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**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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# Changes of Gene Frequencies in Synthetic Corn Populations by Two Methods of Recurrent Selection and Pedigree Selection

**Krisda Samphantharak and Rapeepong Yavilasd**


## ABSTRACT

Forty-five F1C0 corn hybrids were derived from diallel series of 10 selected inbreds from different sources of germplasm. AgSyn1C0 was synthesized from bulked balanced seeds of 45 F1C0. The remnant seeds were tested for their yielding ability in randomized complete block design with 4 replications simultaneously, selected plants in border rows were selfed to obtain S1C0. Forty-five S1C0 from diallel set plus 4 additional S1C0 were planted in R-49 grouped replicated honeycomb design. Top-10 F1C0 (diallel selection) and top-10 S1C0 (S1 selection) were selected from each cycle. Their pedigrees were recorded and frequencies of original lines presented in pedigree of selected lines were used to calculate gene frequencies in derived populations. Pedigree selection was also conducted from each selected S1 set from each cycle. Two cycles of selection were done to obtain AgSyn1C1, AgSyn1C2. Two selected lines from pedigree selection were added to AgSyn1C2 to obtain AgSyn1C2-Sem. Changes of gene frequency patterns by the 3 methods of selection were very similar but diallel selection and pedigree selection retained more genetic diversity than S1 selection. Therefore, there was no clear advantage of recurrent selection over the pedigree selection. However, recurrent selection enhances gene recombination and accumulation of desirable genes in the population while pedigree selection is suitable for accumulation of few desirable genes in single plant. The results suggested that recurrent selection should be a supporting program to provide desirable germplasm to pedigree program. Diallel selection not only retained more genetic diversity but needs only 2 planting seasons to complete a cycle instead of 3 planting seasons as most recurrent selections now generally in use. Therefore, diallel selection should be more effective than S1 selection for the improvement of population per se from which good inbreds could be derived. The results also suggested that genetic diversity of first few cycles of selection drastically dropped when combining ability of the original lines were widely different.


**Key words:** S1 selection, gene frequency, diallel, corn hybrid

## INTRODUCTION

Pedigree selection has been used effectively for the improvement of corn inbreds and hybrids. However, continuous selfing nature of pedigree selection limited gene recombination and accumulation of favorable genes. Eberhart *et al.* (1995) proposed that population improvement should be used as a supporting program of pedigree method to provide desirable germplasm for the improvement of corn inbreds and hybrids. Several modifications of population improvement methods had been proposed with variable results and effectiveness. Duclos and Crane (1968) found that

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S1 selection and testcross selection were equally effective for the improvement of population per se. Similar results were reported by Carrangal *et al*. (1971). Contrarily, Horner *et al*. (1969) found that testcross selection for specific and general combining ability were more effective than S2 selection for the improvement of random mated populations but S2 selection rendered inbreds with better grain yield. However, after four cycles of selection Burton *et al*. (1971) found that response to S1 selection was 10.6 % as compared to only 5.7 % from testcross selection.

Weyhrich *et al*. (1998) conducted a direct comparison of seven population improvement methods ; mass, modified ear-to-row, half-sib with inbred tester, full-sib, S1 progeny, S2 progeny and reciprocal full-sib selection and concluded that S1 and S2 progeny selections were the most effective methods for overall performance of derived populations. Genter and Alexander (1966) suggested that visual selection for desirable agronomic traits plus S1 yield evaluation offers a greater opportunity for effective early generation selection than testcross methods now generally in use. However, Genter and Eberhart (1974) concluded that sources of materials and environmental conditions under which recurrent selection trials are conducted may be as important as the selection methods used.

Despite a wide acceptance of line screening under high plant densities to get tougher inbreds and hybrids (Troyer, 1996) Tokatlidis *et al*. (2001) had proved that selection under nil competition environment in honeycomb design was very effective for the improvement of high yield corn inbreds and hybrids which could adapt to wide ranges of plant densities.

Since potential yield improvement may be counterbalanced by inbreeding depression, this experiment was conducted to study the changes of gene frequencies in responses to S1 selection under nil competion in honeycomb design as compared to diallel and pedigree selections.

## MATERIALS AND METHODS

Ten inbreds from different germplasm sources, Ag17 and Ag18 (Pioneer 3012), Ag6, 25 and 26 (Pioneer 3013), Ag11 and 28 (SW 3853), Ag27 (G5445A), Ag22 (Cargill 919) and Ag24 (Uni-H9728) were intercrossed in a diallel manner. The derived 45 F1C0 were bulked and designated as AgSyn1C0 population. Remnant seeds of each 45 F1C0 were tested for their yielding ability in randomized complete block design with 4 replications, each plot comprised 4 rows of 5 meter long with 0.75 × 0.25 m plant spacing. Five plants in the border rows of each F1C0 were selfed and bulked within each family. Four selected S1C0 families were added to the derived 45 S1C0 families and planted in R-49 grouped replicated honeycomb (Fasoulas and Fasoula, 1995). Forty plants of each S1C0 family were planted in equilateral triangle of side 0.866 m. Single plant selection was done by moving circle selection with selection intensity of 14.3 % (1 out of 7 plants in the circle). Simultaneously, 1 out of 7 plants in the rows was selected by visual selection and selfed to obtained S2 seeds. Ten S1C0 families with highest selection frequencies by moving circle selection were chosen. However, instead of using S1C0 seeds, the S2C0 seeds were diallel crossed to start the following. Remnant seeds of top-10 S2 lines of each cycle were continued selfing for pedigree selection. Pedigree of 10 selected S1s and F1s from each diallel set of each cycle were recorded and the frequency of each original line presented in each population was used to estimate the gene frequencies of each selection cycle. Two cycles of selection were conducted to obtain AgSyn1C1, AgSyn1C2, F1C1, S1C1 and S5 from pedigree selection.

Ten S5s derived from the original S1C0 by pedigree selection were diallel crossed to estimate combining ability of S5 lines. Top-2 general combining ability lines were intercrossed with 10 S1C1 and designated as AgSyn1C2-Sem. (semi-

open end population). All Syn1 populations, AgSyn1C0, AgSyn1C1, AgSyn1C2, and AgSyn1C2-Sem. were tested to compare their yielding ability with SW1C12 and SW5C4.

## RESULTS AND DISCUSSIONS

The original 10 inbred lines were Ag17, Ag18 (pioneer 3012), Ag6, Ag25, Ag26 (pioneer 3013), Ag11, Ag18 (SW3853), Ag27 (G5445A), Ag22 (Cargill 919) and Ag24 (Uni-H9728). Therefore, each source of germplasm contributed a frequency of 20% (2pioneer 3012), 30% (3pioneer 3013), 20% (2SW3853) and 10% (1) each for G5445A, Cargill 919 and Uni-H9728. After the first round of S1 selection, frequencies of Pioneer 3012, Pioneer 3013, SW3853, G5445A, Cargill 919 and Uni-H9728 in top-10 single crosses were 4, 7, 1, 6, 1 and 1 which equal to 20, 35, 5, 30, 5, and 5 percent, respectively and subsequently, after the second round of S1 selection, frequencies of Pioneer 3012, Pioneer 3013, SW3853, G5445A, Cargill 919 and Uni-H9728 in double crosses were 10, 14, 1, 13, 1, and 1 which equal to 25, 35, 2.5, 32.5, 2.5 and 2.5 precent, respectively (Table 1). Simultaneously, frequencies of 10 selected F1C0 and F1C1 from diallel selection calculated from pedigrees of single crosses and double crosses were 10, 40, 15, 25, 5,and 5 percent and 15, 27.5, 5, 37.5, 10 and 5 percent, respectively.

Frequencies of Pioneer 3012, Pioneer 3013 and G5445A were increased steadily by S1 selection from S0C0 to S1C1 with the expense of SW3853, Cargill 919 and Uni-H9728. Similar trend was observed from diallel selection (Table 1). Regardless of selection methods, after one or two cycles of selection, high combining ability lines from Pioneer 3012, Pioneer 3013 and G5445A dominated the gene pool of populations. This finding may explain the previous study of Genter (1973), Duclos and Crane (1968), Ortiz (1963), and Carrangal *et al*. (1971) of which inbred line selection was as effective as testcross selection. Although, different methods of combining ability testing were used, a slightly more balance of gene frequencies by diallel selection of this study also may explain why in certain case, testcross selection was more effective than inbred selection for random mated population per se (Horner *et al*.1973). However, Genter and Eberhart (1974) found that variable results from different

**Table 1** Changes of gene frequencies by S1 selection, diallel selection, and pedigree selection.

| Germplasm source | Original gene frequency (%) | Gene frequency of selected line (%) | | | | |
|---|---|---|---|---|---|---|
| | $S_0C_0$ | $S_1C_0$[1] | $F_1C_0$[2] | $S_1C_1$[1] | $F_1C_1$[2] | $S_5$[3] |
| Pioneer 3012 | 20 | 20 | 10 | 25 | 15 | 25 |
| Pioneer 3013 | 30 | 35 | 40 | 35 | 27.5 | 20 |
| SW 3853 | 20 | 5 | 15 | 2.5 | 5 | 5 |
| G5445A | 10 | 30 | 25 | 32.5 | 37.5 | 35 |
| Cargill 919 | 10 | 5 | 5 | 2.5 | 10 | 10 |
| Uni-H9728 | 10 | 5 | 5 | 2.5 | 5 | 5 |
| Total | 100 | 100 | 100 | 100 | 100 | 100 |

1] Gene frequencies of germplasm after each cycle of S1 selection.
2] Gene frequencies of germplasm after each cycle of diallel selection.
3] Gene frequencies of germplasm from pedigree selection.

methods of selection were largely because of different sources of material under selection and environmental conditions under which selection trials were conducted.

Yields of AgSyn1C1 and AgSyn1C2 were clearly dropped from AgSyn1C0 (Table 2). Horner *et al*. (1969). also found that population yields of the first two cycles were lower than the original population. However, population yields of the more advanced cycles were steadily increased and surpassed the original population after 5 cycles of selection. Results of present study indicated that inbreeding depression had a pronounced effect on yield in the first 1 or 2 cycles of selection. This may be because of the AgSyn1C0 derived from crossing among well selected inbred lines while AgSyn1 of the first 2 cycles were derived from narrow base of segregated S1 lines, causing expression of deleterious genes. However, accumulation of desirable genes in the more advanced cycles should increas yield of random mated populations. In fact, the ultimate purpose was to obtain strong high yield inbreds but not the high yield of random mated population. Selection for inbred lines per se drastically increased inbreeding depression, especially when original germplasm was widely different in combining ability. The poor combining ability lines were discarded in the first few cycles and hence inbreeding among related lines. Variable results of testcross selection were also reported by Sprague *et al*. (1959), Penny and Eberhart (1971) and Genter (1973). Therefore, each selection method worked differently with different populations.

