy, Scanning Electron Microscopy

INTRODUCTION

Microscopy is, particularly, useful in food research development. It provides a clue to understanding the relationship between the product's microstructure and functionality of a food material (Varriano-Marston, 1977).

Confocal Laser Scanning Microscopy (CLSM) has introduced to food research new possibilities for microstructure studies of food systems (Adler *et al*., 1994; Vodovotz *et al*., 1996; Lynn *et al*., 1997). The main advantage of CLSM in food applications, in addition to its optical sectioning capabilities, is that it requires minimal sample preparation: the specimen does not require prior embedding, sectioning, or fixing (Vodovotz *et al*., 1996).

In order to achieve sensitivity and specificity from CLSM application, an appropriate fluorochrome with a proper wavelength of excitation and emission and fluorescence stability, and component stability are necessary. Some fluorochromes used in cereal based products are FITC, fluorolink Cy3, nile blue A, nile red, congo red, sulforhodamine, pyronin Y, pararosaniline and acridine orange (Adler *et al*., 1994; Vodovotz *et al*.,

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1996; Lynn *et al*., 1997).

Scanning Electron Microscopy (SEM) of surface morphology provides stereoscopic images with high magnification. Noodles and pasta samples have been studied by SEM using a liquid nitrogen slush for rapid freezing (Marion *et al*., 1987; Moss, 1985). Moss *et al*. (1987) studied the influence of ingredients and processing variables on the microstructure of several kinds of noodles, including instant noodles by employing SEM, complemented by conventional light microscopy. Microstructure differences at the dough stage of instant noodles were observed to be similar to Cantonese noodles. Microstructure changes affected by additional processes of steaming, frying and final cooking were also reported (Moss *et al*., 1987).

CLSM and SEM were employed to examine the microstructure changes of samples from the production line of instant noodles in this study.

MATERIALS AND METHODS

Confocal laser scanning microscopy

Each of the six samples were randomly taken, immediately following the stage of mixing, compression, reduction, steaming, frying and cooking. The samples were stabilized by the pre-heated chilling to 4°C, not over a period of 3 hours and stored in an air-tight container prior to staining. Wheat flour and mixed dough samples were placed on the cover slips directly. Dough samples taken from compression and reduction were viewed at the volume of approximately 1×3×3 mm^3. Noodle samples taken from steaming, frying and cooking were viewed at 5 mm long. Six specimens were randomly selected for examination.

Triple staining for observing protein, starch and fat simultaneously was modified from the methods of Vodovotz *et al*. (1996); Alder *et al*. (1994); Blonk and van Aalst (1993), respectively and performed on each individual sample. Flourolink Cy3 was directly applied (Amersham Life Science, Inc. IL) for protein marking. Acridine orange (0.01% aq., Sigma Chemical, Poole, Dorset BH17, UK) and sulforhodamine (0.1% aq., Sigma Chemical, Poole, Dorset BH17, UK) were applied directly for starch and lipid, respectively. For low moisture samples (flour and fried noodles), fluorochrome solutions were placed onto the sample and stood for a pre-tested period of 10 minutes before examining. Samples that contained intermediate (mixed or rolled or sheeted dough) to high (cooked noodle) moisture content were viewed immediately after placing fluorochromes.

CLSM examination was performed using Carl Zeiss 410 LSM system attached to an Axiovert 135M-inverted optical microscope fitted with a 100x/1.3 oil Fluar immersion objective lens. The software used to control the microscope was LSM 410 (also supplied by Carl Zeiss). The wavelengths used to generate fluorescence were 488, 514, and 543 nm. The lasers were set at 3.2% of their maximum power. Emission filters 515-565LP, 510, 525, 575-640, 590, 665 and 590-610 were selected. Filter block 1 contained a VHS-510DCLP filter. Cover slips 22×50mm, no.1 were used to support the samples. Each specimen was randomly viewed at 3 areas at ×630 magnification. Optical sections were taken every 1.0 mm through the samples. Forty images were recorded for each series and then 3-D reconstructed by employing the software, Confocal Assistant V 3.1 and LSM Dummy, respectively. The representative 3-D images from each processing step were selected for illustration.

Scanning electron microscopy

Samples for SEM examination were collected, concurrently, with CLSM samples. The specimens were prepared by the method described by Pomeranz and Meyer (1984) and Moss (1985). Samples were immediately frozen in liquid nitrogen and freeze-dried at 0.8 Torr and –50°C, for 18 hours (Edwards, Modulyo 4K Freeze Dryer, England). The freeze-dried specimens were first fractured to expose interior structure, then affixed to aluminum SEM stubs using either double-sided tape with

silver-colloid paint or dental gum with carbon cement for longitudinal sections and cross sections, respectively, at the base of each specimen. Then the prepared specimens were coated with gold using a sputter coater (Balzers SCD 004, Balzers Akteingesellscaft, Leichenstein). The coating was achieved by applying a vacuum of 0.005 mbar and a current of 15 mA for 215 s, resulting in approximately a 20 nm thick coating. The coated specimens were examined with a scanning electron microscope (JSM-5600 LV, Jeol, Japan) operated at an accelerating voltage of 10-15 kV at pertinent magnification (x85-x3000). Photomicrographs were taken on Agfaplan APX 100 Professional 120 film (Agfa, Germany). The representative micrographs from each processing step were selected for illustration.

Formulation of instant noodles and processing

Instant noodle samples were prepared from a dough comprising of wheat flour (78%); water (20%), sodium chloride, phosphates, carbonates and other texture modifiers (2%).

A commercial Australian wheat noodle flour with 87.0% total solid, 10.0% protein, 25.5% wet gluten and 0.5% ash was used in this experiment.

The processing of instant noodles was performed by a continuous production line. Wheat flour was mixed with the dissolved salts and texture modifiers in water for 20 min. The compression and reduction stage were operated by 7 pairs of rollers to achieve a dough thickness of 1.0 mm, the sheeted dough was then proceeded to a sitting roller to obtain a 0.8 mm width of noodle strands. Subsequently, noodles were steamed by being subjected to a saturated steam at 4 bar for either 0, 1 or 3 min in a steaming tunnel, prior to cutting and shaping the noodles into a block of 85.0 g in a mold to convey to a fryer, adjusted at a temperature of 135°C with the speed adjusted for a 50 s frying. The steamed fried noodles were left to ensure cooling before being doubly packed in polyethylene and in vaporized metal polypropylene bags and then stored in cold storage at 4°C.

Cooking of instant noodles (80g) was performed by placing them into 450 ml of boiling water and cooking for 2 min, with occasional stirring. The cooked noodles were then transferred to 600 ml of room temperature water and drained immediately. Excess water was blotted with tissue paper.

RESULTS AND DISCUSSION

Multiple staining was used in order to differentiate protein, starch and oil in the same image. Fluorolink Cy3, acridine orange and sulforhodamine were selected as fluorochromes due to their specificity to protein, starch and oil and different emission wavelengths. Though sulforhodamine possesses a wide range of emission wavelength, one of which produced the same color as protein, plus fluorolink Cy3, it was found to be applicable. Since the morphologies of oil droplets and protein matrix are quite different, differentiation was easily achieved.

CLSM of the wheat flour sample (Figure 1.1) showed the protein bodies, stained yellow, as a compact discrete chunk in irregular shape associated with some of the starch granules. Because of the degree of protein cover, starch granules themselves were not easily identifiable. Some were stained unevenly, unstained sections were black, and slightly stained sections appeared dark green. SEM as clusters of endosperm material of different sizes and shapes (Figure 1.2) characterized the morphology of wheat flour particles. Almost all starch granules were embedded in, or closely packed with protein bodies.

After a reasonably short mixing time with a relatively low amount of added water, CLSM revealed that protein bodies were removed from the starch granules (Figure 2.1).

In addition, a radical change occurred from the mixing, in that, the protein was fused together. The dispersed starch granules were more apparent as displaying a stronger green color when compared

to the previous starch granules in flour particles. The increase in green staining indicates that more aqueous solution had penetrated parts of some granules. The surface morphology of protein bodies, as observed by SEM (Figure 2.2), was smoother and more uniting as compared with that of wheat flour. Starch granules were more noticeable as compared with wheat flour.

After mixing, dough was compressed to form a sheet by repeated passage through pairs of rollers for compression and reduction. The changes in protein morphology occurring during compres-sion was made evident by CLSM and SEM (Figure 3.1 and 3.2). Most of the protein mass was observed as spreading out and the starch granules had been released from the protein mass, resulting in their more intensive green staining (CLSM). After the reduction stage was completed, a greater degree of protein orientation was obvious in both CLSM (Figure 4.1 and 4.2) and SEM as the gluten had developed. Morphology of the starch granules remained the same, as it was, in the original wheat flour.

After entering a steaming chamber charged with saturated steam, noticeable differences of protein strands and starch granules in the noodles were detected. Figure 5.1 revealed a slight aggregation of protein. In steamed noodles, not all the starch granules were fully gelatinized. Besides the intact starch granules, a number of granules displayed a more swollen, irregular shape. Most of the deformed granules exhibited less intensity of fluorescence. Starch gel was also evident as a non-fluorescent dark green mass. SEM micrograph (Figure 5.2) taken at the surface of steamed noodles also revealed various degrees of starch swelling. The shape of starch granules was still discernable and not all the granules were ruptured or fused together. Bubbles and pores were detected. As frying was introduced, the displaying of oil droplets had initiated in CLSM images, (Figure 6.1) ranging from yellow to orange colored and exhibiting protein aggregation. Both CLSM and SEM (Figure 6.2) detected discernable starch granules and protein strands.

After the cooking of instant noodles, clumps of dark, green swollen starch and darker starch gel, accompanied with yellow swollen protein strands were observed in Figure 7.1. A gel structure at the surface of the cooked instant noodles was shown in Figure 7.2.

CONCLUSIONS

Triple labeling of starch, protein and oil examined with CLSM was achieved by an application of specific fluorochromes (acridine orange, fluorolink Cy3 and sulforhodamine) and yielded microscopic observations, which were in agreement with SEM.

CLSM images of wheat flour showed protein bodies in yellow surroundings in almost all of the starch granules that were different in sizes and unevenly stained. Unstained granules were black. Mixing with water produced a radical change, as evident, in the spreading out of the protein matrix.

Staining of starch granules increased, presumably, due to more dye solution penetration. As evident in CLSM and SEM images, orientation of the protein matrix due to compression and subsequent reduction occurred, and the protein strands were more clearly visible. After steaming, the images showed a slightly, aggregated protein and a swollen, irregularly shaped green mass of starch.

SEM confirmed the morphological changing starch granules that were packed into a dense mass. Orange oil droplets were visible in CLSM only after frying. The increase in different degrees of starch swelling and protein aggregation was detected after steaming and frying. As a result of cooking, protein strands and almost all of the starch granules were observed to be swollen. Starch gel was also detected.

It could be concluded that morphology change, the distribution and the inter-relationship of starch granules and protein matrix (protein strands) and the presence of lipids could be

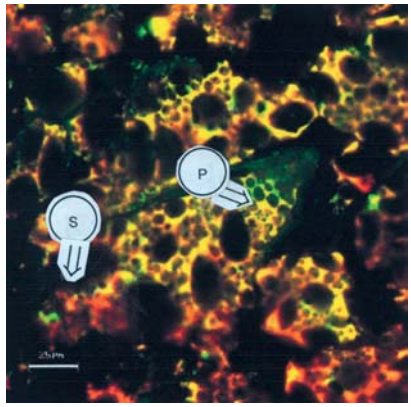

Figure 1.1 CLSM images of wheat flour.

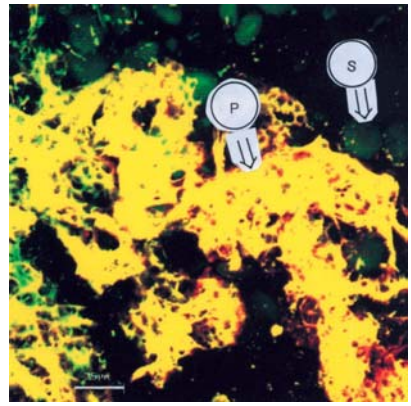

Figure 2.1 CLSM images after mixing.

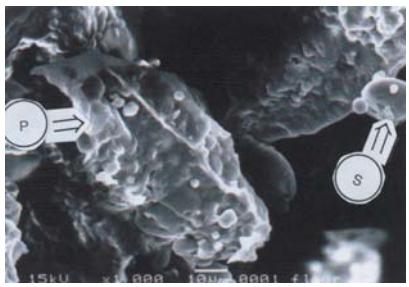

Figure 1.2 SEM micrograph of wheat flour.

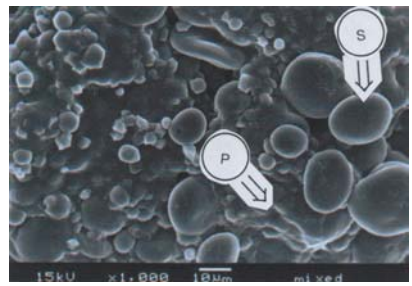

Figure 2.2 SEM micrograph after mixing.

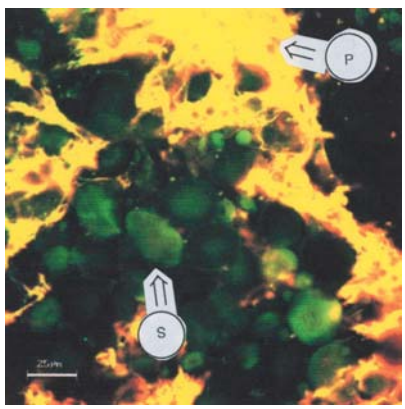

Figure 3.1 CLSM images after compression.

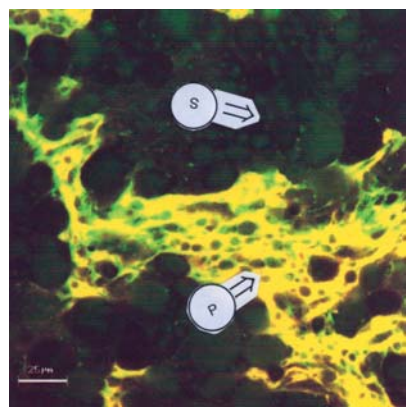

Figure 4.1 CLSM images after reduction.

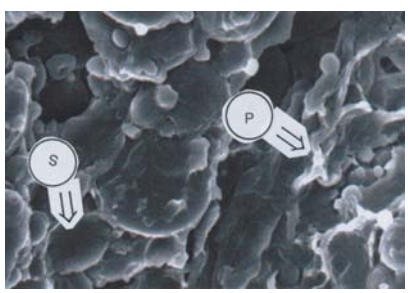

Figure 3.2 SEM micrograph after compression.

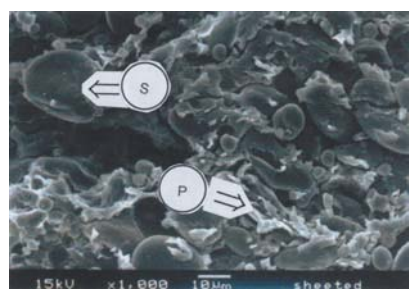

Figure 4.2 SEM micrograph after reduction.

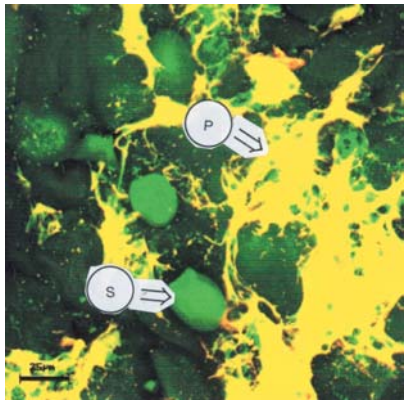

Figure 5.1 CLSM images after steaming.