Selections in Syn2 or Syn3 may slowly decrease genetic variability and reduce inbreeding depression of selected populations but it probably needs more selection cycles to discard deleterious recessive genes and takes longer time for the population to reach the same yield level as of selection in Syn1. SW1-C12 originated from a very broad base population by S1 selection and took more than 20 years (planted 2-3 crops per year) to reach the same yield level as of only few cycles of S1 selection in highly selected narrow-base population from commercial cultivars and improved germplasm. (Table 2).

**Table 2** Yield of Ag-Syn1 populations as compared to SW1-C12 and SW5-C4.

| Selection cycles | Character | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Grain yield | Percent to SW1-C12 | 50% silking | Shelling | Moisture | Plant hight | Ear hight | Foliar dis. |
| | kg/ha | …..%….. | … days… | …….. % …….. | | …… cm …… | | 0-5 |
| C0 | 4,768 a | 108 | 54 a | 82 a | 29 a | 176 b | 96 b | 1.25 ab |
| C1 | 4,293 b | 97 | 54 a | 79 b | 29 a | 174 b | 100 ab | 0.50 b |
| C2 | 4,318 b | 98 | 53 a | 78 b | 29 ab | 177 b | 103 ab | 1.00 ab |
| C2-Sem*. | 4,362 b | 99 | 54 a | 79 b | 29 ab | 173 b | 100 ab | 1.00 ab |
| *Check* | | | | | | | | |
| SW1-C12 | 4,381 b | 100 | 54 a | 78 b | 28 ab | 191 a | 103 ab | 0.75 ab |
| SW5-C4 | 4,406 b | 100 | 54 a | 79 b | 29 b | 196 a | 105 a | 1.50 b |
| Mean | 4425 | _ | 54 | 79 | 29 | 181 | 101 | 1.00 |
| %CV | 3.03 | _ | 1.32 | 1.28 | 2.07 | 3.3 | 4.62 | 54.77 |

* Semi-open end population (Ag-Syn1C2-Sem) dirived from diallel series of 10 S2 lines of Ag-Syn1C1 plus 2 S5 lines from Ag-Syn1C0 by pedigree selection.

**Table 3** Yields at 15 percent moisture and other agronomic traits of top-10 F1 hybrids derived from S5 inbreds extracted from AgSyn1C0 by pedigree method.

| Pedigree | Grain yield | % of Pion. 3012 | Moisture | Shelling | 50% silking | Plant hight | Ear hight |
|---|---|---|---|---|---|---|---|
| | kg./ha. | | ……%…… | | days | …….....cm…….... | |
| Ag210/Ag205 | 8,144 a-c | 105 | 35 a-g | 77 f-m | 54 b-d | 189 c-h | 107 d-f |
| Ag208/Ag204 | 7,847 a-d | 101 | 34 a-j | 78 b-I | 54 c-e | 185 c-k | 100 f-k |
| Ag210/Ag204 | 7,532 a-f | 97 | 33 d-m | 76 f-m | 53 c-f | 184 c-m | 100 f-k |
| Ag201/Ag208 | 7,500 a-f | 97 | 33 d-n | 81 a-d | 54 c-e | 181 g-p | 97 h-n |
| Ag207/Ag203 | 7,404 a-f | 95 | 36 a-d | 82 a-c | 53 d-g | 194 a-f | 110 b-e |
| Ag205/Ag208 | 7,199 b-h | 93 | 33 d-n | 75 I-n | 53 c-f | 183 d-m | 105 d-h |
| Ag209/Ag203 | 7,190 b-h | 93 | 32 g-p | 79 b-h | 52 e-g | 188 c-h | 110 b-e |
| Ag210/Ag203 | 7,150 b-h | 92 | 31 I-r | 77 e-l | 53 d-g | 186 c-j | 112 a-d |
| Ag206/Ag205 | 7,141 b-h | 92 | 33 d-n | 74 k-n | 53 c-f | 203 a | 120 a |
| Ag210/Ag202 | 7,141 b-h | 92 | 31 h-p | 78 b-j | 54 b-d | 194 a-f | 110 b-e |
| *Check :* | | | | | | | |
| Pioneer 3012 | 7,765 a-e | 100 | 29 n-p | 82 ab | 55 a-c | 203 a | 110 b-e |
| CP989 | 6,479 f-m | 83 | 30 m-p | 78 b-I | 56 ab | 196 a-c | 100 f-k |
| Syngenta NK45 | 8,469 a | 109 | 29 p | 79 b-g | 55 a-c | 195 a-d | 94 j-o |
| Cargill 919 | 8,271 ab | 107 | 31 k-p | 83 a | 54 b-d | 172 l-t | 92 k-o |
| *%CV* | *8.84* | _ | *5* | *2* | *1* | 3 | 5 |

The semi-open end population (AgSyn1C2 Sem.) derived from diallel crossed of the same 10 S2 lines of AgSyn1C2 and 2 additional top combining ability S5 lines from pedigree selection yielded more or less the same as AgSyn1C2. Lack of heterosis in AgSyn1C2-Sem indicated that S1selection accumulated the same dominant loci as of S5 lines from pedigree selection. While pedigree selection is more flexible for specific crosses, recurrent selection for inbred per se allows accumulation of additive and dominant genes within population and promotes stronger derived inbreds from advanced populations. Therefore, recurrent selection for inbred per se should be used as gene pool of desirable traits to support the pedigree program especially when single cross hybrid is the ultimate goal of breeding program.

Diallel selection rendered a more balance of germplasm and maintained more genetic variability as compared to S1 selection (Table 1). Moreover, diallel selection allowed a direct test for combining ability among selected inbreds. Therefore, combining ability of the advanced cycles should be increased. Therefore, diallel progeny testing and simultaneously selfing of the diallel set and diallel crossed of selected S1s in the following season to start new cycle of selection should be a logical method for recurrent selection. This method used only 2 seasons per cycle instead of 3 seasons per cycle for S1 and testcross selection methods now generally in use. Gene frequencies of Pioneer 3012, Pioneer 3013, SW 3853, G5445A, Cargill 919 and Uni-H9728 in 10 selected S5s from pedigree selection were 25, 20, 5, 35, 10, and 5 % respectively

(Table 1). The results suggested that pedigree selection rendered a similar pattern of gene frequencies as of that obtained from S1 selection and diallel selection. However, recurrent selection provided a systematic crossing and simultaneous selection of desirable traits and allows a thorough use of germplasm. Therfore, recurrent and pedigree selections should be interplayed for the most effective use of germplasm. Table 3 showed yields and few other agronomic traits of top-10 hybrids ($S_5 \times S_5$) from pedigree selection. The top two hybrids were more or less yielded the same as of check hybrids but significantly higher yield than CP989. The experimental hybrids had higher moisture content and lower shelling percentage and there were no clear advantages on other aspects.

## CONCLUSION

Regardless of selection methods, the ultimate purposes of breeding program are to eliminate of deleterious genes and accumulate desirable genes either for the improvement of pure population or heterogeneous population. However, pedigree selection is still the most widely used for the improvement of inbreds either for direct use or for hybrid production. Although recurrent selection has been proposed for the improvement of combining ability of the heterogeneous population from which good combining ability inbreds could be derived, its effectiveness is obscure because of variable results of the methods. Results from this study as well as previous studies led to the conclusion that recurrent selection in selected narrow base population could be used as supporting system to pedigree method. While pedigree method is more flexible for specific crosses and improvement of few desirable traits, recurrent selection systematically provides a more thorough use and simultaneous selection of desirable traits. Most of recurrent selections now generally in use require 3 steps per cycle but diallel selection requires only 2 steps per cycle, a simultaneous testing of diallel progenies and selfing of the F1s from which derived S1s are diallel crossed to start the new cycle. Beside, diallel selection provides a direct testing for combining ability of selected lines. Comparatively, the present study showed that diallel selection retained more genetic diversity of derived population than S1 selection. Therefore, the method should be an effective system for inbred and hybrid improvement as well as a supporting system to pedigree method.

## LITERATURE CITED

Burton, J.W., L.H. Penny, A.R. Hallauer, and S.A. Eberhart. 1971. Evaluation of synthetic population developed from maize variety (BSK) by two methods of recurrent selection. Crop Sci. 11 : 361-365.

Carangal, V.R., S.M. Ali, A.F. Koble, E.H. Rinke, and J.C. Sentz. 1971. Comparison of S1 with testcross evaluation for recurrent selection in maize. Crop Sci. 11 : 658-661.

Duclos, L.A. and P.L. Crane. 1968. Comparative performance of top crosses and S1 progeny for improving populations of corn (*Zea mays* L.). Crop Sci. 8 : 191-194.

Eberhart, S.A., W. Salhuana, R. Sevilla, and S. Taba. 1995. Principles for tropical maize breeding. Maydica 40 : 339-355.

Fasoulas, A.C. and V.A. Fasoula. 1995. Honeycomb selection designs. Plant Breeding Reviews 13 : 87-139.

Genter, C.F. 1973. Comparison of S1 and testcross evaluation after two cycles of recurrent selection in maize. Crop Sci. 13 : 524-527.

Genter, C.F. and M.W. Alexander. 1966. Development and selection of productive S1 inbred lines of corn (*Zea mays* L.). Crop Sci. 6 : 429-431.

Genter, C.F. and S.A. Eberhart. 1974. Performance of original and advanced maize populations and their diallel crosses. Crop Sci.14 : 881-885.

Horner, E.S.,W.H. Chapman, M.C. Lutrick, and H.W. Lundy. 1969. Comparison of selection based on yield of topcross progenies and S2 progenies in maize. Crop Sci. 9 : 539-543.

Horner, E.S., H.W. Lundy, M.C. Lutrick, and W.H. Chapman. 1973. Comparison of three methods of recurrent selection in maize. Crop Sci. 13 : 485-489.

Ortiz, J. 1963. Evaluation of synthetic population developed from Krug Yellow Dent Corn by two systems of recurrent selection. M.S. Thesis. Iowa State University, Ames.