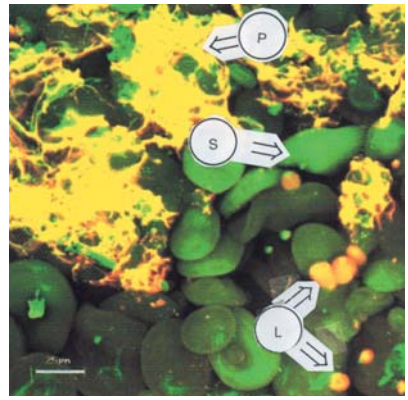

Figure 6.1 CLSM images of instant (fried) noodle.

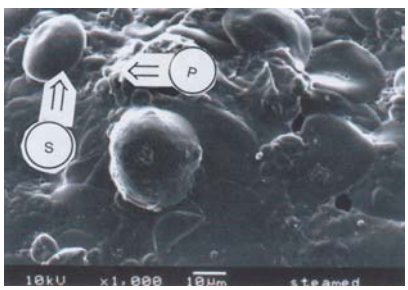

Figure 5.2 SEM micrograph of noodle surface after steaming.

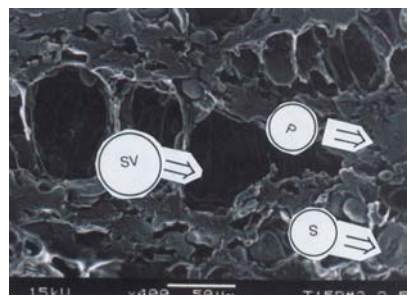

Figure 6.2 SEM micrograph of cross-sectioned instant (fried) noodle.

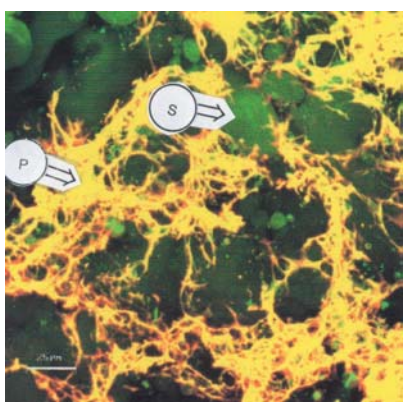

Figure 7.1 CLSM images of cooked instant noodle.

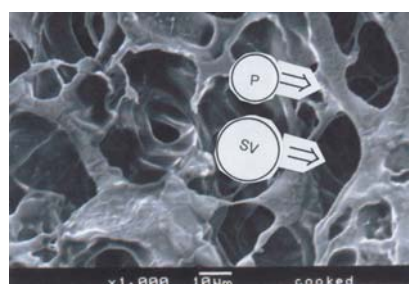

Figure 7.2 SEM micrograph of cooked instant noodle surface.

monitored, successfully, upon an addition of water with an input of mechanical force, upon mixing. The mechanical force, as the dough proceeds through the compression and reduction stage, is subjected to heat with moisture from steaming, heat input and vaporizing of the moisture, as a result of frying and boiling excess water in the final step of cooking. Thus, triple staining via CLSM could be regarded as a potential tool for understanding the effect of processing and the influence of raw materials. It could be expected that this tool would achieve and understanding of the relationship between microstructure information and sensory properties preferred by the consumer, therefore, process optimization would be met.

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Effect of Formaldehyde on the Gel Forming Ability of Fish Meat

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ABSTRACT

The gel forming ability of dorab meat and washed dorab meat with the addition of 0, 25, 50, 75, 100, 200, 300, and 500 ppm of formalin solution was determined. Three levels of heating: (1) 40°C 30 min, (2) 90°C 30 min, and (3) 40°C 30 min followed by 90°C 30 min were used for heating fish meat gel. The SDS-PAGE analysis was also carried out. The results showed that gel force, deformation and gel strength of dorab meat were higher than washed dorab meat with and without formalin addition at all heating temperatures ($P>0.05$). After the addition of formalin solution, the gel force, deformation and gel strength of fish meat gels decreased at all heating temperatures. The addition of formalin at ≥100 ppm affected the gel forming ability of washed dorab meat. Their gel strength was lower than 200 g.cm and formalin smell could be perceived. The fish gels heated at 40°C and 40°C/90°C provided higher gel properties than those heated at 90°C. According to a SDS-PAGE analysis, the protein patterns of samples without the formalin solution correlated to their gel forming ability. Samples with 100, 200, 300, 500 ppm formalin solution showed the loss of myosin heavy chain and actin bands at 90°C. It seemed likely that formaldehyde affected not only the gel forming ability of myofibrillar proteins but also the conformation of these proteins.

Key words : formaldehyde, gel forming ability, fish meat

INTRODUCTION

An important factor affecting the gel forming ability or quality of surimi is the freshness of fish (raw material). Generally, the chemical freshness index of the fish are the K-value, total volatile bases (TVB) and trimethylamine (TMA). Trimethylamine oxide (TMAO) has been naturally found in a large number of marine fish and shellfish. The reduction of TMAO to TMA, dimethylamine (DMA) and formaldehyde (FA) is caused by endogenous enzymes in fish and exogenous enzymes produced by bacteria during fish spoilage. However, several researchers reported that FA correlated to freshness of fish, especially lizard fish. It caused muscle protein denaturation and reduction of gel forming ability (Nozaki *et al*., 1978; Sophonphong and Rungjiratananan, 1993). Moreover, formation of DMA and FA by TMAO decomposition had taken place during storage at -5°C, -20°C and -25°C. (Nozaki *et al*., 1978; Tunhun *et al*., 1996). FA in fish meat can be classified as free form and combined form (Boeri *et al*., 1993). The free form is easily extracted by trichloroacetic acid and determined by Nash's method. The FA combined with proteins (combined FA) could not be extracted. It is known that equal mole of DMA and FA is produced from TMAO (Owusu-Ansah and Hultin, 1986). Thus, the amount of combined FA can be calculated by the following equation.

TMA-O	→	DMA	+	Total FA
$(CH_3)_3N$-O		$(CH_3)_2NH$		HCHO

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equivalent mole (mole DMA ≡ mole total FA) ..... (1)

Total FA = Free FA + Combined FA ... (2)

Therefore the objective of this experiment is to study the effect of formaldehyde, both free and combined forms, on the gel forming ability of fish meat at various heating temperatures.

MATERIALS AND METHOD

Whole fresh dorab (*Chirocentrus dorab*, Forskal), bought from Bangkok fish market organization, were sampled for chemical analysis. Proximate composition and pH were determined by the AOAC (1990) method. TVB and TMAO were estimated by Hasegawa (1992) methods. DMA was analysed by Dyer's method (Dyer and Mounsey, 1945) using copper ammonium reagent and carbondisulfide-toluene solution. FA was determined by Nash's method (Amano *et al*., 1963) using acetylacetone reagent. The rest of the fish was headed, gutted and washed thoroughly in ice water. Fish meat was separated from bone and skin by a deboner machine. This minced meat was separated into 2 portions. The first one was mixed with 3% salt in a Stephan cutter-mixer for gel preparation. This fish meat gel was adjusted to 78% moisture. After mixing, the meat sol was filled into cellulose casings with a diameter of 3.5 cm and a length of 15 cm. Both ends of the casing were fastened tightly. The heating step was carried out at various temperatures of (1) 40°C 30 min, (2) 90°C 30 min, and (3) 40°C 30 min followed by 90°C 30 min. The samples were cooled in ice water and kept at 5°C for 18-24 h before gel evaluation.

The second portion of fish meat was washed in ice water 3 times (10 min each). The ratio of fish meat to ice water was 1 to 5 by weight. The fish meat was pressed by a hydraulic press to discard water and separated into 8 portions. Formalin solution (prepared from formaldehyde 40% w/v Carlo Erba, analysis reagent) was mixed with each portion at concentrations of 0, 25, 50, 75, 100, 200, 300, and 500 ppm, respectively. Each portion of fish meat was left at room temperature for 1 hour and processed for chemical analysis and gel preparation.

SDS-PAGE analysis of fish meat gel and surimi gel

Each gel of 0.5 g was solubilized with 20 ml of 0.05M sodium phosphate buffer (pH 8.0) containing 8M urea, 2%SDS and 10% 2-mercaptoethanol. SDS-PAGE was carried out on 5% polyacrylamide gel (vertical slab gel with 0.75 mm thickness) according to the method of Weber and Osborn (1969). The patterns of protein as polymers, myosin heavy chains (MHC) and MHC breakdown or degraded products were evaluated.

Gel forming ability (MFRD, 1988)

Gel force (g), deformation (mm) and a folding test of fish meat gel were determined. Samples of meat after gel preparation were cut into 2.5 cm thickness. Five pieces of gel were measured for gel force and deformation by a Rheometer with a spherical plunger (5 mm in diameter) at a speed of 60 mm/min. The sample was pressed by a plunger until a break occurred in the surface of sample. The weight exerted on the sample until its breaking point is called force and the depth of breaking point is called deformation.

Sample discs of 25 mm in diameter and 5 mm in thickness were folded and graded in a folding test according to the following scheme:

AA No breakage when folded in quarters

A Slight tear when folded in quarters

B Slight tear when folded in half

C Breakage (but 2 pieces still connected) when folded in half

D Complete breakage into 2 pieces when folded in half

RESULT AND DISCUSSION

The chemical analysis of unwashed and washed dorab meat are shown in Table 1. Protein,

fat, ash content and pH of fresh dorab meat were 17.62%, 1.97%, 1.24% and 6.5, respectively while moisture content was 77.20%. After washing, they changed to 15.21%, 1.34%, 0.38% and 6.8 while moisture was 81.20%. Total volatile bases (TVB) as freshness index was 6.86 mg/100g in unwashed dorab meat and decreased to 4.34 after washing. TMAO of dorab meat also decreased after washing from 13.39 mg/100g to 3.24 mg/100g. Yasui and Lim (1987) concluded that most of the TMAO in the minced meat was washed out, resulting in an increase of meat sol and salt soluble protein extraction.

Hiraoka *et al*. (1995) suggested that combined FA could play an important role in reducing the gel forming ability of fish muscle. The amount of total FA (estimated total FA), free FA and combined FA of fish sample are shown in Table 2. The amount of free FA of unwashed and washed dorab meat were 10.01 ppm and 13.55 ppm while total FA were 36.08 ppm and 38.43 ppm, respectively. Tunhun *et al.* (1996) found that free FA of chub mackerel, rake-gilled mackerel and Indo-Pacific king mackerel was below 1.0 mg/kg and that in silver pomfret was 1.5 mg/kg. After the addition of 0, 25, 50, 75, 100, 200, 300, and 500 ppm formalin solution, almost 80% of these solutions combined with fish meat while the rest was extracted and analysed as free FA. The obtained free FA was 13.55, 16.50, 17.13, 16.82, 18.93, 30.43, 61.92, and 81.94 ppm. Therefore, the combined FA of these samples were 24.88, 46.25, 70.71, 95.91, 119.10, 206.10, 275.23 and 455.96 ppm, respectively (Table 2). Tunhun *et al.* (1996) also reported that the analysed concentration of free FA in some fresh fish correlated with the concentration of the dipping formalin solution.

The gel force of unwashed dorab meat was quite high as 532, 373 and 604 g when heated at 40° C 30 min, 90°C 30 min and 40°C 30 min followed by 90°C 30 min respectively. After washing, the gel force of washed dorab meat was lower than those of unwashed meat at all heating levels ($p<0.05$). This result indicated that some gel-forming enhancing factor such as transglutaminase was removed during washing as also suggested by Seki *et al.*, 1990. The highest gel force of washed meat heated at 40°C 30 min followed by 90°C 30 min was 497 g while the lowest one heated at 90°C 30 min was 293 g. The higher concentration of formalin added, the lower gel force obtained. At low concentrations of 25 and 50 ppm, the gel force was almost the same as the non-added FA meat ($p>0.05$) but decreased drastically when the higher concentrations of FA were added ($p<0.05$). The decrease of gel deformation also observed in samples with high concentrations of FA. In addition, the gel strength of dorab meat gel with and without formalin added at all heating steps correlated to their gel force (Figure 1). The washed dorab meat of 25, 50, 75, 100 and 200 ppm of formalin added still had good gel forming ability at the heating level of 40°C 30

Table 1 Chemical analysis of dorab meat.

Sample	Protein (%)	Fat (%)	Moisture (%)	Ash (%)	pH	TVB (mg/100g)	TMAO (mg/100g)
Dorab meat	17.621/±0.262/	1.97±0.07	77.20±0.27	1.24±0.02	6.5	6.86±0.0	13.39±4.12
Washed dorab meat	15.21±0.06	1.34±0.07	81.20±0.33	0.38±0.03	6.8	4.34±0.13	3.24±0.22

1/ Mean of triplicate determination
2/ Standard deviation

min followed by 90°C 30 min. Their gel strength was higher than 100 g.cm and folding tests were AA. However, formalin smell could be perceived from samples with the addition of formalin from 100 to 500 ppm . Moreover, these samples heated at 90°C 30 min were rejected because of the detection of formalin smell and the low folding test as C. On the other hand the FA smell was detected when free FA and combined FA were >20 and 100 ppm, respectively. Samples with the addition of 200, 300 and 500 ppm formalin heated at 40°C for 30 min were rejected by a folding test score of C while the formalin smell was also detected with the addition of formalin 100-500 ppm. According to Tunhun *et al.* (1996), a formaldehyde smell could be perceived in rake-gilled mackerel and lizard fish dipped in solutions containing more than 500 and 2,000 ml/L formaldehyde, respectively. These results indicated that the increase of FA content in fish meat of more than 20 ppm as free FA or 70 ppm as combined FA would affect the gel forming ability. It has been reported that the reaction of formaldehyde causes

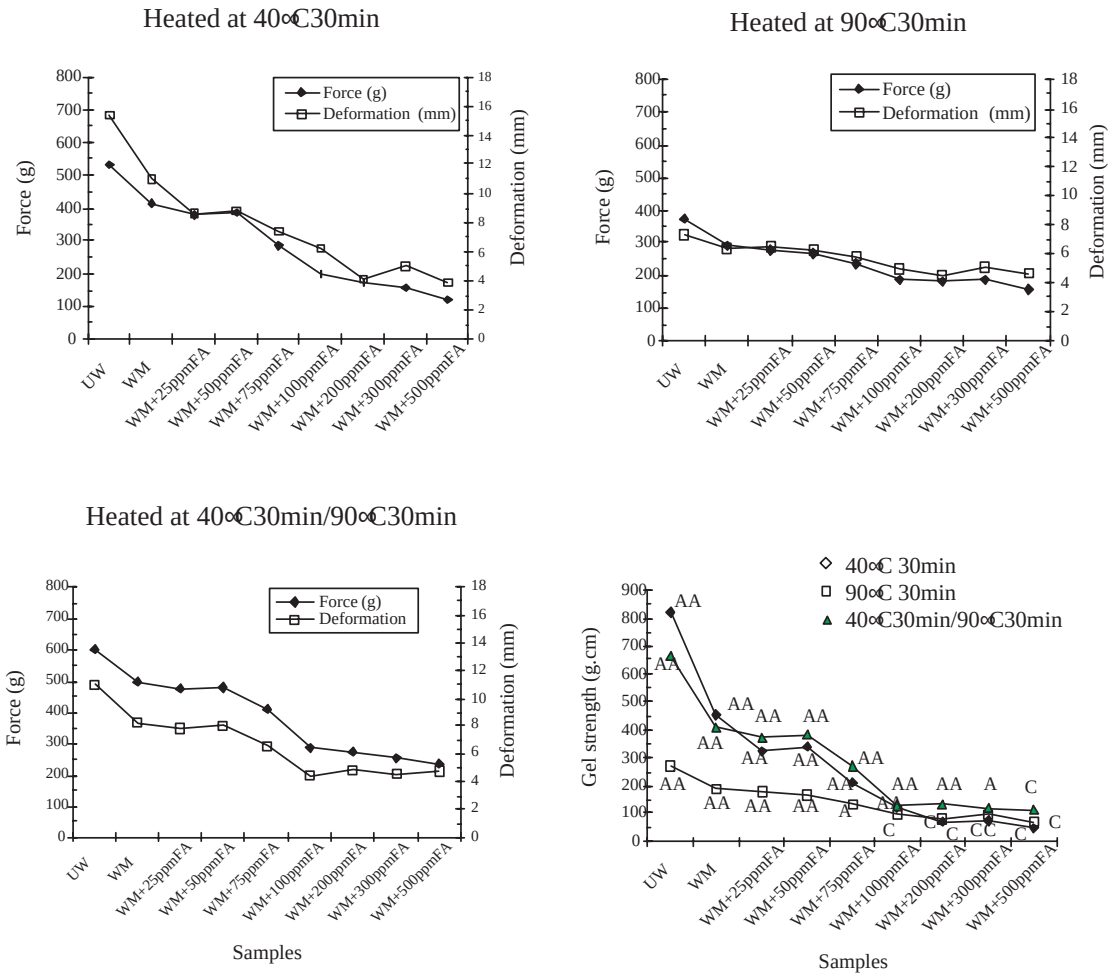
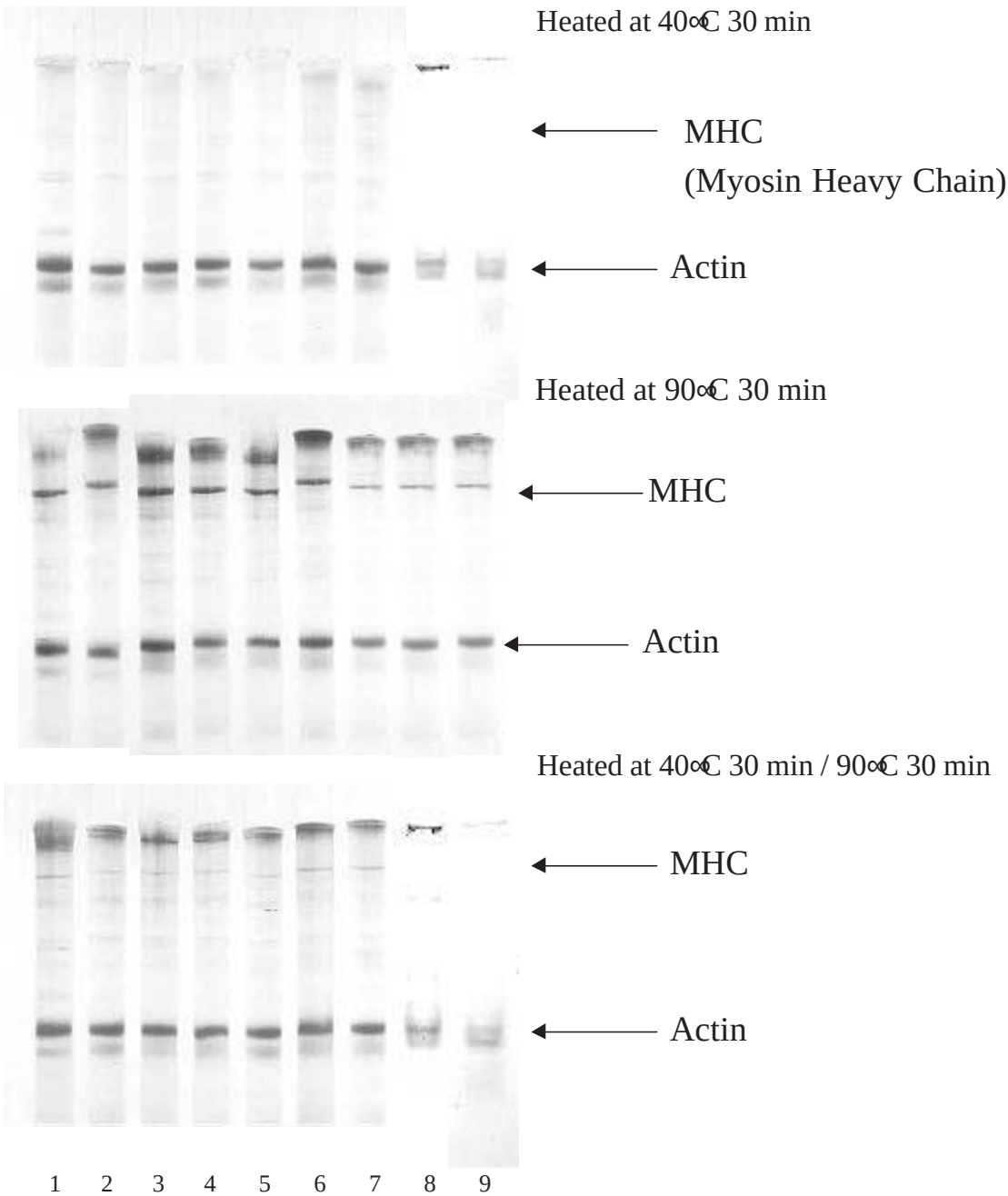


Figure 1 Force, deformation, gel strength and folding tests of dorab meat gel at various heating temperatures.
(UM:unwashed meat, WM:washed meat, FA:formalin solution)



1 Unwashed meat
2 Washed meat (WM)
3 WM+25 ppm formalin sol^{n}(FA)
4 WM+50ppmFA
5 WM+75ppmFA
6 WM+100ppmFA
7 WM+200ppmFA
8 WM+300ppmFA
9 WM+500ppmFA

Figure 2 SDS-PAGE analysis of dorab meat gel at various heating temperatures.

denaturation of proteins (Ang and Hultin, 1989). Sophonphong and Rungjiratananan (1993) concluded that FA content provided a freshness index of fresh lizard fish and also had a good relationship with its gel forming ability.

Figure 2 shows the SDS-PAGE patterns of meat gels heated at (1) 40°C 30 min, (2) 90°C 30 min, and (3) 40°C 30 min followed by 90°C 30 min of unwashed meat, washed meat and washed meat with the addition of several concentrations of formalin solution. Protein patterns of unwashed meat gels were similar to those of washed meat. According to the report by Numakura *et al*.(1990) three main bands appeared on the SDS-PAGE pattern of surimi meat gels were assigned to cross-linked MHC (CMHC) band at the top of the disk gel, the second to MHC and the bottom to actin. The MHC bands were apparent in samples of meat gels heated at 90°C while those of meat gels heated at 40°C and 40°C followed by 90°C almost disappeared. Moreover, the CMHC bands were observed from both of meat gels heated at 90°C and 40°C followed by 90°C but were not observed in that of 40°C. The disappearance of CMHC bands in SDS-PAGE pattern of salted ground meat heated at 40°C were found in Threadfin bream (Lee *et al.*, 1990) and Walleye pollack (Numakura *et al*., 1990). This occurrence could be attributed to the degree of polymerization of CMHC which affected to its solubility and electrophoretic behavior (Nowsad *et al.*, 1993). Therefore, it is likely that CMHC in the samples of 40°C 30 min was not dissolved in the SDS-urea-mercaptoethanol buffer. After the addition of formalin solutions at various concentrations, MHC bands of dorab meat gels disappeared at all heating temperatures. This result correlated to the decrease of their gel forming ability. Especially in washed dorab meat gels addition with 300 and 500 ppm formalin solution, both MHC and Actin bands absolutely disappeared. It seems likely that the action of formaldehyde and heating affected the conformation of the myofibrillar proteins of both actin and MHC.

Table 2 Dimethylamine and formaldehyde concentrations of dorab meat.

Sample	DMA (ppm)	Total FA1/ (ppm)	Free FA (ppm)	Combined FA2/ (ppm)
Dorab meat (Unwashed meat, UW)	16.82	36.08	10.01	26.08
Washed dorab meat (WM + 0 ppm FA)	17.92	38.43	13.55	24.88
WM + 25 ppm FA	17.6	62.75	16.5	46.25
WM + 50 ppm FA	17.64	87.84	17.13	70.71
WM + 75 ppm FA	17.59	112.73	16.82	95.91
WM + 100 ppm FA	17.73	138.03	18.93	119.1
WM + 200 ppm FA	17.03	236.53	30.43	206.1
WM + 300 ppm FA	17.32	337.15	61.92	275.23
WM + 500 ppm FA	17.67	537.9	81.94	455.96

1/ Total FA = Estimated total FA after formalin addition

2/ Combined FA = Total FA - Free FA

CONCLUSION

Formaldehyde, free FA and combined FA at concentrations over 100 ppm, 20 ppm and 70 ppm, respectively, affected the gel forming ability of dorab meat.

The gel strength of dorab meat gel heated at 40°C and 40°C/90°C did not depend on disulfide linkages.

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The Expansion of Inland Shrimp Farming and Its Environmental Impacts in Songkla Lake Basin

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ABSTRACT

Amidst the heightened awareness of environmental issues in the Songkla Lake Basin, widespread concern has also emerged over potential environmental impacts of inland shrimp farming. Outbreaks of disease in the coastal areas and the development of low salinity culture techniques have been major factors behind the migration of shrimp farming into the basin's freshwater areas well inland from the coast. Over a period of 18 years, from 1982 to 2000, shrimp cultivation areas rose dramatically from 3,491 ha to 7,799 ha, equivalent to an increase of 123.4 %. Further analysis has revealed that 3,347 ha, or 77.7 % of the increase in culture areas came from rice fields. The graphic consequence has been well-demonstrated problems of soil and water degradation resulting from the culture operations. Farmed soils possessed several chemical and physical limitations to the establishment of vegetation. The major chemical factors were largely associated with high salinity level and low organic carbon content whereas high bulk density and low saturated hydraulic conductivity were the major physical limitations. Analysis also revealed that salinity levels in soils located within 0, 20, 40, 60 and 100 meters distance from the culture pond were high and well above the suggested critical value of 1.6 mS/cm, indicating that soluble salt could be a limitation to the establishment of plants on these soils. Besides the salinization of soils, the discharge of untreated pond effluents caused deterioration of the quality of waterbodies in close proximity to the pond through the elevation of salinity level, BOD concentration and suspended solid level. The degradation of soil and water quality that occurs could render large areas of productive land unsuitable for arable crop husbandry. Moreover, poor water quality could contribute to outbreaks of disease which, in turn, resulting in a catastrophic collapse of the industry. Management strategy for the reversal of such degradation is discussed.

Key words : shrimp farming, environmental impacts, land use zoning plan, Songkla Lake Basin.

INTRODUCTION

Songkhla Lake Basin covers an area of approximately 8,463 km^2, of which 1,043 km^2 is the lake surface, and stretches southwards for over 150 kilometers along the eastern coast of the Southern Thai Peninsula. The basin consists of three main topographic units: a range of mountains to the west and south of the basin, foothills and terraces, and broad plains on the east and west sides

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of the lake. The lake, which is divided into three distinct but interconnected waterbodies viz. Thale Noi, Thale Luang and Thale Sap Songkla (NEDECO, 1972), is a shallow coastal lagoon formed by interaction of land and ocean processes over geological time (NESDB and NEB, 1985). The lakes connecting to the Gulf of Thailand through a narrow channel outlet are subject to seasonal fluctuations in salinity (Lesaca, 1977). During the dry season, salinity levels in the lake water increase due to intrusion of seawater from the Gulf of Thailand. Salinity level in Thale Sap Songkla may rise to 25 ppt, 15-20 ppt in Thale Luang and almost zero in Thale Noi (DANCED and MOSTE, 1999).

The basin experiences an annual rainfall of approximately 2,000 mm, with a distinct wet season from October to January (DANCED and MOSTE, 1998). More than 100 streams of all sizes drain the basin from the western mountain range into the lakes (Lesaca, 1977). Total annual inflow from streams to the entire lake system is 5,200 million m^3 (Thimakorn and Vongvisessomjai, 1979). Storage in the lakes is 1,681 million m^3 to mean sea level. Only 0.6 % of the basin's total land areas is regarded as having severe erosion, while 4.0 % has moderate erosion and 95.4 % has slight erosion (Tanavud *et al*., 2000). Sediment rate in the lake has been estimated at 1.0 mm yr^{-1} (Tanavud *et al*., 2000).

The economy of the basin is agricultural in nature. Rice is cultivated in the lowlands, while rubber and mixed orchard are cultivated in the terrace and foothills (Tanavud *et al*., 1999). The mountain ranges are covered with evergreen forest. Over recent years several parts of the range have been dedicated as wildlife sanctuaries or national parks. Animal husbandry and fisheries also contribute to the local economy. The Songkhla Lake Basin, which was once the richest and most extensive rice growing area, was formerly described as the rice bowl of southern Thailand (NESDB and NEB, 1985).

It was not until the early 1980s that shrimp cultures for black tiger shrimp (*Penaeus monodon)* were introduced by the government as a means of providing nutrition, improving household incomes and enhancing employment opportunities for the basin's population (Phillips and Barg, 1999). Although it is undeniable that the growth of shrimp culture industry has benefited the social and economic well-being of the people in rural areas, there has also been environmental disruption generating impacts detrimental to the welfare of the rural people (Chin and Ong, 1997). While the establishment of shrimp farming in the basin's freshwater areas is broadly known, there have been no detailed assessments of the nature and actual extent of shrimp cultivation areas, and the environmental impacts generated by the culture industry.

It is in this context that the present study was undertaken. The objectives of the study are (i) to ascertain the nature and areal extent of shrimp cultivation areas in Songkla Lake Basin, and (ii) to evaluate the impacts of shrimp farming on soil and water environments in the areas of operation.

MATERIALS AND METHODS

Areal extent of shrimp farming

To determine the areal extent of shrimp farming in Songkla Lake Basin, Geographic Information Systems (GIS) was used to compile spatially explicit data layers that describe the basin's land use. All spatial analysis operations were performed using PC ArcInfo 3.5.2 and Arcview 3.2 software (ESRI, 1998). To allow for comparisons, land use was determined for two time periods; 1982 and 2000. The data layers selected as input data for PC ArcInfo operations included basin boundary maps and land use maps. In ArcInfo GIS, maps can be converted into a digital format by tracing them with a digitizer. In the present study, basin boundaries were created in ArcInfo GIS by

digitizing from the 1:50,000 topographic map produced by the Royal Thai Survey Department. The 1982 land use coverage was generated in ArcInfo GIS by digitizing from a paper map displaying 1982 land use at a scale of 1:50,000 prepared by the Department of Land Development. The 2000 land use coverage was digitized from a paper map visually interpreted from the 1:50,000 Landsat TM images for Songkla Lake Basin acquired in 2000. Ground truthing was also conducted to assist in the imagery classification and validate the final results. Following the preparation of land use coverages for the two dates, the areas of each type of land use for each period were calculated using the TABLES and CALCULATE commands in PC ArcInfo. Changes in land use between the two inventories were determined by overlaying land use coverages between the two dates using PC OVERLAY's UNION and INTERSECT commands. The acreage of land areas within 50 m and 100 m from the edge of the shrimp ponds were determined using PC OVERLAY's BUFFER and CLIP commands.

Impacts of shrimp farming on soil resources

To assess the impacts of shrimp farming on soil resources, a shrimp farm, situated at Tambon Kootao in Hat Yai District (7º 06/ N and 100º 27/ E), was selected as a study site. The site, previously devoted to rice cultivation, is located adjacent to Khlong U-Taphao river approximately 25 km inland from the coast. To enable a direct comparison between soils "before" and "after" shrimp farming, soil samples were taken from pond bottoms and original soils in adjacent rice fields. These soils are designated as farmed soils and pre-farmed soils respectively in this study. According to the Department of Land Development (1973), both farmed soils and pre-farmed soils belong to Rangae series and are classified as Thapto-Histic Tropic Fluvaquents. At each sampling location, three replicate samples of disturbed and undisturbed soils at a depth of 15 cm were collected.