Penny, L.H. and S.A. Eberhart.1971. Twenty years of reciprocal recurrent selection with two synthetic varieties of maize (*Zea mays* L.). Crop Sci. 11 : 900-903.

Sprague, G.F.,W.A. Russell, and L.H. Penny. 1959. Recurrent selection for specific combining ability and type of gene action involved in yield heterosis in corn. Agron. J. 51 : 392-394.

Tokatlidis, I.S.,M. Koutsika-Sotiriou, and A.C. Fasoulas. 2001. The development of density independent hybrids in maize. Maydica 46 : 21-25.

Troyer, A.F., 1996. Breeding widely adapted, popular maize hybrids. Euphytica 92 : 163-174.

Weyhrich, R.A., K.R. Lamkey, and A.R. hallauer. 1998. Responses to seven methods of recurrent selection in the BS11 maize population. Crop Sci. 38 : 308-321.

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# Callus Induction and Plant Regeneration from Mature Embryos of Glutinous Rice (*Oryza sativa* L.) Cultivar TDK1

**Souvanh Thadavong[1], Prapa Sripichitt[1], Wasana Wongyai[1] and Peeranuch Jompuk[2]**


## ABSTRACT

The present study was conducted to determine the optimum *in vitro* conditions for callus induction and plant regeneration from mature embryos (seeds) of glutinous rice cultivar TDK 1. It was revealed that embryos cultured on Murashige and Skoog (MS) agar medium supplemented with 2 mg/l 2,4-D and 500 mg/l L-proline under light condition produced the highest percentage of callus formation (96.91 %). The average size of callus produced was large (6.02 mm). The calli dehydrated by placing in petridishes with covers for 7 days under light condition before transferring onto regeneration medium generated higher frequency of shoot regeneration than the calli cultured on regeneration medium without dehydration. The most suitable medium for plant regeneration from dehydrated calli was MS agar medium supplemented with 1 mg/l IAA, 4 mg/l BA and 800 mg/l casein hydrolysate which induced the highest percentage of calli forming shoots (45.00 %) and each callus produced the largest number of shoots (average 15 shoots). The shoots were rooted at the highest rate (100 %) when transferred onto MS agar medium supplemented with 1 mg/l NAA. The complete plantlets were thereafter transplanted to grow under greenhouse condition. They were morphologically normal and fertile.


**Key words:** callus, regeneration, embryo, glutinous rice

## INTRODUCTION

Laos is the largest producer and comsumer of glutinous rice in the Asian. Approximately 85 % of annual rice production in Laos is glutinous rice. TDK 1 has proven to be the most popular variety of glutinous rice characterized by good tillering ability, highly nitrogen response and resistance to brown planthopper. However, TDK1 gives moderate yield with susceptibility to green leafhopper and poor in grain milling quality when they are grown under dry season with irrigated condition (Schiller *et al*., 1999). Therefore, breeding program to develop new varieties that are superior to TDK 1 in both yield and grain quality is required.

Rice improvement using biotechnology such as somaclonal variation, *in vitro* selection, production of doubled haploid lines from anther culture, and genetic transformation might be an alternative way for achieving desirable rice varieties. However, the basic prerequisite for the potential use of biotechnology in rice improvement is the regeneration ability of cell, tissue and organ of rice plant. Successful regeneration of plant tissue culture mainly depends on genotype, explant type, medium composition, plant growth regulator and culture environment (Khanna and Raina, 1998).

There were numerous reports on callus

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formation and plant regeneration from mature seeds of indica rice especially the non-glutionus type (Vajrabhaya *et al*., 1986; Raina *et al*., 1987; Chowdhry *et al*., 1993; Sripichitt and Chewasestatham, 1994; Burikam *et al*., 2002). However, information of plant regeneration from tissue and organ culture of glutinous rice is scarcely found.

The objectives of this study were to find a suitable medium and culture condition for callus induction and plant regeneration from mature seeds (embryos) of glutinous rice variety TDK 1. Mature seed was chosen as the explant in this experiment because it was tolerant to surface sterilization and easy to be handled. Moreover, mature seed exhibited high potential of plant regeneration and low rate of albinism (Ogewa *et al*., 1982).

## MATERIALS AND METHODS

### Experiment 1. Callus induction

Mature seeds of TDK 1 (Tha Dok Kham 1) were dehusked and surface sterilized by soaking in 70 % ethanol for 5 min and in 15 % clorox containing a wetting agent "Tween 20" for 20 min followed by three rinses in sterile distilled water. Aseptic seeds were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) at the concentrations of 0, 1, 2, 3, 4 and 5 mg/l, 3 % sucrose and 0.7 % agar, pH = 5.8. Seed cultures were incubated at 25°C under dark and light condition illuminated by 2,000 lux of cool-white fluorescent light with 16hrs photoperiod for 4 weeks. After 4 weeks of culture, the number of seeds forming callus and the size of callus [(width + length)/2)] were recorded to determine the optimum concentration of 2,4-D for callus induction from the seeds.

### Experiment 2. Enhancing callus formation

Mature seeds were cultured on MS medium supplemented with 2,4-D at the optimum concentration (result from experiment l) in combination with 0.3 and 0.5 mg/l kinetin (K), 50 and 100 mg/l L-tryptophan (Tryp), 300 and 500 mg/l casein hydrolysate (CH), 300 and 500 mg/l L-proline (P), 10 and 15 % (v/v) coconut water (CW). Three percent of sucrose and 0.7 % of agar were also added to the medium. Seed cultures were incubated at 25°C under dark and light condition (as previously mentioned in experiment l) for 4 weeks. Four weeks after culture, the number of seed forming callus (%) and the size of callus [(width + length)/2] were recorded to investigate the kind and concentration of supplements to 2,4-D which can enhance the frequency of callus formation.

### Experiment 3. Plant regeneration

Four-week-old calli derived from seeds cultured on suitable callus induction medium (result from experiment 2) were divided into 3 groups. The first group, calli were cut into 0.5 mm pieces and cultured immediately on regeneration medium. The second group, calli were dehydrated by placing on the single layer of filter paper in petridishes and sealed by plastic film (parafilm). The petridishes were kept at 25°C under dark condition for 7 days. The third group, calli were dehydrated by the method previously described and kept at 25°C under light condition for 7 days.

Non-dehydrated and dehydrated calli under dark and light conditions were cultured on MS medium supplemented with 1 mg/l indoleacetic acid (IAA) in combination with benzyladenine (BA) or kinetin at the concentrations of 1,2,3 and 4 mg/l, 3 % sucrose and 0.7 % agar. The cultures were incubated at 25°C under light condition. Four week after culture, the number of calli forming shoots and the number of shoots per callus were counted to determine the optimum combination of IAA and BA or kinetin for plant regeneration from seed-derived calli.

**Experiment 4. Enhancing plant regeneration capacity**

Dehydrated calli under light condition were cultured on MS medium added with the optimum combination of IAA and BA or kinetin (result from experiment 4) and supplemented with 20 and 40 mg/l adenine sulfate (AS), 50 and 100 mg/l L-tryptophan, 500 and 800 mg/l casein hydrolysate, 1 and 2 g/l yeast extract (YE) and 10 and 20 % coconut water. Three percent of sucrose and 0.7 % of agar were also added to the medium. The cultures were incubated at 25°C under light condition. Four weeks after culture, the number of calli forming shoots and the number of shoots per callus were counted to verify the kind and concentration of supplements which could enhance the frequency of plant regeneration.

**Experiment 5. Root induction**

Regenerated shoots were rooted by culturing on MS medium without growth regulators or supplemented with ∝-naphthaleneacetic acid (NAA) at the concentration of 0.5, 1, and 2 mg/1,3 % sucrose and 0.7 % agar. Two weeks after culture, the number of shoot forming roots and the number of roots per shoot were recorded to investigate the suitable medium for root induction.

## RESULTS AND DISCUSSION

**Callus induction**

Seeds of glutinous rice variety TDK 1 cultured on MS medium without 2,4-D could not form callus but germinated to be seedlings. Whereas seeds cultured on MS medium supplemented with 2,4-D developed shoots after 2-3 days of culture but later the shoots ceased to grow further. Calli were observed at the base of the shoots about one week of culture. Calli obtained were initiated from dividing cells of scutellum and mesocotyl of the embryos by the induction of 2,4-D (Maeda, 1980). Callus proliferation was continued until the 4$^{th}$ week of culture. Then the number of seeds forming callus and the size of callus were determined. It was found that the seeds cultured on MS medium supplemented with 2,4-D at every concentration produced callus with relatively high frequencies of 80.00 to 98.43 % under light and dark condition (Table 1). However, seeds cultured under light condition gave higher average frequency of callus formation (92.03 %) and larger average size of callus (3.88 mm) than those cultured under dark condition (87.02 % and 3.44 mm). In addition, calli formed under light condition were mostly embryogenic with creamy, dry and compact appearance, while non-embryogenic calli with white, wet and friable characters were found predominantly under dark condition. It was documented that embryogenic callus displayed higher frequency of plant regeneration than the non-embryogenic one (Nabors *et al*., 1983; Siriwardana and Nabors, 1983). Janet and Seabrook (1980) reported that callus cultured under light condition showed higher proliferation and plant regeneration because light induces morphogenesis process and green spot formation of callus.

Considering the effects of 2,4-D at various concentrations, it was shown that 2 mg/l 2,4-D gave the highest percentage of callus formation (98.43 %) and largest size of calus (5.07 mm) when the seeds were cultured under light condition. Whereas under dark condition, seeds cultured on the medium supplemented with 1 mg/l 2,4-D exhibited the highest frequency of callus formation (94.64 %) and largest amout of callus (4.11 mm). It is well known that 2,4-D is the most suitable auxin for callus induction of rice in tissue culture. However, the optimum concentration of 2,4-D varied depending on the explant source and genotype of rice (Raina, 1989). Our result revealed that 2,4-D at the concentrations of 1-2 mg/l were suitable for callus production from rice seed which was in accordance with many previous reports (Vajrabhaya *et al*., 1986; Raina *et al*., 1987; Sripichitt and Cheewasestatham, 1993; Burikam *et al.*, 2002).