The disturbed soil samples were air-dried, passed through a 2 mm sieve, and analyzed for pH, electrical conductivity, organic matter content, total nitrogen, available phosphorus, exchangeable potassium, and texture. Measurements of pH and electrical conductivity (EC) were made on a 1:5 soil/de-ionized water suspension using a glass electrode, and a conductivity cell and direct reading meter respectively. Soil organic matter content was measured using the Walkley-Black technique (Nelson and Sommers, 1982). The Kjeldahl method was used for the assessment of total nitrogen (Bremner and Mulvaney, 1982). Available phosphorus was measured by the Bray-2 method (Bray and Kurtz, 1945). Exchangeable potassium was extracted using ammonium acetate and determined by atomic absorption spectrophotometry (Thomas, 1982). The particle distribution of each soil sample was determined by the hydrometer method (Gee and Bauder, 1986) and the results are expressed as percentage sand, silt, and clay using the USDA size classification. Bulk densities were measured gravimetrically on three replicate undisturbed cores, with core dimensions of 50 mm in diameter by 50 mm in height. Particle density of the solids was determined by the method of Blake and Hartge (1986). Porosity was calculated from the bulk density and particle density (McIntyre, 1974a). Plant available water was evaluated as the difference in water content held at 0.01 MPa and 1.5 MPa (McIntyre, 1974b). The significance of the differences between farmed soils and pre-farmed soils in regard to chemical and physical properties was evaluated using analyses of variance (ANOVA) and the least significant difference test (LSD) procedures (Gomez and Gomez, 1984). Significance was at the $P = 0.05$ level unless otherwise noted.

In addition, three replicate samples of disturbed soils were taken from the top 15 cm at 0, 20, 40, 60, 80 and 100 meters distances from the

culture pond. These samples were analyzed for electrical conductivity (EC) using a conductivity cell and direct reading meter.

Impacts of shrimp farming on water resources

In order to elucidate the impacts of shrimp culture on water resources, three sampling sites were established in proximity to a shrimp pond. The sites included source water from which the water was taken to fill the pond enclosures, pond water in which *Penaeus monodon* was cultivated and receiving water immediately outside the ponds in which pond water was discharged. Samplings were carried out during the fourth months of the growout period. At each sampling location, three replicate water samples were collected in 0.75 litre polyethylene bottles from the middle of the water column. The samples were immediately analyzed for dissolved oxygen (DO) using a dissolved oxygen meter (HACH model DO 175). The rest of the water samples were stored on ice and transferred to the laboratory for further analyses. pH and electrical conductivity (EC) were measured using a microprocessor pH meter (WTW model pH 537) and microprocessor conductivity meter (WTW model LF 137), respectively. Turbidity was determined using a turbidimeter (HACH model 2100). Estimates of total suspended solids (TSS) were obtained from the mass of materials retained on Whatman No. 42 filter paper. Analysis of nitrate nitrogen, orthophosphate, and biological oxygen demand (BOD) was performed by the Department of Aquatic Science, Prince of Songkla University, as per methods outlined in the American Public Health Association (1989).

RESULTS AND DISCUSSION

Areal extent of shrimp farming in Songkla Lake Basin

It was not until the early 1980s that Songkla Lake Basin experienced a substantial growth of the shrimp culture industry (Flaherty *et al*., 1999). In 1982, areas devoted to shrimp farming covered an estimated 3,491 ha, equivalent to 0.47 % of the total area of the basin (Table 1). At that time, the development of shrimp farming was limited to a relatively narrow band of coastal land in Ranote District (Figure 1). This is because large volume of seawater are needed to fill the pond enclosures for raising shrimp and to offset losses from water seepage and evaporation during the growth-period (Szuster and Flaherty, 2000). Over a period of 18 years, from 1982 to 2000, shrimp culture areas dramatically rose from 3,491 ha to 7,799 ha, equivalent to an increase of 123.4 % (Table 1). Given that all of the shrimp ponds in the basin are currently in operation and a hectare of pond yields 6 metric tonnes of shrimp per annum, the basin's shrimp production increased from 20,946 metric tonnes in 1982 to 46,794 metric tonnes in 2000. This represents an annual increase of 1,436 metric tonnes. In 2000, the basin's annual shrimp production accounted for about 19.5 % of the country's total production. If a typical price for a metric ton of shrimp is $ US 6,950 (C.P. Group, 2000), a total estimate of $ US 325,218,300 can be obtained in 2000. It is interesting to note that a farmer with one hectare of his holding devoted to the shrimp culture would have a gross annual income of $ US 41,700. This is 165 times the income of a typical rice farmer in the basin, assuming that a hectare of rice fields yields 2.34 metric tonnes and a typical price for a metric ton of rice is $ US 108.09 (Office of Agricultural Economics, 2001). This economic analysis of shrimp production, however, does not take into account its long-term adverse social and environmental impacts associated with the farming activity (Flaherty *et al*., 2000).

In 2000, it was found that new cultivation areas have emerged along the estuaries of the main rivers some distance upstream from the coast and/or the Songkhla lakes (Figure 2). The establishment

of shrimp farming in the basin's freshwater environment has occurred as a result of outbreaks of disease along the coast and the development of low salinity culture techniques for shrimp cultivation (Flaherty *et al*., 2000). The growth of low-salinity shrimp culture in freshwater areas, which is referred to as inland shrimp farming in this paper, has generated widespread concern over the degradation of soil and water resources in the areas of operation.

Coincident with an increase in shrimp culture areas has been a decrease in rice growing areas and mangrove forests. Indeed, between 1982 and 2000, mangrove forests in the basin declined from 3,221 ha to 406 ha, which represents a decrease of 87.4 % (Table 1). Hussain (1995) reported that large areas of mangrove forests in the South China Sea countries have already been converted into shrimp farms and the process is continuing. The depletion of mangroves contributes to the loss of habitat and nursery area for aquatic species as well as reduces shoreline stability during storms (IUCN, 1983; Field, 1995). Likewise, rice growing areas also reduced from 208,599 to 164,209 ha, representing a decrease of 21.3 %. It should be noted, however, that the biggest changes in land use in Songkla Lake Basin between 1982 and 2000 were in forested and rubber areas (Table 1).

An overlay of land use coverage for 1982 with that for 2000 revealed that 3,347 ha, or 77.7 % of the increase in shrimp farm areas in 2000, came from rice fields in 1982 (Table 2). The establishment of shrimp culture operations in the rice growing areas of southern Thailand has generated widespread concern over the salinization of rice fields adjacent to culture ponds (Flaherty *et al*., 1999).

It has been reported that seepage of saline water and discharge of pond effluents can increase salinity level in soils up to 100 meters from the edge of the shrimp ponds (MOSTE, 1999). If the soils within 50 meters radius of the ponds are taken as affected areas, it can be estimated through the use of PC OVERLAY commands in PC ArcInfo that soil subject to salinization impacts was 2,271 ha (Table 3). Further analysis also revealed that 1,127 ha, equivalent to 49.6 %, of the affected soils, is rice fields. In addition, if the soils within 100 meters radius of the pond are taken as affected areas, land area subject to salinization is increased to 4,138 ha (Table 3), of which 1,977 ha, or 47.8 %, of the affected areas is rice fields. If the figure of 1,977 ha is taken as the affected rice fields, then 4,626 metric tonnes of rice, equivalent to a value of approximately $ US 500,024 would have been lost through salinization of soils, assuming a hectare of rice fields yields 2.34 metric tonnes and the price for a metric ton of rice is $ US 108.09. It should be noted that productivity losses due to salinization effects on soils may last several years.

Impacts of shrimp farming on soil resources

Chemical and physical characterization of the pre-farmed and farmed soils was conducted to allow a comparison between the two soils in regard to their properties as well as to define potential limitations to plant growth in these soils. The pH values for both pre-farmed and farmed soils were 4.30 and 4.98, respectively (Table 4). The possible reasons for this could be that the culture ponds were built on sites where soils are acid sulfate soils. The low pH levels in these two soils may have resulted from sulfuric acid generated by exposed pyrite. Acidity of pond bottom soils may subsequently reduce the pH of the pond water (Zweig *et al*., 1999; Boyd and Zimmermann, 2000) which, in turn, reduces growth and survival of cultured shrimps and decreases natural food production (algae growth) within the culture ponds (Poernomo and Singh, 1982). Generally, lime is applied to the pond bottom soils to raise the alkalinity of pond water, thereby removing carbon deficiencies, which limit phytoplankton growth (Boyd and Bowman, 1997). Hence, the significantly greater pH in farmed

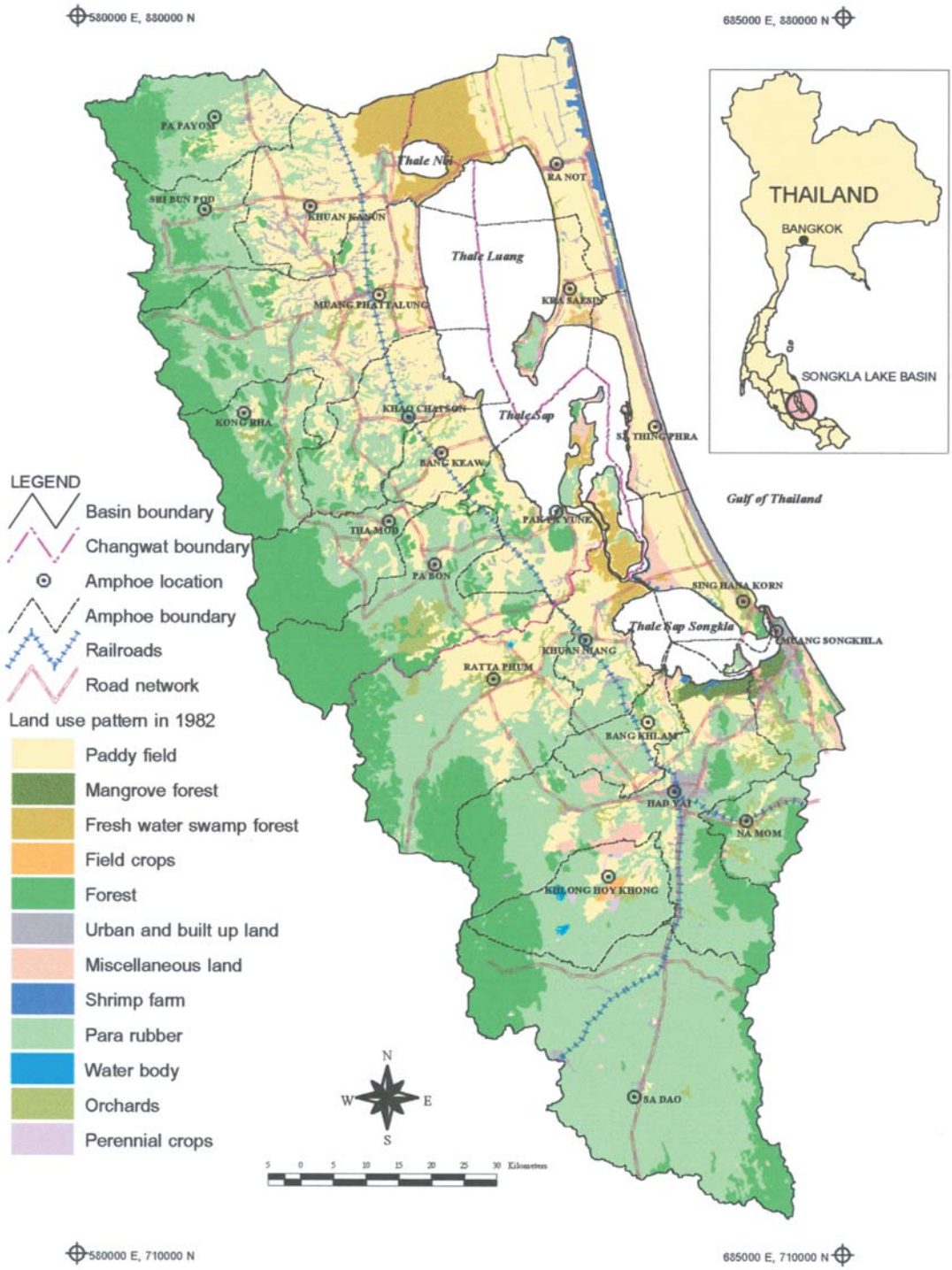

Figure 1 Land use map of Songkla Lake Basin in 1982.

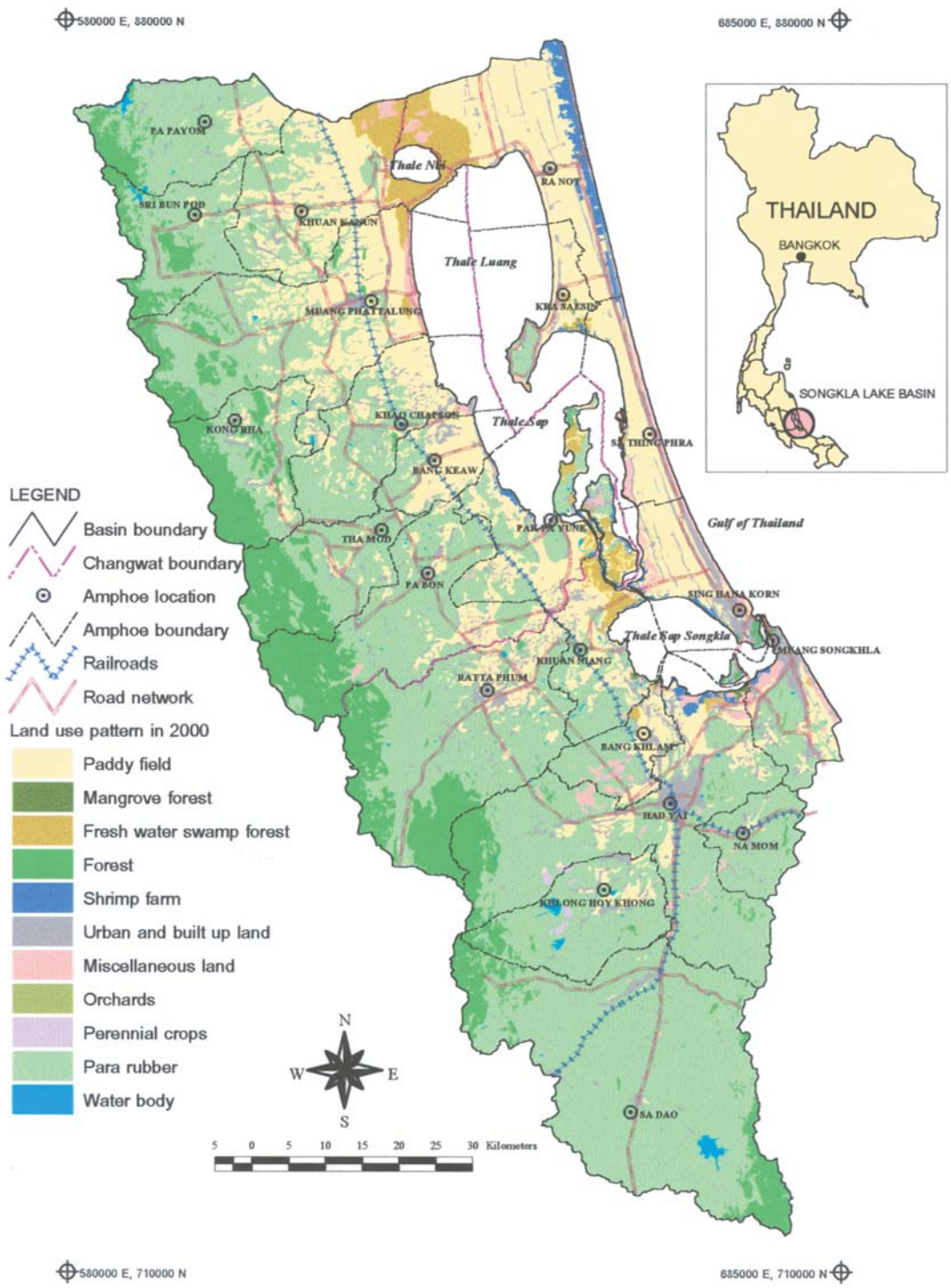

Figure 2 Land use map of Songkla Lake Basin in 2000.

soils compared with that in pre-farmed soil can be explained by this. The EC is recognized as a useful parameter for appraising soil salinity in relation to plant growth. High salinity inhibits plant growth by reducing the osmotic pressure gradient between the plant and soil solutions, restricting the ability of the plant to take up water (Moore, 1998). It should be noted that the EC value for the farmed soils was significantly higher compared to levels in the pre-farmed soils (Table 4), most likely as a result of deposition and accumulation of salts from seawater that are brought in to support the operation of the shrimp farms (Szuster and Flaherty, 2000). With EC value higher than the suggested critical value of 1.6 mS/cm, plants grown on the farmed soils would be restricted by soluble salt (Hunt and Gilkes, 1992). This finding is consistent with the work of Szuster and Flaherty (2000) who reported that salinization can occur through the deposition and accumulation in salts in soils located immediately beneath the pond enclosure.

The salinization effects on soils in the vicinity of shrimp farm were also evident in this study. As seen in Table 5, salinity levels in soils located within 0, 20, 40, 60, 80 and 100 meters distance from the edge of the culture pond were high and well above the suggested critical value of 1.6 mS/cm, indicating that soluble salt levels within these soils limit plant growth. High salinity levels in these soils could be attributed to the seepage of saline water from the culture ponds and/or the discharge of saline pond effluents directly into adjacent waterbodies as well as land areas immediately adjacent to the ponds (Szuster and Flaherty, 2000). In addition, shrimp farmers often remove accumulated sediment deposits that remain on the pond bottoms after harvest and dispose to vacant lands in the vicinity of shrimp farms (Miller *et al*., 1999). Salt leaches from this sediment can salinize nearby soil and groundwater resources (Boyd and Tucker, 1998). However, the distance of the salt-affected areas from the ponds should be assessed with caution. This is because the physical properties of soils that influence the volume and

Table 1 Estimates of land use and land use change in Songkla Lake Basin between 1982 and 2000.

Land use categories	Area* (ha)		
	1982	2000	Change
1. Forest	146,568	86,225	- 60,343
2. Rubber	292,610	404,531	+ 111,921
3. Rice field	208,599	164,209	- 44,390
4. Fruit orchards	21,412	126	- 21,286
5. Perennial crops	1,196	881	- 315
6. Shrimp farming	3,491	7,799	+4,308
7. Mangrove forest	3,221	406	- 2,815
8. Fresh water swamp forest	24,821	18,682	- 6,139
9. Miscellaneous	7,725	1,908	- 5,817
10. Water bodies	1,334	2,632	+ 1,298
11. Urban / built up area	30,990	54,568	+ 23,578
Total	741,967	741,967	

* excludes lake surface

rate of water flow through soil pores varies spatially (Scott, 2000). However, the high salinity levels of soils in the immediate vicinity of the ponds are of sufficient concern to warrant an initiation of environmentally sound shrimp cultivation practice that reduce concentrations of potential pollutants in pond effluents and/or a closure of all the shrimp ponds operating in freshwater areas. The salinization of soils generated by shrimp cultivation has also been reported by Csavas (1995), Tookwinas *et al*. (!997) and Im-Erb *et al*. (2001).

The organic carbon figure for farmed soils is significantly lower than that for pre-farmed soils (Table 4). The low organic carbon level in farmed soils was likely to be the result of the removal of surface soils that were subsequently used for the construction of pond embankments when ponds are initially constructed. With organic carbon contents lower than 1 %, farmed soils would be expected to have unstable structure (Moore, 1998). Further, the shortage of organic carbon in farmed soils curtails the supply of available nitrogen and phosphorus (Pulford, 1991). Higher content of organic carbon in pre-farmed soils could be attributed to vegetation remains. According to Landon (1991), CEC values for pre-farmed and farmed soils are considered to be medium and low, respectively. The greater organic carbon content in pre-farmed soil accounts, in general, for its high value of CEC.

The total nitrogen in farmed soils, which was significantly lower than that in pre-farmed soils, was lower than the critical level of 0.15 % (Moore, 1998). Likewise, phosphorus concentration in farmed soils falls below the 4 mg/kg minimum threshold (Landon, 1991), suggesting a probable

Table 2 The acreage of land areas under different uses in 1982 that were converted to shrimp farming in 2000.

Year	1982								2000
Land use	Rice	Rubber	Mangrove	Orchards	Shrimp	Swamp	Urban	Miscellaneous	Shrimp
Area (ha)	3,347	161	466	25	2,836	370	267	327	7,799

Table 3 Areas affected by shrimp farming in 2000.

Land use types in 2000	Affected areas (ha)	
	50 meters from pond edge	100 meters from pond edge
1. Rice fields	1,127	1,977
2. Forest	-	-
3. Rubber	83	176
4. Mangrove forests	12	28
5. Fresh water swamp	232	456
6. Urban/built up area	440	805
7. Water bodies	1	4
8. Miscellaneous	376	693
Total	2,271	4,139

deficiency. The higher concentrations of total nitrogen and available phosphorus in pre-farmed soils may have resulted from a combination of the fertilizers and vegetation remains on rice fields The potassium levels in both soils were found to be higher than critical values of 0.15 meq/100 g, and therefore deficiency was unlikely (Landon, 1991). To ensure successful establishment of plants on the farmed soils after cessation of shrimp farming, the shortage of both nitrogen and phosphorus has to be rectified by applying chemical fertilizers, maintaining soil organic matter, and growing nitrogen-fixing species.

The physical characteristics of the soils collected from farmed soils and pre-farmed soils are presented in Table 5. The farmed soils and pre-farmed soils had clay percentage of 31.4 and 57.8 and their textural classes were clay loam and clay, respectively. According to Boyd and Bowman (1997), pond soils should contain 20-30 % clay-size particles to provide a barrier to seepage. Dry bulk density, porosity and hydraulic conductivity are important parameters determining the transport of air and water into the rooting zone (Rowell, 1994). The higher the soil bulk density the more compacted the soil and the lower the porosity (Scott, 2000). The significantly higher bulk density in farmed soils indicated that compaction has occurred, probably as a result of tractor wheel passage under wet field conditions during pond construction (Batey, 1988). Compaction problems in abandoned farmed lands could be a major factor in rehabilitation failure and alleviation requires good mechanical loosening practices. The air-filled porosity of farmed soils was below the critical value of 10 %, indicating that oxygen supply could become limiting to root growth (Mullins, 1991). In addition, farmed soils had lower values of available water percentage than the pre-farmed soils (Table 5), apparently as a result of higher bulk density and lower organic carbon contents. However, the available water values for both soils are above the acknowledged critical threshold value of 12 %, indicating that both soils would retain sufficient moisture in most years to allow satisfactory plant growth. According to Hunt and Gilkes (1992), values of saturated hydraulic conductivity for both soils were considered to be extremely slow, and hence seasonal waterlogging could be a problem in plants grown in these soils.

Laboratory characterization of farmed soils have demonstrated that the practice of inland shrimp farming contributes to the loss of soil quality in the area of operation. The degradation of lands that were used for shrimp production poses a serious threat to the welfare of the local population who are reliant upon this resource for their livelihoods. Since in most cases land which has been made derelict by shrimp farming was in agricultural use, rehabilitation to arable farming after the conclusion of shrimp cultivation is perhaps the most common land use objective. However, the cost of rehabilitating erstwhile farmed lands could be substantial (Department of Land Development, 1999) and may take several years (Bhatta and Bhat, 1998). In this regard, relevant government agencies should support further research studies on farmed land rehabilitation that can be achieved at affordable costs and in a timely manner.

Impacts of shrimp farming on water resources

The properties of source water, pond water, and receiving waters are presented in Table 6. The pH value for pond water was significantly higher than that for source water or receiving waters. The high pH value in pond water could be attributed to the application of lime to raise the pH level of acidic pond water in order to improve survival, reproduction and growth of shrimp (Boyd, 1990; Boyd and Bowman, 1997). According to the water quality standard for coastal aquaculture established by the Department of Fisheries (1994), the ideal pH levels for shrimp cultivation range from 6.5 - 8.5. As seen in Table 6, pond water had a salt content of

4.40 ppt, which was significantly higher than that of the source water. The higher salinity level in the pond water was attributed to seawater added to them to adjust salinity to an optimum level for cultivating shrimp (Tsai, 1989). According to Szuster and Flaherty (2000), the salt levels for the low-salinity shrimp culture should be in the range of 4 to10 ppt. The exchange of pond water with outside water to maintain good water quality and the usual practice of discharging saline pond water directly into adjacent receiving waters at harvest probably accounted for the higher salinity levels in receiving waters compared with the source water.

Pond water turbidity was significantly higher than that for source water or receiving waters. Eroded sediments from the pond sides, uneaten

Table 4 Physical and chemical properties of pre-farmed and farmed soils.

Soil properties	Pre-farmed soils	Farmed soils
1. Chemical properties		
PH	4.30a	4.98b
EC (mS/cm)	0.23a	2.04b
Organic carbon (%)	3.69a	0.26b
CEC (meq/100 g soil)	16.64a	5.71b
Total nitrogen (%)	0.30a	0.10b
Available Phosphorus (mg/kg)	7.23a	1.38b
Exchangeable Potassium (meq/100g soil)	0.26a	0.22a
2. Physical properties		
Texture	Clay	Clay loam
Sand (%)	16.01a	24.89b
Silt (%)	26.18a	43.74b
Clay (%)	57.82a	31.37b
Bulk density (g/cm^3)	1.15a	1.62b
Particle density (g/cm^3)	2.73a	2.76a
Air-filled porosity at field capacity (%)	27.94a	8.14b
Plant available water (%)	16.06a	12.60b
Saturated hydraulic conductivity (m/day)	0.19a	0.01b

*Within a column, means followed by the same letter within each properties are not significantly different at the 0.05 level of significance.

Table 5 Mean electrical conductivity of soils taken from 0, 20, 40, 60, 80 and 100 meters distances from the edge of the culture pond. Numbers in brackets represent Standard Deviation.

Distance from pond edge	0 m	20 m	40 m	60 m	80 m	100 m
Electrical conductivity (mS/cm)	5.24 (0.08)	4.42 (0.01)	5.10 (0.01)	4.05 (0.01)	5.65 (0.01)	5.10 (0.02)

feed and plankton probably accounted for the higher turbidity values of the pond water (Boyd *et al*., 1994). The turbidity value of pond water was high and well above the maximum permissible concentrations of 400 mg/l recommended by Chaiyakam and Predalumpabut (1994), indicating high pollution potential of discharge from the ponds. DO value for pond water was significantly higher than that for source water or receiving waters (Table 6). The DO value for pond water was high and well above the required minimum of 4.0 ppm (Department of Fisheries, 1994). This was likely the result of the use of paddlewheel aerators to maintain the pond's optimum oxygen level (Department of Fisheries, 1999). BOD concentrations for pond water were significantly higher than that for source water (Table 6), most likely due to the decay of uneaten feed, vegetation and plankton (Lee, 1997) and plankton respiration (Seim, *et al*., 1997). Even though receiving waters had significantly lower BOD concentrations than pond water, its BOD value exceeded the acceptable limits of 10 mg/l recommended by the Department of Fisheries (1994). Assimilative capacity of receiving waters and flush by the wet season flood probably accounted for the significantly lower BOD value in receiving waters compared to value in the pond water (Predalumpaburt and Chaiyakam, 1994). It should be noted that high concentrations of BOD in pond water and receiving waters found in this study are similar to those reported by Pongthanapanich (1999).

The TSS values for pond water and receiving water were significantly higher than that for source water (Table 6). The major sources of suspended solids in pond water and pond effluents are suspended soil particles and particulate organic matter resulting from live plankton and detritus (Boyd and Tucker, 1998). The observed increment in the TSS values in receiving waters compared to that in source water was probably a direct consequence of the discharge pond effluents. The concentrations of nitrate in pond water were significantly higher than the corresponding values for source water (Table 6). Similarly, a significantly higher concentration of orthophosphate was also recorded in pond water, compared with the source water. Elevated levels of these two nutrients in the pond water were most likely a result of the fertilizers applied, decomposed feeds and faecal matter in the culture ponds (Boonsong and Eiumnoh, 1995; Bhatta and Bhat, 1998). However, concentrations of nitrate and orthophosphate recorded in pond water and receiving waters were quite low (Chaiyakam and Predalumpabut, 1994; Boyd and Tucker, 1998), indicating that eutrophication and excessive growth of algae and aquatic plants may not be a problem (Zweig *et al*., 1999; Boyd and Zimmermann, 2000).

It is apparent from the water analyses that shrimp farming activities deteriorated the quality of waterbodies in close proximity to the culture ponds, most likely through the discharge of saline pond effluents rich in TSS and BOD during water exchange to maintain the growing environment, and draining of grow-out ponds at harvest. Deterioration in the quality of waterbodies could affect options for crop irrigation (Dierberg and Kiattsimkul, 1996) and generate conflicts in resource use between rice farmers and shrimp producers (Szuster and Flaherty, 2000). In addition, water quality deterioration could contribute to outbreaks of disease which, in turn, result in a catastrophic collapse of the industry (Corea *et al*., 1998).

In conclusion, the results of the present study have clearly indicated that the expansion of inland shrimp farming in the Songkla Lake Basin, while bringing considerable economic benefits, has also brought about environmental and social costs. Shrimp culture operations perturb the long-term viability of the basins biophysical environments, primarily through the losses of soil and water quality. The seawater added to the culture

ponds to obtain suitable pond salinity level for raising shrimp increased soluble salt levels of pond bottom soils. In addition, seepage of saline water from the culture ponds raised salinity levels of surface soils in the immediate vicinity of the ponds beyond tolerable limits for crop production, generating conflicts with the rice farmers. Moreover, the release of large quantities of untreated pond effluents directly into waterbodies in close proximity to the culture ponds causing deterioration of its quality. The perturbation in the freshwater environment and the conflicts in resource use between rice farmers and shrimp producers that occur pose a direct threat to the welfare of the local population. Thus, the practice of inland shrimp farming must be prohibited in order to restore the right to a healthy environment and the livelihood of current as well as future generations of the basin's population.

Approaches to the problem

To address the expansion of inland shrimp farming that threatened the environmental sustainability, the Royal Thai Government imposed the ban on shrimp farming in the country's freshwater areas in July 1998 (National Statistical Office, 1999; Miller *et al*., 1999). Moreover, the Department of Land Development has created a zoning map to designate fresh water areas where shrimp farming is not permitted (Anecksamphant, 2001). With this zoning plan, relevant government departments would be able to oversee and control inland shrimp cultivation in order to mitigate its impacts on the freshwater environment. However, the effort to enforce the zoning plan by the government departments has been largely ineffective (Szuster and Flaherty, 2000). This has allowed the small-scale shrimp farmers, who believe that shrimp culture provides a means by which they can obtain farm incomes many times higher than that provided by rice farming, to operate in the freshwater areas using culture practices that degrade the soil and water environments. The enforcement capacity of responsible agencies must, therefore, be strengthen through increased budget and personnel, improved technologies such as Geographic Information Systems and remote sensing, and better coordination between local government agencies. In addition, it should be recognized that the most crucial points in making the zoning plan successful is the full and active participation of the general public, Tambon

Table 6 Water properties in source water, pond water and receiving waters.