**Table 1** Callus formation of mature embryos cultured on MS medium supplemented with various concentrations of 2,4-D under light and dark condition. Each treatment comprises 70 replications.

| 2,4-D (mg/l) | Light | | Dark | |
|---|---|---|---|---|
| | % Seed forming callus | Size of callus (mm)[1] | % Seed forming callus | Size of callus (mm)[1] |
| 0 | 0.00 d[3] | 0.00 d | 0.00 e | 0.00 c |
| 1 | 93.87 b | 4.65 ab | 94.64 a | 4.11 a |
| 2 | 98.43 a | 5.07 a | 90.83 b | 4.00 a |
| 3 | 85.50 c | 2.96 c | 84.61 c | 2.61 b |
| 4 | 87.93 c | 2.67 c | 85.00 c | 2.67 b |
| 5 | 94.40 b | 4.04 b | 80.00 d | 3.83 a |
| Average[2] | 92.03 | 3.88 | 87.02 | 3.44 |

1 Size of callus = (width + length)/2

2 Control is not included

3 Means within column followed by the same letter are not significantly different at 95 % level of confidence by DMRT

**Enhancing callus formation**

Seeds were cultured on MS medium added with 2 mg/l 2,4-D in combination with various kinds and concentrations of organic substances under light and dark conditions to promote callus formation. It was observed that under light condition organic supplements enhanced callus induction and size of callus from 88.14 % and 4.34 mm (with no supplement) to 89.78 % and 4.79 mm (Table 2). However, organic supplements could not promote callus production under dark condition. It is clearly shown that light condition had stimulatory effect on callus proliferation from embryos of this glutinous rice variety.

Comparing the effects of various kinds and concentrations of organic supplements on enhancing callus formation, it was found that proline supplemented to the medium at the concentration of 500 mg/l under light condition promoted the highest percentage of seeds forming callus (96.91 %) and large size of callus (6.02 mm) (Figure 1A). However, the other organic supplements including 0.5 mg/l kinetin, 100 mg/l tryptophan and 10 % coconut water could enhance callus formation as well. Although 2,4-D alone induced callus formation from rice embryos (Vajrabhaya *et al*., 1986; Raina *et al*., 1987; Sripichitt and Cheewasestatham, 1994), some organic substances such as kinetin, tryptophan, casein hydrolysate, proline and coconut water added to the callus induction medium containing 2,4-D could enhance the efficiency of callus formation (Siriwardana and Nabors, 1983; Vajrabhaya *et al*., 1986; Sripichitt and Cheewasestatham, 1994; Rueb *et al*., 1994). Especially, proline was found to be the most effective supplement for callus promotion in this experminet. This is because proline is a kind of amino acid and is used as a precursor in the process of metabolism and cell division (Santos *et al*., 1996). Moreover, calli of TDK 1 derived from seeds cultured on the medium supplemented with proline, tryptophan and kinetin were mostly embryogenic calli. This is in agreement with the previous works showing many organic substances added to the callus induction medium including proline, tryptophan, casein hydrolysate, coconut water and kinetin increased the frequency of

**Table 2** Callus formation of mature embryos cultured on MS medium supplemented with 2 mg/l 2,4-D and various kinds and concentrations of organic substances under light and dark conditions. Each treatment comprises 70 replications.

| Supplement to 2 mg/l 2,4-D | Light | | Dark | |
|---|---|---|---|---|
| | % Seed forming callus | Size of callus (mm)[1] | % Seed forming callus | Size of callus (mm)[1] |
| No supplement | 88.14 a[3] | 4.34 bc | 95.90 a | 3.86 a |
| K 0.3 mg/l | 88.70 a | 4.29 bc | 92.77 a | 4.05 a |
| K 0.5 mg/l | 93.56 a | 5.83 a | 81.54 a | 3.69 a |
| Trypt 50mg/l | 89.27 a | 4.25 bc | 87.65 a | 3.48 a |
| Trypt 100mg/l | 96.34 a | 4.97 ab | 91.43 a | 3.55 a |
| CH 300 mg/l | 78.68 b | 3.34 c | 90.13 a | 3.66 a |
| CH 500mg/l | 87.97 a | 4.37 bc | 83.55 a | 2.98 a |
| P 300mg/l | 89.29 a | 3.63 c | 89.47 a | 3.64 a |
| P 500mg/l | 96.91 a | 6.02 a | 88.94 a | 3.58 a |
| CW 10 % | 90.59 a | 6.05 a | 86.78 a | 3.14 a |
| CW 15 % | 86.49 a | 5.21 ab | 82.07 a | 3.68 a |
| Average[2] | 89.78 | 4.79 | 87.43 | 3.54 |

1 Size of callus = (width + length)/2

2 No supplement is not included

3 Means within column followed by the same letter are not significantly different at 95 % level of confidence by DMRT

embryogenic callus formation (Siriwardana and Nabors, 1983; Armenia and Futsuhara, 1992; Chowdhry *et al*., 1993; Rueb *et al*., 1994).

Callus proliferation was carried out to be used in the other experiments by culturing the embryos on MS medium supplemented with 2 mg/l 2,4-D and 500 mg/l proline under light condition.

**Plant regeneration**

After the calli were transferred onto regeneration medium for 1 week, green spots and roots were induced from the calli. Thereafter the green spots were developed to be shoots or plantlets on the 2nd week of culture. Plant regeneration of calli observed in this experiment was the result of organogenesis process because shoots and roots were not originated simultaneously from the same cells. Shoots were developed firstly followed by root formation at the base of some shoots. The process of plant regeneration from rice calli were classified into organogenesis (Abe and Futsuhara, 1989) and embryogenesis (Ling *et al*., 1983) of which shoots and roots were developed simultaneously from the same originated cell.

Non-dehydrated calli cultured on MS medium without growth regulator or supplemented with 1 mg/l IAA and various concentration of either kinetin or BA formed green spots with varying frequencies from 6.86 to 37.47 % (Table 3). However, low frequencies of calli (0-5.0 %) of which green spots gave rise to shoots were observed. Among them calli cultured on MS medium supplemented with 1 mg/l IAA and 1 mg/l BA gave the highest percentage of shoot regeneration (5 %) and largest number of shoots per callus (1.66 shoots/callus).

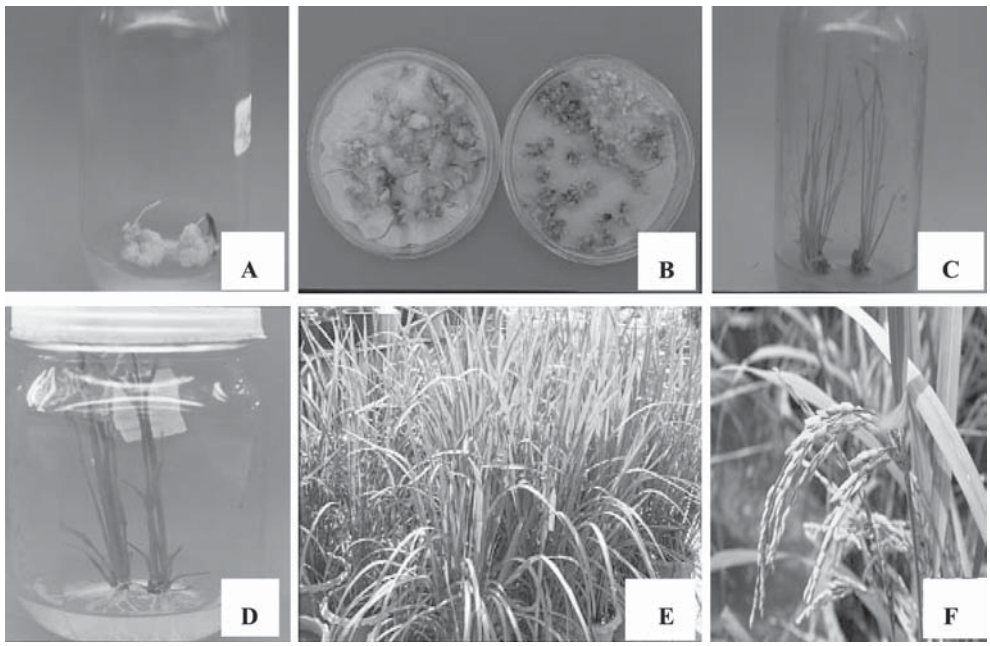


**Figure 1** Callus formation and plant regeneration from mature embryos of TDK 1.
(A) Callus formation of mature embryos cultured on MS medium containing 2 mg/l 2,4-D and 500 mg/l proline.
(B) Non-dehydrated calli (left) and dehydrated calli under light condition (right).
(C) Shoot regeneration form dehydrated calli (under light condition) cultured on MS medium containing 1 mg/l IAA, 4 mg/l BA and 800 mg/l casein hydrolysate.
(D) Root formation of regenerated shoots cultured on MS medium containing 1 mg/l NAA.
(E) Regenerated plants transplanted in the greenhouse showing normal morphological characters and well seed setting (F).

Calli dehydrated for 7 days under dark condition when culturing on every kind of regeneration medium including control could develop green spots with varying degree from 10.17 to 52.00 % (Table 4). Nevertheless, relatively low percentages of calli (0-11.26 %) developed shoots from green spots were recovered. Calli cultured on MS medium added with 1 mg/l IAA and 4 mg/l BA exhibited the highest rate of shoot regeneration (11.26 %) and largest number of shoots per callus (3.75 shoots/callus) comparing with the calli cultured on other kinds of regeneration medium.

Calli dehydrated for 7 days under light condition (Figure 1 B) formed green spots with the frequencies from 25.00 to 50.00 % when culturing on regeneration medium (Table 5). Relatively high frequencies of calli forming shoots (0-37.00 %) were achieved. Calli cultured on MS medium supplemented with 1 mg/l IAA and 4 mg/l BA showed the highest regeneration ability (37.00 % calli forming shoots and 12.30 shoots/callus) among the calli cultured on all kinds of regeneration medium.