Properties	Source water	Pond water	Receiving water
pH	6.61a	8.77b	6.84a
Salinity (ppt)	0.10a	4.40b	4.47b
Turbidity (NTU)	38.77a	70.83b	16.67c
DO (mg/l)	4.53a	6.52b	5.16c
BOD (mg/l)	4.00a	26.33b	13.67c
TSS (mg/l)	336.67a	5,860.00b	6,283.33b
Nitrate (mg/l)	0.009a	0.188b	0.005a
Orthophosphate (mg/l)	0.018a	0.028b	0.024a

- Within a column, means followed by the same letter within each property are not significantly different at the 0.05 level of significance.

Administration Organization, NGO and local farmers in the planning, implementation and refinement of this zoning plan. Cooperative and collaborative relationship with these parties will help ensure the protection of the soil and water quality in freshwater areas that represent the agricultural heartland of the Songkla Lake Basin.

Of equal importance to the zoning plan is an improvement in shrimp producers' awareness of the adverse environmental impacts that inland shrimp farming could have on their communities and/or on their own production in return. The development of public awareness programme to educate, inform and warn shrimp raisers about the potential environmental consequences of shrimp farming would be one the critical elements for longer-term environmental stability and sustainability of the culture industry in the Songkla Lake Basin.

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Agricultural Production Forecasting Using Planning Distribution Model (PDM):A Case Study of the Nam Oon Project

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ABSTRACT

For forecasting agricultural production to design cropping patterns and to utilize water effectively, a computerized agricultural production monitoring system named PDM was selected. The Nam Oon irrigation project was chosen as a case study. PDM was used for agricultural production forecasting in the Nam Oon project for ten cultivation seasons from the 1995 wet season to the 2000 dry season. The factors affecting flow in the canal and the agricultural production forecasting equation were also investigated. In the wet season, the highest efficiency was achieved when 60% of the command area was planted with rice because of high production comparing to planted area and lowest insufficient water area. In dry season, command area can be increased about 100% comparing to the past for rice, field crop, and farm crop area because of sufficient water. Water management index of early wet season was less than 1 and end of wet season was greater than 1 while in dry season was greater than 1. It means that planted area in dry season can be increased.

Key word : agricultural production forecasting, Nam Oon irrigation project, Planning Distribution Model (PDM)

INTRODUCTION

Normally irrigation projects are planned to manage water during the wet season and the dry season before cultivation. In the dry season the area of land that can be cropped is determined by the amount of water in storage. In the wet season rain is used for cropped land. This style of planning is easy but it does not take into account the yield of agricultural products.

Nam Oon irrigation project is the part of Somkam river basin. This irrigation has the project area 203,000 rai, the irrigable area 185,800 rai, and the irrigated area 185,800 rai, which divided to the left irrigable area 63,300 rai and the right irrigable area 122,500 rai (Nam Oon irrigation project includes the left irrigable area, the right irrigable area, and Nam Oon colony). This research study in the left and right irrigable area. The length of the left main canal and the right main canal are 28.040 km. and 45.700 km., respective that share water by gravity. The average annual rainfall is 1,400 mm. And the water storage of Nam Oon reservoir is 520 million $m.^3$. Nam Oon irrigation project in Sakon Nakhon province was selected for the study. The purposes of this research are:

1. to forecast the effect of water deficit and water-logging on agricultural production by using the Planning Distribution Model (PDM).

2. to analyze the water management index: the balance between water delivery and water requirement.

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3. to determine the equation relating evapotranspiration and cropping area.

Biological and Irrigation Engineering Dept. (1995) developed the Planning Distribution Model (PDM). This application was developed to perform simulations of water distribution and crop yield response for irrigation and other use in complex branching canal and drainage networks. Crop water requirements are computed based on specified cropping patterns and weather information, and simulated flows are routed through the system from a main supply source, groundwater aquifer, downstream source, and open drains as show in figure 1. The model is intended primarily for use in planning and training activities, but it can also be applied to design and analysis studies of agricultural irrigation systems.

MATERIALS AND METHODS

Mathematical modeling

In simulation the PDM can generate system flow requirements based on calculated crop water needs, specified hydrographs for municipal and industrial nodes, and conveyance losses. Daily water balance calculations are performed for soil water and canal reach storage in the supply and drainage system. Options allow water to be taken from a combination of the supply system, drainage system, groundwater resources, and multiple external sources. Various features can be enabled and disabled from one simulation to another to quickly examine the effects of different conditions on overall water management. To calculate the evapotranspiration, the mathematical modeling is based on the Pemman-Matheith Equation (Allen, 1989).

The porosity of the soil is used to estimate the salinity of the saturation extract, Ec_e, which is used in crop yield reduction calculation. The runoff coefficient determines the fraction of surface-applied water that enters the soil. If the runoff coefficient is 0.2, for example, then 20% if the surface-applied water runs off, and 80% infiltrates. Surface-applied water includes inflow from all sources (supply and drainage system, conjunctive use of deep aquifer water, and precipitation) except shallow groundwater upflux. The specific yield of the soil is used by the model to convert between volumes of water and changes in the depth to the shallow water table.

The leaching fraction is defined as (USBR 1993):

$$LF = \frac{EC_{iw}}{EC_{dw}} = \frac{D_{dw}}{D_{iw}}$$

Where LF is the leaching fraction (from 0 to 1.0); EC_{iw} is the electrical conductivity of the irrigation water (ds/m); EC_{dw} is the electrical conductivity of the drainage or deep percolation, water (ds/m); D_{dw} is the depth of drainage water (m); and D_{iw} is the depth of infiltrated water from the soil surface (m). This value is used in the model to estimate the salinity of the drainage (deep percolation) water at the bottom of the crop root zone, and to determine the additional depth of water to apply in irrigations to maintain a favorable root zone salt balance.

Crop yield response due to deficit and water-logging can be estimated by the model. Calibration parameters can be specified for each growth stage-establishment, vegetative, flowering, yield formation, and ripening-of each crop type for yield reduction due to deficit. The deficit yield reduction equation is adapted from R.W. Hill, et. Al. (1987) and written as follows:

$$Y_{rel} = 100 \prod_{i=1}^{n} \left(\frac{ET_a}{ET_m} \right)_i^{\lambda_i}$$

where Y_{rel} is the relative yield (%); ET_a is actual transpiration (mm./day); ET_m is the maximum potential transpiration; λ is a fitted exponent (a calibrated value); and the subscript i refers to the growth stage (the PDM uses five growth stages, so, n = 5). This equation is applied at the end of each growth stage by using the cumulating values of ET_a

and ET_m during the stage. However, when the ratio of ET_a to ET_m is greater than one hundred minus the specified threshold value the relative yield for that state is assumed to be 100%. ET_a and ET_m reset to zero at the end of each stage of each crop type in a command area.

Simulation

The simulation of each month determined evapotranspiration, agricultural production and water management index from June 1995 to May 2000. The first year of simulation was from June 1995 to May 1996 which were split into wet season and dry season. Similarly the second year (from June 1996 to May 1997), the third year (from June 1997 to May 1998), the fourth year (from June 1998 to May 1999) and the fifth year (from June 1999 to May 2000) were split into wet season and dry season too.

Monthly means of effective rainfall and runoff coefficient were come from daily value. This value was determined by trial and error, which base on a concept of the study of effective rainfall and runoff coefficient at Nam Oon irrigation project ago, because they depend on the land use and climate each year. The result from this simulation may be used in the next season for the Nam Oon irrigation project. The simulation can be calculating the daily value for the study's year by continual processing.

Case studies are created for the wet season

Table 1 Size of cultivated area for each of the case studies for the wet seasons. (1995-1999)

Case	% of irrigation Area	Cultivated area for each year (rai) 1995	1996	1997	1998	1999
R1	100	203,000	203,000	203,000	203,000	203,000
R2	95	192,850	192,850	192,850	192,850	192,850
R3	90	185,700	185,700	185,700	185,700	185,700
R4	85	172,550	172,550	172,550	172,550	172,550
R5	80	162,400	162,400	162,400	162,400	162,400
R6	75	152,250	152,250	152,250	152,250	152,250
R7	70	142,100	142,100	142,100	142,100	142,100
R8	65	131,950	131,950	131,950	131,950	131,950
R9	60	121,800	121,800	121,800	121,800	121,800

Table 2 Percentage of cultivated area and case studies.

Case studies	Percentage of cultivated area (%)	Note
D1	+25	- It is increase 25% from past cultivated area.
D2	+50	- It is increase 50% from past cultivated area.
D3	+75	- It is increase 75% from past cultivated area.
D4	+100	- It is increase 100% from past cultivated area.
D5	-25	- It is decrease 25% from past cultivated area.
D6	-50	- It is decrease 50% from past cultivated area.
D7	-75	- It is decrease 75% from past cultivated area.

and the dry season. In the wet season, rice only is cultured in the irrigation area (203,000 rai). In the dry season, the irrigation area is planted with a range of crops-rice, vegetables, melon, corn, groundnut, and soybean.

In the wet season, nine case studies for each year were set up by decreasing the rice area in 5% decrements from 100% to 60% of the cultivated area as shown in Table 1. In the dry season, seven case studies for each year were set up for cultivated areas ranging from 75% below the past cultivated area to 75% above this area, in steps of 25%, as shown in Table 2 and Table 3.

Calibration

The water recognizes at the measurement structure are compared with the result of the simulation from wet season 1995 to dry season

Table 3 Size of cultivated area in each of the case studies for dry season. (1996-2000)

Year	Kind of crop	Past of cultivated area (rai)	Cultivated area for each case studies (rai)						
			D1	D2	D3	D4	D5	D6	D7
1996	rice	507.00	633.75	760.50	887.25	1014.00	380.25	253.50	126.75
	vegetables	9589.76	11987.20	14384.64	16782.08	19179.52	7192.32	4794.88	2397.44
	melon	1724.75	2155.94	2587.13	3018.31	3449.50	1293.56	862.38	431.19
	corn	374.75	468.44	562.13	655.81	749.50	281.06	187.38	93.69
	groundnut	1614.00	2017.50	2421.00	2824.50	3228.00	1210.50	807.00	403.50
	soybean	335.00	418.75	502.50	586.25	670.00	251.25	167.50	83.75
1997	rice	587.00	733.75	880.50	1027.25	1174.00	440.25	293.50	146.75
	vegetables	4475.90	5594.88	6713.85	7832.83	8951.80	3356.93	2237.95	1118.98
	melon	1889.80	2362.25	2834.70	3307.15	3779.60	1417.35	944.90	472.45
	corn	523.10	653.88	784.65	915.43	1046.20	392.33	261.55	130.78
	groundnut	586.50	733.13	879.75	1026.38	1173.00	439.88	293.25	146.63
	soybean	392.00	490.00	588.00	686.00	784.00	294.00	196.00	98.00
1998	rice	4545.00	5681.25	6817.50	7953.75	9090.00	3408.75	2272.50	1136.25
	vegetables	4590.95	5738.69	6886.43	8034.16	9181.90	3443.21	2295.48	1147.74
	melon	1769.50	2211.88	2654.25	3096.63	3539.00	1327.13	884.75	442.38
	corn	874.50	1093.13	1311.75	1530.38	1749.00	655.88	437.25	218.63
	groundnut	723.00	903.75	1084.50	1265.25	1446.00	542.25	361.50	180.75
	soybean	7.00	8.75	10.50	12.25	14.00	5.25	3.50	1.75
1999	rice	4703.00	5878.75	7054.50	8230.25	9406.00	3527.25	2351.50	1175.75
	vegetables	5913.75	7392.19	8870.63	10349.06	11827.50	4435.31	2956.88	1478.44
	melon	1819.25	2274.06	2728.88	3183.69	3638.50	1364.44	909.63	454.81
	corn	1621.50	2026.88	2432.25	2837.63	3243.00	1216.13	810.75	405.38
	groundnut	1330.00	1662.50	1995.00	2327.50	2660.00	997.50	665.00	332.50
	soybean	96.00	120.00	144.00	168.00	192.00	72.00	48.00	24.00
2000	rice	4398.00	5497.50	6597.00	7696.50	8796.00	3298.50	2199.00	1099.50
	vegetables	6334.00	7917.50	9501.00	11084.50	12668.00	4750.50	3167.00	1583.50
	melon	843.00	1053.75	1264.50	1475.25	1686.00	632.25	421.50	210.75
	corn	1335.75	1669.69	2003.63	2337.56	2671.50	1001.81	667.88	333.94
	groundnut	889.50	1111.88	1334.25	1556.63	1779.00	667.13	444.75	222.38
	soybean	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

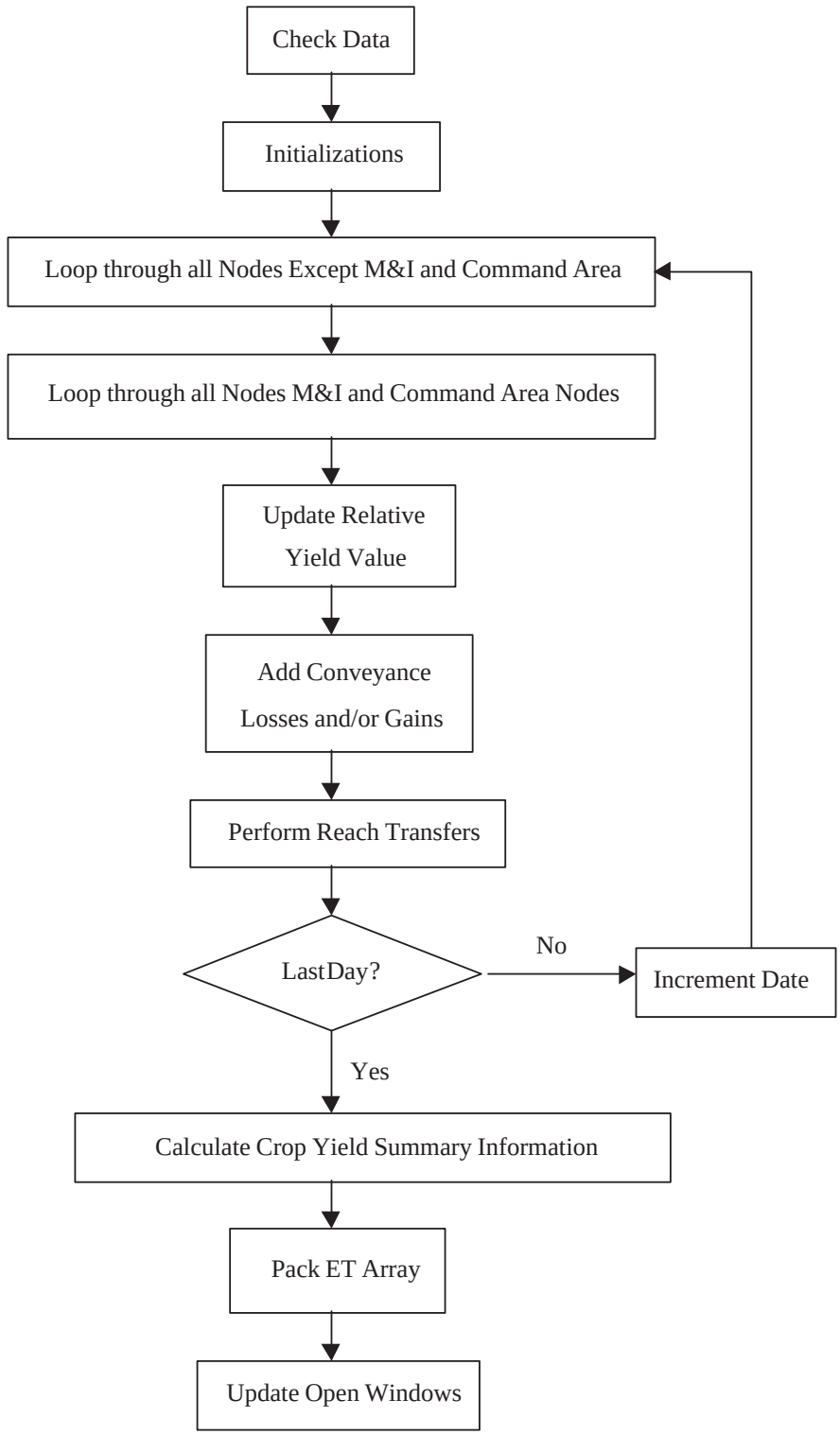

Figure 1 Flow chart of calculating in PDM.