Comparing the non-dehydrated calli, dehydrated calli for 7 days under dark and light condition, it was revealed that calli dehydrated under light condition showed the highest average percentage of calli forming green spots (38.07 %) and shoots (12.80 %) and largest average number of shoots per callus (4.26 shoots/callus) (Table 5)

**Table 3** Plant regeneration from non-dehydrated calli cultured on MS medium supplemented with 1mg/l IAA and various concentrations of either K or BA.

| Growth regulator (mg/l) | No. of calli cultured | No. of calli forming | | | No. of shoots/ callus |
|---|---|---|---|---|---|
| | | Green spot (%) | Roots (%) | Shoots (%) | |
| 0 (control) | 19 | 37.47 a[1] | 68.00 d | 0.00 d | 0.00 e |
| IAA : K | | | | | |
| 1:1 | 35 | 13.83 d | 54.83 e | 3.84 b | 1.28 c |
| 1:2 | 29 | 33.88 a | 57.69 e | 0.00 d | 0.00 e |
| 1:3 | 36 | 22.48 c | 54.76 ef | 0.00 d | 0.00 e |
| 1:4 | 22 | 25.02 b | 50.00 f | 4.54 a | 1.51 b |
| IAA:BA | | | | | |
| 1:1 | 39 | 10.54 e | 96.42 a | 5.00 a | 1.66 a |
| 1:2 | 33 | 10.78 e | 73.68 c | 0.00 d | 0.00 e |
| 1:3 | 40 | 12.83 de | 76.66 c | 2.50 c | 0.08 d |
| 1:4 | 32 | 6.86 f | 84.61 b | 0.00 d | 0.00 e |
| Average | | 19.29 | 68.51 | 1.76 | 0.50 |

[1] Means within column followed by the same letter are not significantly different at 95 % level of confidence by DMRT

**Table 4** Plant regeneration from dehydrated calli (under dark condition) cultured on MS medium supplemented with 1mg/l IAA and various concentrations of either K or BA.

| Growth regulator (mg/l) | No. of calli cultured | No. of calli forming | | | No. of shoots/ callus |
|---|---|---|---|---|---|
| | | Green spot (%) | Roots (%) | Shoots (%) | |
| 0 (control) | 38 | 34.52 d[1] | 40.00 e | 0.00 d | 0.00 c |
| IAA : K | | | | | |
| 1:1 | 64 | 40.62 c | 35.40 g | 0.00 d | 0.00 c |
| 1:2 | 58 | 43.98 b | 37.50 fg | 5.29 c | 1.76 b |
| 1:3 | 62 | 43.34 b | 63.80 b | 0.00 d | 0.00 c |
| 1:4 | 64 | 50.12 a | 70.00 a | 9.79 b | 3.26 a |
| IAA:BA | | | | | |
| 1:1 | 66 | 35.89 d | 32.50 h | 6.06 c | 2.02 b |
| 1:2 | 58 | 10.27 e | 48.00 c | 0.00 d | 0.00 c |
| 1:3 | 60 | 10.17 e | 39.50 ef | 0.00 d | 0.00 c |
| 1:4 | 70 | 52.00 a | 45.70 d | 11.26 a | 3.75 a |
| Average | | 35.65 | 45.82 | 3.60 | 1.19 |

[1] Means within column followed by the same letter are not significantly different at 95 % level of confidence by DMRT

**Table 5** Plant regeneration from dehydrated calli (under light condition) cultured on MS medium supplemented with 1mg/l IAA and various concentrations of either K or BA.

| Growth regulator (mg/l) | No. of calli cultured | No. of calli forming | | | No. of shoots/ callus |
|---|---|---|---|---|---|
| | | Green spot (%) | Roots (%) | Shoots (%) | |
| 0 (control) | 32 | 37.50 c[1] | 80.00 c | 0.00 e | 0.00 d |
| IAA : K | | | | | |
| 1:1 | 40 | 40.00 c | 56.25 de | 0.00 e | 0.00 d |
| 1:2 | 40 | 40.00 c | 55.55 ef | 0.00 e | 0.00 d |
| 1:3 | 40 | 45.00 b | 55.55 ef | 7.69 d | 2.56 c |
| 1:4 | 46 | 29.63 d | 52.63 f | 11.11 c | 3.70 c |
| IAA:BA | | | | | |
| 1:1 | 32 | 30.55 d | 84.61 b | 0.00 e | 0.00 d |
| 1:2 | 40 | 25.00 e | 90.00 a | 29.40 b | 9.80 b |
| 1:3 | 42 | 45.00 b | 58.82 d | 30.00 b | 10.00 b |
| 1:4 | 42 | 50.00 a | 41.17 g | 37.00 a | 12.30 a |
| Average | | 38.07 | 63.84 | 12.80 | 4.26 |

[1] Means within column followed by the same letter are not significantly different at 95 % level of confidence by DMRT

followed by calli dehydrated under dark condition giving an average of 35.65 % and 3.60 % calli forming green spots and shoots and an average of 1.19 shoots/callus (Table 4) and non-dehydrated calli exhibiting an average of 19.29 % and 1.76 % calli forming green spots and shoots and an average of 0.50 shoots/callus (Table 3), respectively. Interestingly, calli dehydrated under light condition performed the highest plant regeneration ability (50.00 % and 37.00 % calli forming green spots and shoots and 12.30 shoots/callus) when they were cultured on MS medium containing 1 mg/l IAA and 4 mg/l BA (Table 5).

Dehydration of calli for 7 day under dark and light condition before transferring to regeneration medium promoted plant regeneration capacity comparing to the non-dehydrated calli. A similar stimulatory effect of dehydration on plant regeneration of rice callus has been manifested in several previous studies (Tsukahara and Hirosawa, 1992; Sripichitt and Cheewasestatham, 1994; Jain *et al*., 1996; Burikam *et al*., 2002). Gray (1987) suggested that callus has lost water from the cells to become at quiescent stage during dehydration and reabsorped water and nutrients when transferring to regeneration medium which resulted in higher capacity of plant regeneration. Whereas, Rueb *et al*. (1994) demonstrated that non-dehydrated callus released the excess water from the cells to the surface of callus which obstructed the regeneration process of the embryogenic callus. The result showed that dehydration of callus under light condition increased the frequency of plant regeneration when compared to dehydration under dark condition. Armenia and Futsuhara (1992) suggested that regeneration process of callus depended not only on genotype and suitable regeneration medium but also on the light.

The suitable medium for plant regeneration from dehydrated calli under dark and light condition in this experiment was MS medium supplemented with 1 mg/l IAA and 4 mg/l BA. It is well recognized

that regeneration of callus to become shoots (plantlets) or roots depends mainly on the proportion of auxin and cytokinin in the medium. Shoot regeneration is achieved in the medium supplemented with low ratio of auxin to cytokinin while root formation preferred the medium with high ratio of auxin to cytokinin. Thus, increasing the concentration of BA in the medium reduced the ratio of auxin to cytokinin and consequently enhanced the frequency of shoot regeneration. Although kinetin is a kind of cytokinin, it induced lower rate of shoot regeneration than BA in this study.

**Enhancing plant regeneration capacity**

Dehydrated calli (under light condition) were cultured on MS medium containing 1 mg/l IAA and 4 mg/l BA (result of the previous experiment) in conjuction with various kinds and concentrations of organic substances to increase the frequency of plant regeneration. It was found that organic supplements including tryptophan, casein hydrolysate, yeast extract and coconut water induced higher percentage of calli forming green spot and shoots and number of shoots per callus than those of the control (no supplement) (Table 6). Especially, casein hydrolysae at the concentration of 800 mg/l gave the highest frequency of calli forming green spots (80 %) and shoots (45 %) and largest number of shoots per callus (15 shoots/callus) (Figure 1 C) followed by 500 mg/l casein hydrolysate exhibiting 75 % and 40 % calli forming green spots and shoots and 13.3 shoots/callus and 2,000 mg/l yeast extract showing 57.14 % and 35.71 % calli forming green spots and shoots and 11.90 shoots/callus, respectively. There were several reports demonstrating that casein hydrolysate, yeast extract, tryptophan and proline could promote the efficiency of plant regeneration from rice callus (Ling *et al*., 1983; Raina *et al*., 1987; Sahrawat and Chand,

**Table 6** Plant regeneration from dehydrated calli (under light condition) cultured on MS medium supplemented with 1mg/l IAA, 4mg/l BA and various kinds and concentrations of organic substances.

| Supplement to 1mg/l IAA+4mg/lBA | No. of calli cultured | No. of calli forming | | | No. of shoots/ callus |
|---|---|---|---|---|---|
| | | Green spot (%) | Roots (%) | Shoots (%) | |
| No supplement | 57 | 20.24 j[1] | 30.62 a | 4.73 g | 1.57 f |
| AS 20 mg/l | 31 | 29.00 i | 16.12 d | 0.00 h | 0.00 f |
| AS 40 mg/l | 33 | 72.72 c | 30.30 a | 0.00 h | 0.00 f |
| Tryp 50 mg/l | 32 | 62.50 d | 25.00 b | 0.00 h | 0.00 f |
| Tryp 100 mg/l | 20 | 50.00 f | 30.00 a | 10.00 f | 3.30 e |
| CH 500 mg/l | 20 | 75.00 b | 10.00 e | 40.00 b | 13.30 b |
| CH 800 mg/l | 20 | 80.00 a | 8.00 f | 45.00 a | 15.00 a |
| YE 1,000 mg/l | 24 | 37.50 h | 8.33 f | 29.16 d | 9.72 c |
| YE 2,000 mg/l | 28 | 57.14 e | 3.57 g | 35.71 c | 11.90 b |
| CW 10 % | 30 | 36.66 h | 23.33 c | 16.00 e | 5.30 d |
| CW 20 % | 20 | 40.00 g | 5.00 g | 15.00 e | 5.00 d |
| Average | | 50.97 | 17.29 | 17.78 | 5.91 |

[1] Means within column followed by the same letter are not significantly different at 95 % level of confidence by DMRT

**Table 7** Root formation of regenerated shoots cultured on MS medium supplemented with various concentrations of NAA.

| NAA (mg/l) | No. of shoots cultured | No. of shoots forming roots | % Root formation |
|---|---|---|---|
| 0 | 39 | 36 | 92.30 |
| 0.5 | 63 | 62 | 98.41 |
| 1.0 | 56 | 56 | 100.00 |
| 2.0 | 53 | 53 | 100.00 |

1997). However, the beneficial effect of organic substances on plant regeneration is not clearly know.

**Root induction**

Regenerated shoots when transferring onto MS medium containing NAA at the concentrations of 0.5-2 mg/l formed roots with the higher frequencies (98-100 %) than those cutured on MS medium without NAA. Especially, NAA at the concentrations of 1-2 mg/l induced the highest percentage of shoots forming roots (100 %) (Figure 1 D). Moreover, shoots produced higher number of roots and longer root length when culturing on rooting medium containing NAA. Auxin such as NAA, IAA and IBA are generally used for regulating cell elongation, cell division, formation of adventitious roots and callus initiation and growth when added to the medium at appropriate concentrations. NAA and IBA have been reported to induce roots from regenerated shoots of rice (Sahrawat and Chand, 1998; Burikam *et al*., 2002). The complete plantlets obtained were transferred to grow in pots under greenhouse condition until maturity (Figure 1 E). They were morphologically normal and could set seeds well (Figure 1 F).