2000. The calibration were base on the weekly accuracy which the error are the value between –13.281% to 13.558%. The variable of the effective rainfall and the runoff coefficient in this simulation can be changing the water consume by crop, which are present at the measurement structure then this research can be determinates the effective rainfall and the runoff coefficient from the calibration.

RESULTS AND DISCUSSIONS

Monthly mean effective rainfall and runoff coefficient

Monthly mean effective rainfall and runoff coefficient were determined by the trial and error method, based on weather conditions in the Nam Oon irrigation area. These results are presented in the following table.

Performance analysis of water requirement with PDM

PDM was used to calculate the water requirements in the Nam Oon irrigation project for wet seasons (Jun.-Nov. 1995, Jun.-Nov. 1996, Jun.-Nov. 1997, Jun.-Nov. 1998, and Jun.-Nov. 1999) and dry seasons (Dec. 1995 -May 1996, Dec. 1996 -May 1997, Dec. 1997 -May 1998, Dec. 1998 -May 1999, and Dec. 1999 -May 2000). In the wet season, water requirements are highest in July because prepare the land, on the other hand, in November less water is used because the rice has ripened. In the dry season, water requirements vary with type of crop and number of crops planted for each year. Most water is used in March, while less is used in May.

Table 4 Monthly mean effective rainfall and runoff coefficient for the wet season.

Month	1995		1996		1997		1998		1999	
	Effective rainfall	Runoff coefficient	Effective rainfall	Runoff coefficient	Effective rainfall	Runoff coefficient	Effective rainfall	Runoff coefficient	Effective rainfall	Runoff coefficient
Jan.	0.58	0.170	0.64	0.175	0.67	0.170	0.67	0.185	0.65	0.195
Jul.	0.56	0.170	0.60	0.180	0.54	0.170	0.62	0.190	0.62	0.185
Aug.	0.49	0.165	0.53	0.175	0.43	0.165	0.56	0.190	0.50	0.185
Sep.	0.55	0.165	0.51	0.180	0.52	0.165	0.50	0.190	0.50	0.200
Oct.	0.60	0.160	0.55	0.180	0.60	0.160	0.61	0.190	0.67	0.190
Nov.	0.70	0.160	0.61	0.160	0.60	0.160	0.65	0.180	0.70	0.200

Table 5 Monthly mean effective rainfall and runoff coefficient for dry season.

Month	1996		1997		1998		1999		2000	
	Effective rainfall	Runoff coefficient	Effective rainfall	Runoff coefficient	Effective rainfall	Runoff coefficient	Effective rainfall	Runoff coefficient	Effective rainfall	Runoff coefficient
Dec.	0.70	0.155	0.70	0.160	0.60	0.155	0.70	0.170	0.70	0.195
Jan.	0.70	0.155	0.72	0.150	0.60	0.150	0.70	0.160	0.70	0.180
Feb.	0.68	0.150	0.70	0.150	0.64	0.160	0.70	0.160	0.70	0.165
Mar.	0.68	0.155	0.67	0.160	0.64	0.160	0.75	0.155	0.70	0.160
Apr.	0.66	0.160	0.72	0.160	0.68	0.160	0.68	0.160	0.68	0.160
May.	0.66	0.160	0.70	0.160	0.70	0.160	0.66	0.160	0.68	0.160

Performance analysis of agricultural product with PDM

PDM was used to estimate agricultural production for each crop. Agricultural production forecasts for the wet season and the dry season are presented in terms of the crop yield area. For the wet season, agricultural production forecasts are shown in Table 6, which present yield area and ratio-yield area divide by planted area- for each case studies from 1995-1999. For the dry season the agricultural production forecast is displayed in Table 7.

Performance analysis of water management index with PDM

The water management index is the ratio of applied to required water for each command area over the simulation period. The value is equal to unity (1.0) when the application exactly matches the estimated requirement, and is greater than unity when too much water has been applied. In the wet season, Monthly mean water management indexes are high because water for culture comes from rainfall and irrigation watering. The indexes for the dry season are less because water for culture comes from only irrigation watering.

Performance analysis of equation

Equations of relationship between evapotranspiration and cropping area for rice in wet season, rice in dry season, vegetables, melon, corn, groundnut, and soybean are shown in Table 8. This equation has positive slope so that evapotranspiration varies directly with cropping area.

CONCLUSION

The Nam Oon irrigation project has the potential to supply irrigation watering to support water demand. In the dry season, the cropping area can be increased by 100%, compared to past cropping area, for rice, field crop, and farm crop area because of sufficient water. In the wet season, the highest

Table 6 Yield area for wet season (1995-1999).

Case studies		R1	R2	R3	R4	R5	R6	R7	R8	R9
Planted area (rai)		203,000.00	192,850.00	182,700.00	172,550.00	162,400.00	152,250.00	142,100.00	131,950.00	121,800.00
1995	Yield area (rai)	123,830.00	115,710.00	109,620.00	106,981.00	102,312.00	106,575.00	103,733.00	100,282.00	97,440.00
	Ratio	0.61	0.60	0.60	0.62	0.63	0.70	0.73	0.76	0.80
1996	Yield area (rai)	144,130.00	144,637.50	146,160.00	141,491.00	136,416.00	127,890.00	119,364.00	112,157.50	103,530.00
	Ratio	0.71	0.75	0.80	0.82	0.84	0.84	0.84	0.85	0.85
1997	Yield area (rai)	127,890.00	123,424.00	118,755.00	117,334.00	115,304.00	115,710.00	110,838.00	106,879.50	102,312.00
	Ratio	0.63	0.64	0.65	0.68	0.71	0.76	0.78	0.81	0.84
1998	Yield area (rai)	123,830.00	119,567.00	113,274.00	106,981.00	102,312.00	98,962.50	96,628.00	101,601.50	102,312.00
	Ratio	0.61	0.62	0.62	0.62	0.63	0.65	0.68	0.77	0.84
1999	Yield area (rai)	129,920.00	127,281.00	124,236.00	127,687.00	128,296.00	124,845.00	117,943.00	110,838.00	102,312.00
	Ratio	0.64	0.66	0.68	0.74	0.79	0.82	0.83	0.84	0.84

Table 7 Yield area for dry season (1996-2000).

Years	Case studies	Rice		Garden		Melon		Corn		Groundnut		Soybean	
		Yield area (rai)	Ratio	Yield area (rai)	Ratio	Yield area (rai)	Ratio	Yield area (rai)	Ratio	Yield area (rai)	Ratio	Yield area (rai)	Ratio
1996	D1	633.75	1.00	4,914.75	0.41	1,164.21	0.54	356.01	0.76	1,573.65	0.78	318.25	0.76
	D2	760.50	1.00	5,753.86	0.40	1,371.18	0.53	432.84	0.77	1,839.96	0.76	376.88	0.75
	D3	887.25	1.00	7,048.48	0.42	1,569.53	0.52	511.53	0.78	2,174.87	0.77	457.28	0.78
	D4	1,014.00	1.00	8,055.40	0.42	1,793.74	0.52	577.12	0.77	2,421.00	0.75	522.60	0.78
	D5	380.25	1.00	2,876.93	0.40	957.24	0.74	222.04	0.79	1,053.14	0.87	190.95	0.76
	D6	253.50	1.00	1,917.96	0.40	758.89	0.88	142.41	0.76	742.44	0.92	123.95	0.74
	D7	126.75	1.00	935.00	0.39	370.82	0.86	73.08	0.78	363.15	0.90	61.98	0.74
1997	D1	733.75	1.00	2,182.00	0.39	1,984.29	0.84	470.79	0.72	623.16	0.85	362.60	0.74
	D2	880.50	1.00	2,685.54	0.40	2,381.16	0.84	564.95	0.72	738.99	0.84	435.12	0.74
	D3	1,027.25	1.00	3,133.13	0.40	2,778.01	0.84	659.11	0.72	882.69	0.86	507.64	0.74
	D4	1,174.00	1.00	3,580.72	0.40	3,174.86	0.84	753.26	0.72	1,008.78	0.86	580.16	0.74
	D5	440.25	1.00	1,342.77	0.40	1,176.41	0.83	282.48	0.72	369.50	0.84	217.56	0.74
	D6	293.50	1.00	917.56	0.41	774.83	0.82	188.32	0.72	246.33	0.84	145.04	0.74
	D7	146.75	1.00	469.97	0.42	387.41	0.82	99.39	0.76	123.17	0.84	72.52	0.74
1998	D1	5,681.25	1.00	2,123.32	0.37	1,769.50	0.80	743.32	0.68	759.15	0.84	6.65	0.76
	D2	6,817.50	1.00	2,685.71	0.39	2,176.49	0.82	918.23	0.70	910.98	0.84	8.19	0.78
	D3	7,953.75	1.00	2,972.64	0.37	2,539.24	0.82	1,040.66	0.68	1,062.81	0.84	9.68	0.79
	D4	9,090.00	1.00	3,489.12	0.38	2,937.37	0.83	1,241.79	0.71	1,214.64	0.84	10.92	0.78
	D5	3,408.75	1.00	1,273.99	0.37	1,074.98	0.81	432.88	0.66	433.80	0.80	3.68	0.70
	D6	2,272.50	1.00	849.33	0.37	734.34	0.83	292.96	0.67	292.82	0.81	2.38	0.68
	D7	1,136.25	1.00	459.10	0.40	376.02	0.85	161.78	0.74	155.45	0.86	1.37	0.78

Table 7 Yield area for dry season (1996-2000).

Years	Case studies	Rice		Garden		Melon		Corn		Groundnut		Soybean	
		Yield area (rai)	Ratio	Yield area (rai)	Ratio	Yield area (rai)	Ratio	Yield area (rai)	Ratio	Yield area (rai)	Ratio	Yield area (rai)	Ratio
1999	D1	5,878.75	1.00	665.30	0.09	886.89	0.39	587.80	0.29	581.88	0.35	22.80	0.19
	D2	7,054.50	1.00	798.36	0.09	709.51	0.26	705.36	0.29	638.40	0.32	25.92	0.18
	D3	8,230.25	1.00	931.42	0.09	604.90	0.19	879.67	0.31	744.80	0.32	30.24	0.18
	D4	9,406.00	1.00	1182.75	0.10	545.78	0.15	908.04	0.28	771.40	0.29	34.56	0.18
	D5	3,527.25	1.00	266.12	0.06	504.84	0.37	352.68	0.29	349.13	0.35	13.68	0.19
	D6	2,351.50	1.00	177.41	0.06	363.85	0.40	259.44	0.32	232.75	0.35	7.68	0.16
	D7	1,175.75	1.00	103.49	0.07	181.93	0.40	133.78	0.33	119.70	0.36	3.84	0.16
2000	D1	5,497.50	1.00	316.70	0.04	389.89	0.37	701.27	0.42	489.23	0.44	0.00	0.00
	D2	6,597.00	1.00	475.05	0.05	328.77	0.26	761.38	0.38	520.36	0.39	0.00	0.00
	D3	7,696.50	1.00	443.38	0.04	309.80	0.21	841.52	0.36	575.95	0.37	0.00	0.00
	D4	8,796.00	1.00	506.72	0.04	320.34	0.19	961.74	0.36	640.44	0.36	0.00	0.00
	D5	3,298.50	1.00	190.02	0.04	202.32	0.32	400.72	0.40	286.87	0.43	0.00	0.00
	D6	2,199.00	1.00	126.68	0.04	122.24	0.29	273.83	0.41	191.24	0.43	0.00	0.00
	D7	1,099	1.00	63.34	0.04	61.12	0.29	140.25	0.42	95.62	0.43	0.00	0.00

Table 8 Equations of relationship between evapotranspiration and cropping area.

Type of crop	Equations	Condition
Rice (in wet season)	Y= 0.0006X + 101.21	X > 0.00
Rice (in dry season)	Y= 0.0013X - 0.0055	X > 4.23
Vegetables	Y = 0.0081X - 0.2022	X > 24.96
Melon	Y = 0.0287X + 0.0070	X > 0.00
Corn	Y = 0.0041X + 0.0489	X > 0.00
Groundnut	Y = 0.0116X - 0.1461	X > 12.59
Soybean	Y = 0.0006X + 0.0018	X > 0.00

Note: Y is evapotranspiration (MCM)
X is cropping area (rai)

efficiency was achieved when 60% of the command area was planted with rice because of the high ratio of crop yield area to crop planted area. Effective water management can be indicated by the water management index. Equations of relationship between evapotranspiration and cropping area are used to design cropping patterns to increase the efficiency of water management. Planning Distribution Model (PDM) is an effective model for water management.

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Preparation of Carbon Nanotubes by Nickel Catalyzed Decomposition of Liquefied Petroleum Gas (LPG)

Apisit Songsasen and Paranchai Pairgreethaves

ABSTRACT

A method is proposed for the preparation of carbon nanotubes by means of decomposition of liquefied petroleum gas (LPG) using zeolite-supporting nickel as catalyst. The catalyst was prepared by deposition-precipitation method. The carbon nanotubes, which observed by Raman spectrometer and transmission electron microscope (TEM), are approximately 30 nm in diameter and 300-600 nm long. The reaction temperature and the LPG:H_2 ratio have an effect on the amount of carbon nanotubes formed. The highest amount of carbon nanotubes was obtained by using LPG:H_2 ratio of 9:1, the reaction temperature of 1,100°C and the reaction time of 2 hours.

Key words: carbon nanotubes, nickel catalyst, decomposition of LPG.

INTRODUCTION

Carbon nanotubes have increasingly been studied in recent years owing to their important properties and a wide variety of potential applications. The carbon nanotubes may be used, for example, as catalyst support or semiconductor (Ajayan *et al*., 1995; Eric *et al*., 1996; Lago *et al*., 1995; Sloan *et al*., 1997). Three main methods are currently used for synthesis of carbon nanotubes, *i.e.* carbon arc synthesis; chemical vapor deposition (CVD) or catalyzed decomposition of hydrocarbon; and ion bombardment (Ebbesen, 1994). The general reaction for catalyzed decomposition of hydrocarbon, such as methane, may be written as follows:

$$CH_4 + H_2 \xrightarrow{\text{catalyst}} C + 3H_2 \quad \text{..... (1)}$$

The catalyzed decomposition of methane at 600°C using $MgNiO_2$ as catalyst (Cui *et al*. 1999), and the catalytic pyrolysis of benzene at 1200°C using ferrocene as catalyst (Cheng *et al*. 1998) gave carbon nanotubes on a large scale.

In this letter we report the preparation of carbon nanotubes using zeolite-supporting nickel as catalyst and LPG as the carbon source. The effects of the reaction temperature and the LPG:H_2 ratio on the amount of carbon nanotubes formed had also been investigated.

MATERIALS AND METHOD

Preparation of Zeolite-supporting Nickel Catalyst

The catalyst was obtained by deposition-precipitation method, using $Ni(NO_3)_2 \cdot 6H_2O$ (May and Baker No.2725) as starting nickel compound and $NaHCO_3$ (Unilab No.F8K440) as precipitating agent. Zeolite (Fluka No.96096) 36 g was suspended in a 170-cm^3 aqueous solution of 0.4M $Ni(NO_3)_2$ with constant stirring at 1,100 rpm. A 136-cm^3 aqueous solution of 0.8M $NaHCO_3$ was added to

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the suspension at room temperature, and the slurry was stirred for 3 hours. The precipitate was filtered, washed with water, and then dried for 23 hours at 383 K, followed by calcination of the precipitate under air for 6 hours at 723 K. The catalyst obtained was characterized by X-ray diffractometer, XRD (Phillips X'pert).