## CONCLUSIONS

1. The appropriate medium for callus induction from mature embryos of glutinous rice cultivar TDK 1 was MS agar medium added with 2 mg/l 2,4-D and 500 mg/l L-proline.

2. Dehydrated calli showed higher capacity of shoot regeneration than non-dehydrated calli.

3. The suitable medium for plant regeneration from dehydrated calli (under light condition) was MS agar medium supplemented with 1 mg/l IAA, 4 mg/l BA and 800 mg/l casein hydrolysate.

4. Root induction was achieved when the regenerated shoots were transferred onto MS agar medium containing 1 mg/l NAA.

## LITERATURE CITED

Abe, T. and Y. Futsuhara 1989. Selection of higher regenerative callus and change in isozyme pattern in rice (*Oryza sativa* L.). Theor. Appl. Genet. 78 : 648-652.

Armenia, B.M. and Y. Futsuhara. 1992. Histological observations on plant regeneration in rice (*Oryza sativa* L.) calli. Japan J. Breed. 42 : 33-41.

Burikam, S., P. Sripichitt, W. Kositratana, and S. Attathom. 2002. Improved frequencies of embryogenic calli induction and plantlet regeneration in mature embryos of KDML 105, and elite Thai indica rice. Thai J. Agric. Sci. 35 : 83-97.

Chowdhry, C., A.K. Tyagi, N. Maheshwari, and S.C. Maheswari. 1993. Effect of L-proline and L-tryptophan on somatic embryogenesis and plantlet regeneration of rice (*Oryza sativa* L.

cv. Pusa 169). Plant Cell, Tissue and Organ Cult. 32 : 357-361.

Gray, D.J. 1987. Quiescence in monocotyledonous and dicotyledonous somatic embryos induced by dehydration. Hortscience 22 : 810-814.

Jain, R.K., S. Jain, and R. Wu. 1996. Stimulatory effect of water stress on plant regeneration in aromatic indica rice varieties. Plant Cell Rep. 15 : 449-454.

Janet, E. and A. Seabrook. 1980. Laboratory culture, pp. 1-17. *In* J. Staba (ed.). Plant Tissue Culture as a Source of Biochemical. CRC Press, Inc., Boca Raton.

Khanna, H.K. and S.K. Raina. 1998. Genotype x culture media interaction effects on regeneration response of three indica rice cultivars. Plant Cell, Tissue and Organ Cult. 52 : 145-153.

Ling, D.H., W.Y. Chen, M.F. Chen, and Z.R. Ma. 1983. Somatic embryogenesis and plant regeneration in an interspecific hybrid of *Oryza*. Plant Cell Rep. 2 : 169-171.

Maeda, E. 1980. Organogenesis and cell culture in rice plants under sterile condition (part I). JARQ 14 : 4-8.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant 15 : 473-497.

Nabors, M.W., J.W. Heyser, T.A. Dykes, and K.J. Demott. 1983. Long duration, high frequency regeneration from cereal tissue cultures. Planta 157 : 385-391.

Ogewa, M.S., S. Yoshida, G.S. Cabuslay, Y.H. Chun, and K. Suenaga. 1982. Induction and selection of salt tolerant mutant rices by tissue culture. IRRI Saturday Seminar. December 4, 1982.

Raina, S.K., P. Sathish, and K.S. Sarma. 1987. Plant regeneration from *in vitro* cultures of anthers and mature seeds of rice (*Oryza sativa* L.) cv. Basmati-370. Plant Cell Rep. 6 :43-45.

Raina, S.K. 1989. Tissue culture in rice improvement : status and potential. Adv. Agron. 42 : 339-398.

Rueb, S., M. Leneman, R.A. Schilperoort, and L.A.M. Hensgens. 1994. Efficient plant regeneration through somatic embryogenesis from callus induced on mature rice embryos (*Oryza sativa* L.). Plant Cell, Tissue and Organ Cult. 36 : 259-264.

Sahrawat, A.K. and S. Chand. 1998. Effects of L–tryptophan, L-proline, and activated charcoal on plant regeneration in indica rice (*Oryza sativa* L.) IRRN 23 (3) : 10-12.

Santos, M.A., T. Camara, P. Rodriguez, I. Claparols, and J.M. Torne. 1996. Influence of exogenous proline on embryogenic and organogenic maize callus subjected to salt stress. Plant Cell, Tissue and Organ Cult. 47 : 59-65.

Schiller, J.M., S. AppaRao, Hatsadong, and P. Inthapanya. 1999. Glutinous rice varieties of Laos, their improvement, cultivation, processing and consumption. Chapter written for proposed FAO publication "Speciality rices in the world : Breeding production and marketing" . 35 p.

Siriwardana, S. and M.W. Nabors. 1983. Tryptophan enhancement of somatic embryogenesis in rice. Plant Physiol. 73 : 142-146.

Sripichitt, P. and P. Cheewasestatham. 1994. Plant regeneration from embryo derived callus of aromatic rice (*Oryza sativa* L.) variety Khao Dawk Mali 105. Kasetsart J. (Nat. Sci.) 28 : 27-37.

Tsukahara, M. and T. Hirosawa. 1992. Simple dehydration treatment promotes plantlet regeneration of rice (*Oryza sativa* L.) callus. Plant Cell Rep. 11 : 550-553.

Vajrabhaya, M., O. Tunvachkul, and T. Vajrabhaya. 1986. Effects of auxin and cytokinin on plant regeneration from rice callus. Sci. Res. Chula. Univ. 11 : 113-115.

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# Geographical Diversity and its Influence on Rice Yield

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## ABSTRACT

Experiments were conducted at two different geographical locations, the Asian Institute of Technology, Thailand and the Cereal Institute, Thessaloniki, Greece to compare tillering and yield of locally recommended rice varieties. Rice varieties RD23 and KDML105 of Thailand gave the same number of tillers at harvest and delayed planting (September 14) showed higher number of tillers/hill compared to early planting though there were no significant variations of panicles/m2 observed among different planting dates. The variety RD23 resulted in significantly higher grain yield (3.7 t/ha) compared to KDML105 (2.9 t/ha). The variety KDML105 showed higher 1000-grain weight (26.3 g) with longer duration (125 days) and lower HI (0.27) compared with RD23. Planting up to August 14 resulted in higher grain yield and 1000-grain weight, but delayed planting showed lower yield as well as grain weight. The indica type rice variety Olympiada showed significantly higher number of tillers/hill (19.8) at harvest compared with other japonica-typed varieties. The variety Maratelli resulted in lowest grain yield (5.7 t/ha). Indica typed variety showed higher panicles/m2 (319) with higher HI (0.57) but less 1000-grain weight (24.0 g) compared with other japonica typed varieties. Tropical rice showed lower grain yield, HI and maturity duration compared with temperate rice.


**Key words:** geographical variations, rice, tiller, yield

## INTRODUCTION

There is a 'hunger belt' in the world, located between 30 °N latitude and 30 °S latitude, centering around the equator. There are about 2.7 billion people living in the hunger belt; 60% of them suffer from mal-nutrition and 20% are starving. It is possible to improve the area through increasing the rice production in the hunger belt (Matsushima, 1980).

Rice has a wide physiological adaptability and is grown successfully in tropics, subtropics and temperate region. It requires a fairly high temperature, ranging from 20 to 40 °C. The optimum temperature appears to be about 30°C for the daytime maxima and 20°C for the nighttime maxima (Sreenivasan, 1985). Remarkable diversity exists in rice because of its long history of cultivation and selection under diverse climatic, edaphic and biotic environments; frequently in geographically separated areas. Most of the world's rice is grown in the tropics and the extreme latitude in which it is grown is in temperate region. Yoshida (1981) reported that rice can be grown between 53 °N to 35 °S latitude, below sea level to elevations above 2,000 m, and in upland conditions to 5 m water depth.

There are 111 rice-growing countries in the world of which three countries produce an average yield of 6 t/ha or more, 17 countries produce 4 t/ha

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or more, 78 countries produce 3 t/ha or less of which 57 countries produce 2 t/ha or less and 13 countries produce less than 1 t/ha. Temperate countries produce higher yield compared with tropical areas where the average rice paddy yield of Europe was 5.1 t/ha and that of Asia was 4.0 t/ha (Anon, 2000). Productivity of rice does not only vary between one country to another, but also within the same country based on the different agro-ecological zones and production system used. The maximum potential yield of modern rice varieties are about 13 t/ha in tropical environments and 15 t/ha in temperate regions as reported by Tran (1997).

An attempt was therefore undertaken to compare the tillering and yield parameters of some locally recommended rice varieties under two geographically diverse locations.

## MATERIALS AND METHODS

Two experiments were conducted with rice (*Oryza sativa* L.) of which Experiment 1 was done at Asian Institute of Technology, Thailand (14°04' N latitude and 100 °37' E longitude, 2 m altitude) and Experiment 2 was conducted at experimental station of the Cereal Institute in Kalochori, Thessaloniki, Greece (40°33' N latitude and 23°00' E longitude, 0 m altitude). The weather data of the study areas were presented in Figure 1. Experiment 1 was conducted during July to December, 1999 in an acid sulfate soil (Rangsit Series) with heavy clay (65%) in texture, pH 4.3, organic carbon 1.32%, total nitrogen 0.18%, total phosphorus 0.049%, total potassium 0.69% and bulk density 1.17 g/cc. The experiment was laid out in a split plot design having varieties as the main plot and planting dates as the sub plot with three replications. There were two varieties (KDML105 – photoperiod-sensitive and RD23 - photoperiod-insensitive) and five planting dates (July 15, July 30, August 14, August 30 and September 14). The unit plot size was 4 m × 3 m. Thirty-day old nursery seedlings were transplanted with 25 cm × 25 cm spacing having three seedlings/hill. At final land preparation, basal fertilizer which consisted of 30% of the N (75 kg/ha), P at the rate of 30 kg/ha and K at the rate of 16.6 kg/ha was applied and incorporated to the soil. The remaining N was top-dressed in two equal splits at active tillering and before panicle initiation.

Experiment 2 was conducted during May to October, 2000. The condition of the soil was silty loam (Aquic Xerotluvents) with a pH of 7.5 and 1.6% organic matter. Randomized complete block design was used with four replications. There were seven different locally recommended rice varieties viz. Olympiada, Selenio, San Andrea, Senia, Roxani, Ariete and Maratelli used in the experiment. The studied varieties came from Greece (Olympiada and Roxani), Italy (Ariete, Maratelli, San Andrea and Seleni) and Spain (Senia). All the varieties were japonica type except Olympiada, which was indica type. The planting of seeds in pots was done on May 4, 2000, while the transplanting of the seedlings on the field as recommendation (l/hill) at June 12, 2000. The spacing was maintained as 25 cm × 25 cm. The field was fertilized with 75 kg N/ha (in three installments), 17 kg P/ha and 31 kg K/ha that was applied by hand broadcasting. The first 25 kg/ha of N and the whole amount of phosphorus and potassium were applied before transplanting. The second application of N (30 kg/ha) was done at the early beginning of tillering stage and the third one (20 kg/ha) was done before panicle initiation.

Both experiments were kept free from weeds by hand weeding. The water level was maintained at about 10 cm (in depth) throughout the growth period, except 15 days before harvesting. Tiller numbers were counted at 15 and 45 days after transplanting (DAT) and at harvest from pre selected hills and finally averaged as their number/hill. Only those tillers having three or more leaves were considered for counting. Grain yield was determined from inner rows leaving boarders and adjusted to 14% moisture content. Grain moisture content was measured with a digital moister tester. Analysis of Variance (ANOVA) was done for the data using

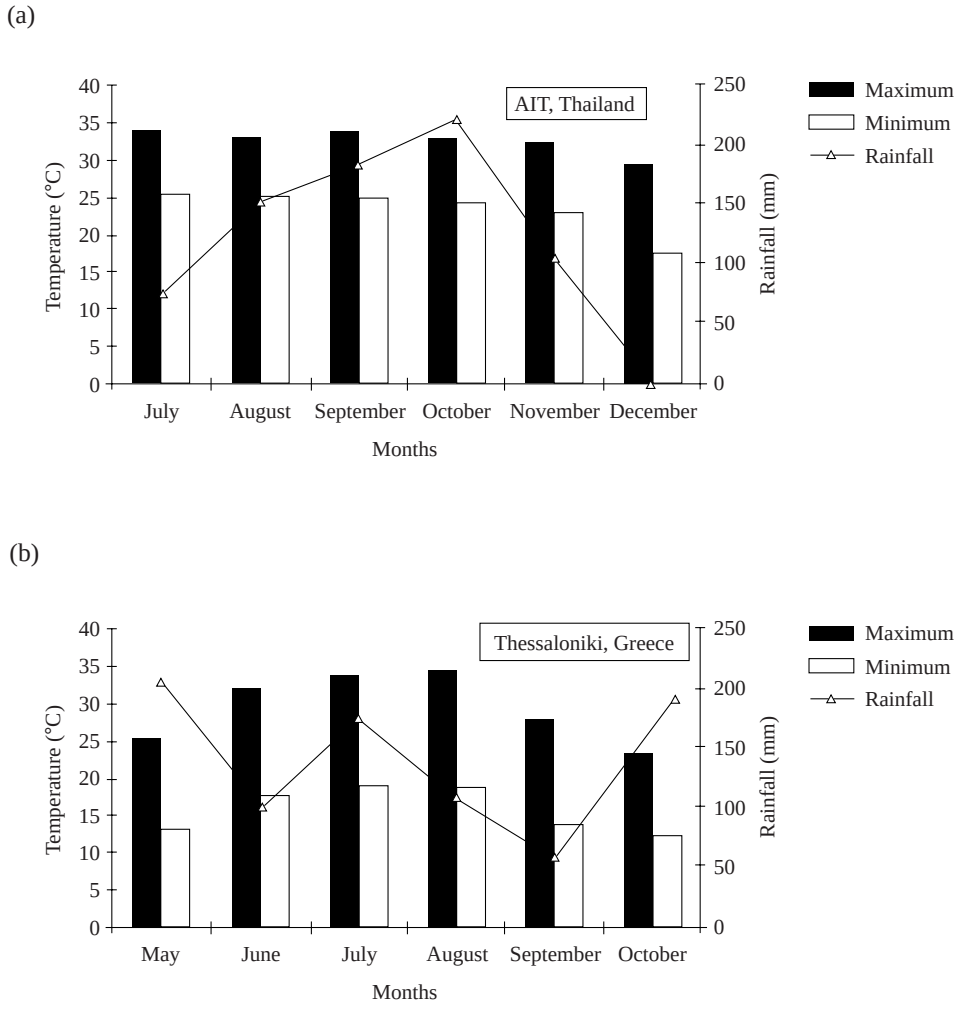


**Figure 1** Weather condition of (a) AIT, Thailand and (b) Thessaloniki, Greece during study period.

IRRISTAT statistical package. Treatment means were separated using Fisher's protected Least Significant Difference (LSD) test (P = 0.05) (Steel and Torrie, 1980).

## RESULTS AND DISCUSSION

### Tiller production

Experiment 1

At 15 DAT, the tiller number was significantly influenced by planting dates, but the same as varieties. Delayed planting showed higher tiller production compared with early planting. September 14 planting produced 16 tillers/hill that was same as August 30 planting (13 tillers/hill) and July 15 planting gave the lowest number (7) of tillers/hill. At 45 DAT, varieties and planting dates did not show any significant variations in tiller production. At harvest, though the variety RD23 showed 23% higher number of tillers compared with KDML105, they were statistically similar. For planting dates, September 14 planting gave significantly highest number of tillers (19 tillers/hill) compared with other planting dates (Table 1).

Experiment 2

The indica type rice variety Olympiada

showed significantly highest number of tillers/hill at 15 and 45 DAT as well as at harvest. Among the japonica type varieties, Selenio and Roxani produced higher number of tillers/hill (Table 2).

Above results showed that irrespective of variety, there were 27.5 tillers/hill found in tropical climate at 45 DAT (planting 3 seedlings/hill) whereas it was only 11.6 in temperate climate (planting single seedling/hill). The tiller mortality rate at harvest was 47% in tropical climate and only 5% in temperate climate. Though higher mortality rates, tropical rice varieties gave 32% higher number of tillers/hill at harvest compared to temperate rice. Tiller mortality takes place towards the maturity due to several reasons like nutrients, light, temperature, plant density and cultivar (Wu *et al*., 1998).

**Growth duration, yield attributes and yield**

Experiment 1

The variety and planting date as well as their interactions were found significant for flowering

**Table 1** Tillering pattern of transplanted rice at Asian Institute of Technology, Thailand.

| Treatment | Tiller production, no./hill | | | |
|---|---|---|---|---|
| | 15 DAT | 30 DAT | 45 DAT | At harvest |
| Variety | | | | |
| KDML105 | 10 | 20 | 23 | 13 |
| RD23 | 12 | 26 | 32 | 16 |
| LSD 0.05 | ns | ns | ns | ns |
| Planting date | | | | |
| July 15 | 7 | 15 | 26 | 14 |
| July 30 | 9 | 22 | 28 | 13 |
| August 14 | 11 | 27 | 27 | 13 |
| August 30 | 13 | 24 | 27 | 16 |
| September 14 | 16 | 28 | 29 | 19 |
| LSD 0.05 | 2.9 | 5.7 | ns | 3.1 |

**Table 2** Tillering pattern of transplanted rice at Thessaloniki, Greece.

| Treatment | Tiller production, no./hill | | | |
|---|---|---|---|---|
| | 15 DAT | 30 DAT | 45 DAT | At harvest |
| Olympiada | 6 | 19 | 20 | 20 |
| Selenio | 6 | 15 | 14 | 12 |
| San Andrea | 4 | 8 | 8 | 8 |
| Senia | 5 | 12 | 12 | 9 |
| Roxani | 3 | 10 | 12 | 12 |
| Ariete | 3 | 8 | 8 | 9 |
| Maratelli | 2 | 7 | 7 | 7 |
| LSD 0.05 | 0.9 | 1.6 | 1.7 | 1.9 |

and maturity dates. July 15 planting of photosensitive variety KDML105 needed significantly longer maturity duration (150 days) and the lowest duration (107 days) for the same variety planted on September. The maturity duration for the other variety RD23 ranges between 119 to 123 days. The duration of the vegetative phase increases greatly when the photoperiod sensitive varieties are subjected to photoperiods longer than the critical daylength. On the other hand, duration of the vegetative phase in weakly or non photoperiodic sensitive varieties does not increase much even if the varieties are subjected to long photoperiods (Anon., 1970). Therefore changes observed with different planting dates for KDML105 variety could be attributed to its photoperiod sensitivity. Planting date and variety had no influence on effective number of panicles/$m^2$. The variety KDML105 showed higher grain weight but lower HI compared with RD23. Delayed planting of both the varieties resulted in lower grain weight (Table 3). The higher grain weight of early planting might be due to the distribution of a greater portion of assimilates into developing panicles. Early planting and the variety KDML105 revealed higher grain weight than late planting and variety RD23 as reported by Biswas (2001).

The variety and planting date as well as their interaction showed significant influence on grain yield. The highest yield (4.9 t/ha) was given by July 15 planting of RD23 and the lowest yield (2.0 t/ha) from KDML105 planted on September (Figure 2a). The superior grain yield of RD23 than KDML105 was reported by Biswas and Salokhe (2001). The lower number of tillers per hill of KDML105 might be responsible for its lower grain yield.