Preparation of Carbon Nanotubes

A 30-cm long stainless steel tube reactor (3/4 inch in diameter) was located in a furnace as illustrated in Figure 1. Zeolite-supporting nickel catalyst 100 mg was placed in the middle of the reactor tube. Nitrogen gas was flown through the reactor in order to flush out any gases inside and to prevent the entering of gases from outside. The reaction was carried out at various temperatures of 500, 600, 700, 800, 900, 1,000, and 1,100°C. The volume ratio of LPG:H_2 was varied: 1:9, 3:7, 5:5, 7:3, and 9:1, with the total flow rate of the LPG-H_2 at 100 ml/min. The reaction time was varied: 30, 60, 90, 120, 150, 180, 210, and 240 minutes. The reaction was quenched by replacing the LPG-H_2 with N_2. The carbon nanotubes were separated from the nickel catalyst by dissolving the product in 40% HF solution. The nanotubes were characterized by Raman spectrometer (RENISHAW, System 2000) using argon laser source, and by transmission electron microscope, TEM (JEOL, JEM1220).

RESULTS AND DISCUSSION

The X-ray powder diffraction (XPD) spectrum of the zeolite-supporting nickel catalyst is shown in Figure 2. This spectrum shows 36 peaks at 16.20, 17.98, 19.21, 20.76, 21.39, 22.56, 23.18, 23.72, 25.08, 25.94, 26.03, 26.87, 27.15, 27.84, 28.34, 29.63, 30.05, 30.71, 31.24, 31.72, 32.46, 33.70, 34.59, 35.05, 36.21, 39.25, 41.77, 42.71, 44.16, 47.69, 48.91, 50.83, 53.24, 61.43, 62.04 and 63.33 degrees, which indicated the presence of zeolite in the catalyst. This spectrum also shows 3 peaks at 37.37, 43.50 and 62.67 degrees, which indicated the presence of NiO (Schmahl *et al*., 1964).

The catalyzed decomposition product was confirmed to be carbon via the characteristic peaks displayed in the Raman spectrum, Figure 3. A sharp peak at 1580 cm^{-1} and a broad weak peak at 1350 cm^{-1} indicate the sp^2 and sp^3 hybridization of carbon, respectively (Dresselhaus *et al*., 1995).

The products obtained were mainly carbon

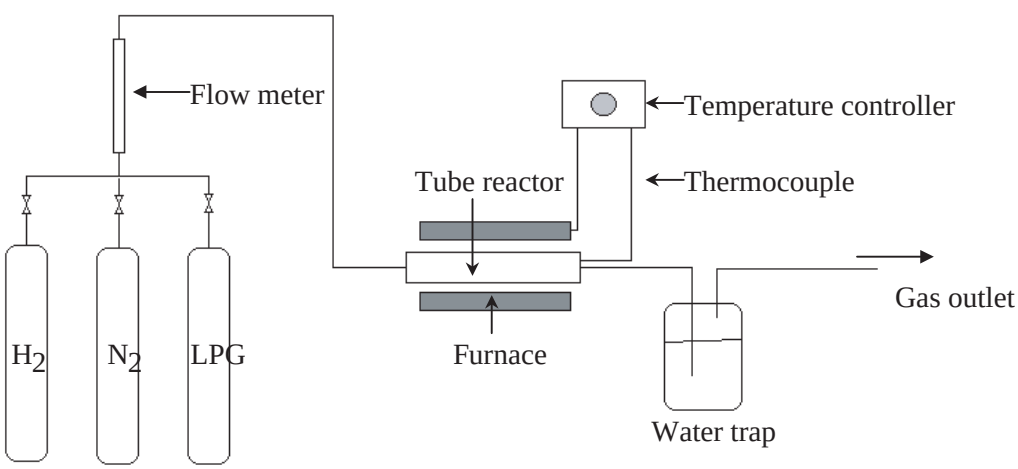

Figure 1 Schematic diagram of the apparatus used for the preparation of carbon nanotubes.

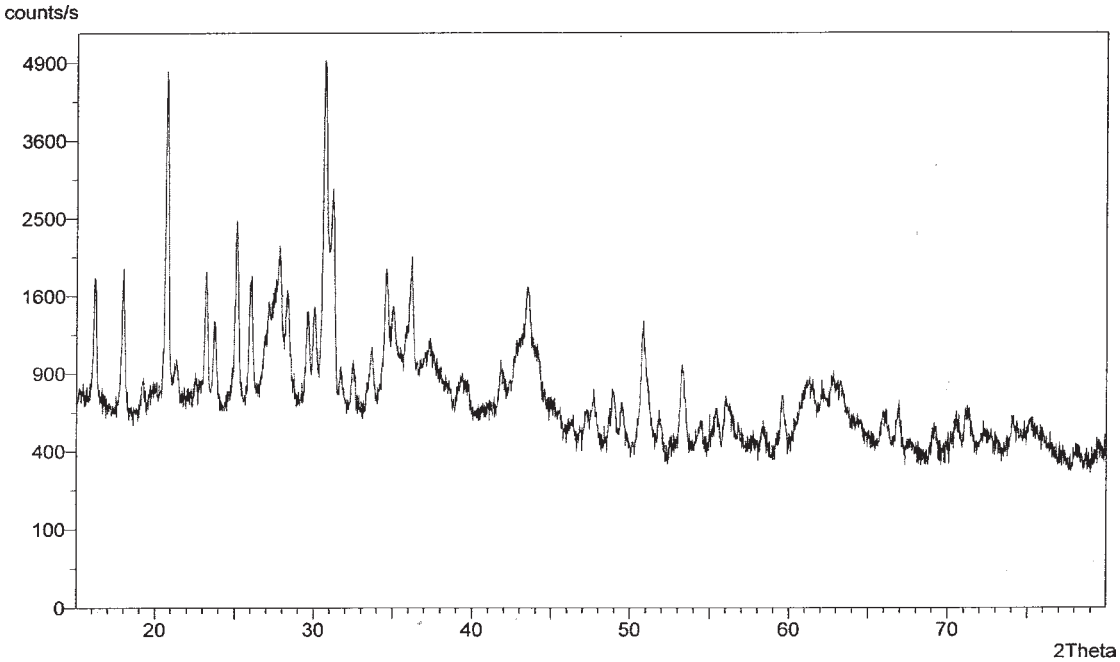

Figure 2 X-ray powder diffraction (XPD) spectrum of the zeolite-supporting nickel catalyst.

nanotubes when the reaction was carried out at 1,000°C and the LPG:H_2 ratio was 3:7. The transmission electron micrographs in Figure 4a and Figure 4b and 4c show the carbon nanotubes before and after being separated from the nickel catalyst, respectively. The nanotubes obtained were single-wall carbon nanotubes of approximately 30 nm in diameter and 300-600 nm long.

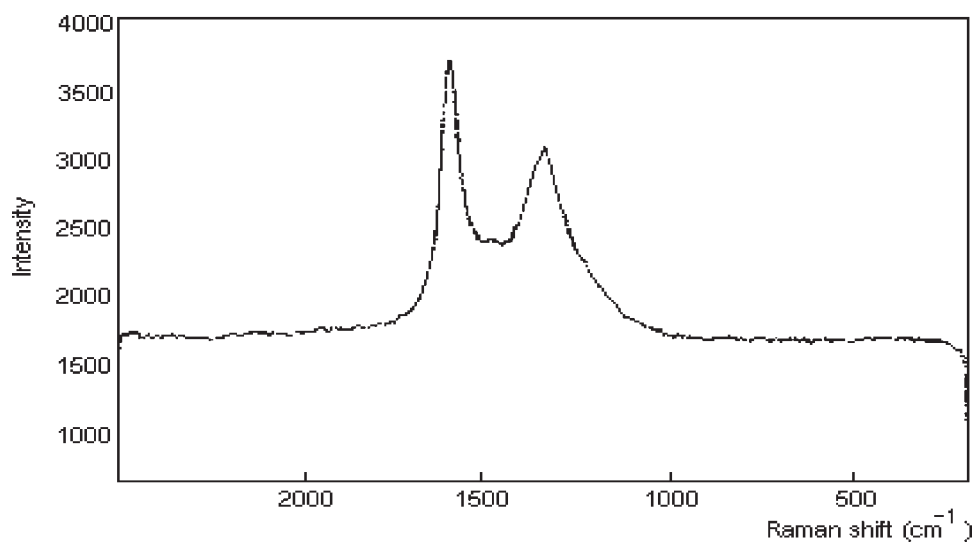

Figure 3 Raman spectrum of the carbon nanotubes.

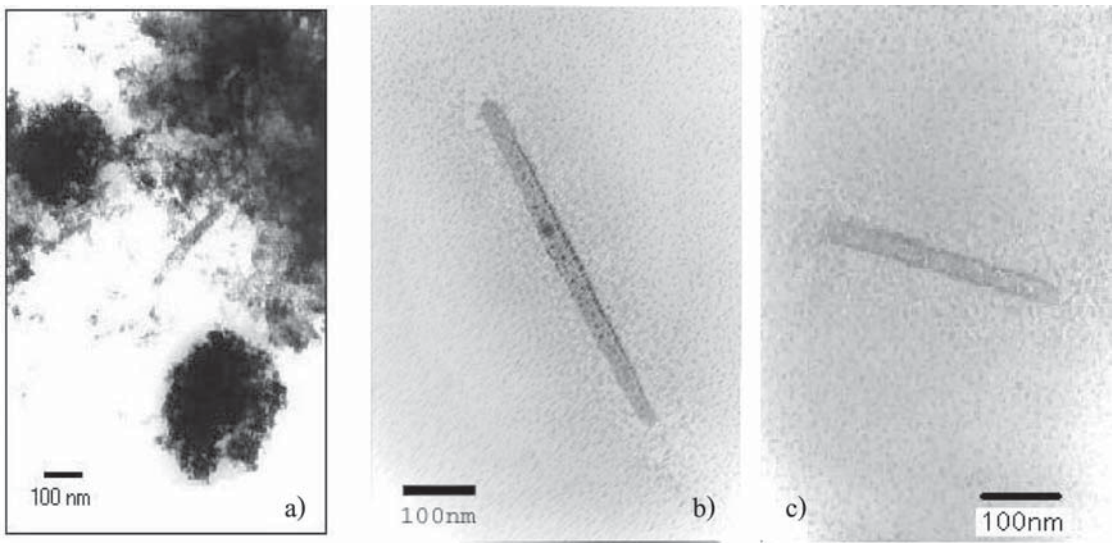

Figure 4 Transmission electron micrographs.
(a) Carbon nanotubes before being separated from Ni catalyst.
(b) and (c) Carbon nanotubes after being separated from Ni catalyst.

Effect of LPG:H_2 ratio on the production of carbon nanotubes

The carbon nanotubes were produced at 1,100°C. The amount of nickel catalyst was 100 mg and the reaction time was 2 hours. The LPG:H_2 ratio was varied as 1:9, 3:7, 5:5, 7:3 and 9:1, and the total flow rate was 100 ml/min. In this experiment, the amount of carbon nanotubes formed increases with the increase of LPG:H_2 ratio as shown in Figure 5. This may be because the quantities of H_2 have an effect on the diffusion of carbon species, which decompose from LPG, into the nickel catalyst. At the low LPG:H_2 ratio (the large quantities of H_2), the rate of carbon diffusion into the bulk (apart

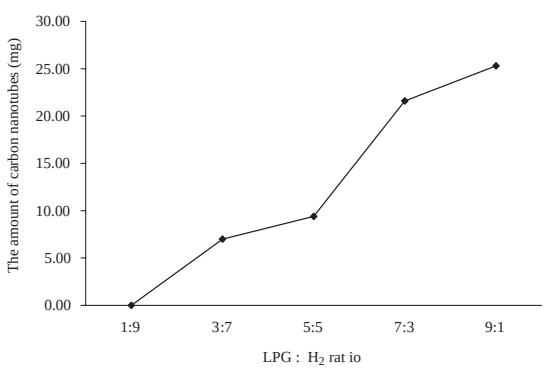

Figure 5 The effect of the LPG:H_2 ratio on the production of carbon nanotubes.
(reaction temperature = 1,100°C, reaction time = 2 hours)

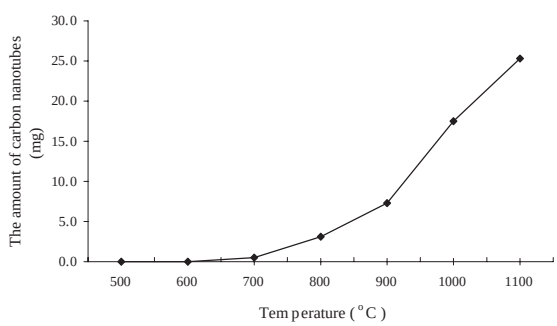

Figure 6 The effect of temperature on the production of carbon nanotubes.
(LPG:H_2 = 9:1 , reaction time = 2 hours)

from the nickel catalyst) may be faster than the rate of carbon growth on nickel catalyst, so small amount of carbon can be formed.

Effect of temperature on the production of carbon nanotubes

The carbon nanotubes were produced by using LPG:H_2 ratio of 9:1, and the total flow rate was 100 ml/min. The amount of nickel catalyst was 100 mg and the reaction time was 2 hours. The temperature was varied as 500, 600, 700, 800, 900, 1,000 and 1,100°C. In this experiment, the amount of carbon nanotubes formed increased with increase of the reaction temperature as shown in Figure 6. This may be because the reaction temperature had an effect on the decomposition of LPG. When the reaction temperature was higher, the LPG may be decomposed easier and many carbon species can be formed, so the amount of carbon nanotubes can be greatly increased. In this experiment, we could not prepare the carbon nanotubes at temperature above 1,100°C because the furnace has maximum temperature at 1,200°C.

Effect of reaction time on the production of carbon nanotubes

The carbon nanotubes were produced at 1,100°C by using LPG:H_2 ratio of 9:1, and the total flow rate was 100 ml/min. The amount of nickel catalyst was 100 mg. The reaction times were varied as 30, 60, 90, 120, 150, 180, 210, and 240 minutes. In this experiment, the amount of carbon nanotubes formed was almost constant (about 20 mg) with increase of the reaction time as shown in Figure 7. This may be due to the limitation of catalyst active site, used for the carbon growth.

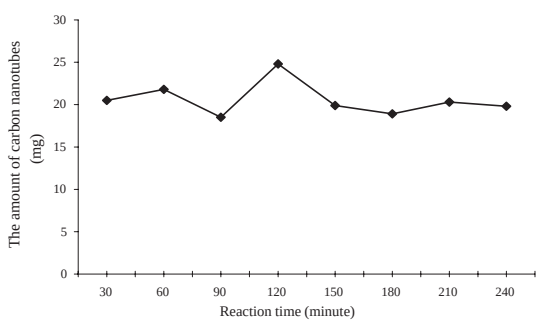

Figure 7 The effect of reaction time on the production of carbon nanotubes. (reaction temperature = 1,100°C, LPG:H_2 = 9:1)

CONCLUSION

The catalyzed decomposition of hydrocarbon using zeolite-supporting nickel catalyst and liquefied petroleum gas (LPG) can prepare carbon nanotubes, which have approximately 30 nm in diameter and 300-600 nm long. The amount of carbon nanotubes formed increases with the increase of the reaction temperature and the LPG:H_2 ratio. The method proposed can be adopted to prepare carbon nanotubes at yield was obtained by using LPG:H_2 ratio of 9:1 at the reaction temperature of 1,100°C and the reaction time of 2 hours. If the conditions are improved further, the yield of carbon nanotubes may be raised to a higher level.

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