**Experiment 2**

The variety Roxani and Olympiada needed longer duration compared with other varieties. The maturity duration of all the tested varieties of temperate climate in Greece were higher than the varieties planted on the tropical climate in Thailand. Approximately 30 days were needed to mature in tropical climate but it was about 60 days in temperate climate irrespective of their types. Tanaka and Vergara (1967) reported temperature

**Table 3** Growing period, panicles/$m^2$, 1000-grain weight and harvest index of transplanted rice at AIT, Thailand.

| Treatment | Flowering date | Maturity date | Panicles /$m^2$ | 1000-grain weight, g | Harvest index |
|---|---|---|---|---|---|
| Variety | | | | | |
| KDML105 | 97 | 125 | 201 | 26.3 | 0.27 |
| RD23 | 91 | 121 | 234 | 24.4 | 0.42 |
| LSD 0.05 | 0.8 | 1.7 | ns | 1.48 | 0.04 |
| Planting date | | | | | |
| July 15 | 108 | 136 | 201 | 26.6 | 0.36 |
| July 30 | 100 | 129 | 209 | 26.2 | 0.34 |
| August 14 | 91 | 120 | 205 | 25.9 | 0.36 |
| August 30 | 86 | 117 | 238 | 23.5 | 0.34 |
| September 14 | 85 | 114 | 233 | 24.5 | 0.34 |
| LSD 0.05 | 1.7 | 0.9 | ns | 1.15 | ns |

as an important factor that dominates for about 30 days from flowering to maturity in the tropics but for 65 days in temperate regions. The variety Olympiada (indica type) gave significantly higher panicle numbers/$m^2$ (319) and Maratelli, the lowest (118/$m^2$). The varieties Roxani and San Andrea resulted in heavier weights, whereas the 1000-grain weight of Olympiada was lowest (24.0 g). The higher grain weight of japonica type varieties over indica type was reported by Ntanos and Koutroubas (2000). The HI of Ariete and Olympiada were significantly higher and on the other hand lowest HI was revealed in Roxani and Maratelli (Table 4). The indica type variety Olympiada gave the highest grain yield (9.1 t/ha) and the lowest yield by Maratelli of japonica type variety (5.7 t/ha). Tran (1997) reported that because of the more favorable climatic conditions, such as long day-length, high solar radiation and low night temperature in the temperate regions and Mediterranean climate zones, rice yield is usually superior to that of rice grown in humid tropical regions. There were no variations of grain yield observed among japonica type varieties (Figure 2b).

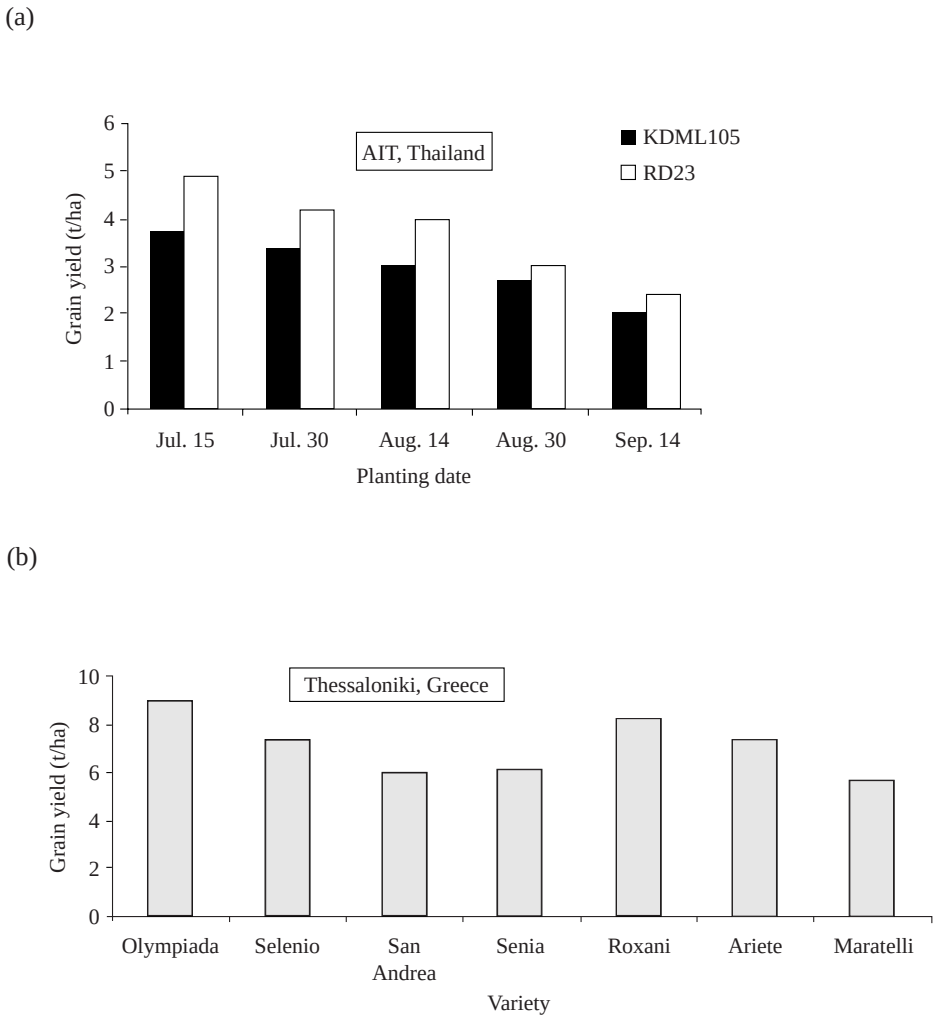


**Figure 2** Grain yield of rice at (a) AIT, Thailand and (b) Thessaloniki, Greece.

**Table 4** Growing period, panicles/m2, 1000-grain weight and harvest index of transplanted rice at Thessaloniki, Greece.

| Treatment | Flowering date | Maturity date | Panicles /$m^2$ | 1000-grain weight, g | Harvest index |
|---|---|---|---|---|---|
| Olympiada | 104 | 162 | 319 | 24.0 | 0.57 |
| Selenio | 91 | 146 | 189 | 28.0 | 0.54 |
| San Andrea | 91 | 146 | 122 | 39.2 | 0.50 |
| Senia | 94 | 151 | 144 | 36.0 | 0.51 |
| Roxani | 103 | 164 | 189 | 40.0 | 0.46 |
| Ariete | 94 | 149 | 150 | 35.0 | 0.57 |
| Maratelli | 92 | 147 | 118 | 34.0 | 0.45 |
| LSD 0.05 | - | - | 30.4 | 0.70 | 0.01 |

## CONCLUSION

Understanding the various components of grain yield and how to improve them will help in increasing rice production by raising the current yield level. Above results showed that rice grain yield in temperate region was much more higher compared to tropical region. Temperate rice had the advantage of longer maturity duration that was almost double than tropical rice. Grain weight of japonica rice was higher than tested indica rice. The HI of temperate rice was also higher compared to tropical rice. Maturity duration, tiller mortality rate, grain weight and harvest index were the important parameters those reflected on the better performance of rice in temperate region than tropical region.

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## LITERATURE CITED

Anonymous. 1970. Rice Production Manual (Revised Edition). International Rice Research Institute, Los Banos.

Anonymous. 2000. Feeding the world, Facts and Figures. http.//www.riceweb.org.

Biswas, P.K. and V.M. Salokhe. 2001. Effects of Planting date, variety, tiller separation and plant density on yield of transplanted rice. J. Agric. Sci., Cambridge. 137 (3) 279-287.

Biswas, P.K. 2001. Tiller dynamics and yield of parent and clonal plant of transplanted rice. Doctor of Technical Science dissertation. Asian Institute of Technology, Thailand.

Matsushima, S. 1980. Rice Cultivation for the Million. Japan Scientific Society Press, Tokyo.

Ntanos, D.A. and S.D. Koutroubas. 2000. Competition of Barnyardgrass with Rice Varieties. J. Agronomy and Crop Science 184 : 241-246.

Sreenivasan, P.S. 1985. Agro-climatology of rice in India, pp. 213-214. *In* Rice Research in India, ICAR, New Delhi.

Steel, R.G.D. and J.H. Torrie. 1980. Principles and Procedures of Statistics. A Biometrical

Approach (2nd edn.) McGraw-Hill, kugakusha Ltd. Tokyo, New Delhi. 633 p.

Tanaka, A. and B.S. Vergara. 1967. Growth habit and ripening of rice plants in relation to the environmental conditions in the Far East. Int. Rice Comm. Newsl. (Spec. issue) : 26-42.

Tran, D.V. 1997. World rice production main issues and technical possibilities. Cahiers Options Mediterraneennes 24(2) : 57-69.

Wu, G.W., L.T., Wilson, and A.M. McClung 1998. Contribution of rice tillers to dry matter accumulation and yield. Agron. J. 90(3) : 317-323.

Yoshida, S. 1981. Fundamentals of Rice Crop Science. International Rice Research Institute, Los Banos, Philippines. 269 p.

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# Arginine Enhancement of Cell Dissociation in Suspension-Cultured of Aromatic Rice Cells (*Oryza sativa* L. var Khao Dawk Mali 105)

**Nitsri Sangduen and Pranot Klamsomboon**


## ABSTRACT

Suspension culture of *Oryza sativa* L. var. Khao Dawk Mali 105 was initiated from embryogenic region of callus derived from mature seeds cultured in the modified $N_6$ medium supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.25 mg/l kinetin, 2 % sucrose, 5 mM proline and various nitrogen sources. Three types of suspension-cultured medium (SM) with the supplementation of inorganic nitrogen ($SM_1$), or organic nitrogen plus arginine ($SM_2$), or inorganic nitrogen plus arginine ($SM_3$) were used.The suspension-cultured of rice callus in the SM gave cytoplasmically dense cell and homogeneous aggregation cell size larger than 1 mm, while those in the $SM_2$ produced cytoplasmically clear cell and aggregated cell size smaller than 1 mm. The addition of arginine to SM with inorganic nitrogen ($SM_3$) seemed to combine the desirable characters of finely dispersed cell aggregate and cytoplasmically dense cell. It was revealed by scanning electron micrographs indicating a difference in intercellular layer while cultured in 1 mM arginine containing SM medium was less deeply embedded in intercellular layer than in arginine-free medium. A finely dispersed cell suspension was obtained from rice callus tissue in SM supplemented with 1mM arginine. These results suggested that the suspension-cultured medium with inorganic nitrogen plus arginine should be suitable for generating an established suspension cell line for selection experiment as well as protoplast isolation.


**Key words:** arginine, cell aggregation, suspension culture, rice, Khao Dawk Mali 105

## INTRODUCTION

A strategy in the field of plant cell culture is to isolate single cell without removal of cell walls, permitting selection of cells with wall and the control of cell growth and differentiation by synchronization of the culture. Because the single cells with walls may regenerate their normal walls more easily than those without walls (Hayashi *et al*., 1986) and grow normally to form clonal colonies and embryos. This objective should be useful for genetic manipulation via selection of transformants, mutation selection, superinduction model for gene expression (Bostock *et al*., 1999) and be a major contribution to plant biotechnology.

Hayashi and Yoshida (1988) reported that a combination of colchicine and galacturonan induced single cell separation in suspension-cultured soybean cells. The effects of colchicine and galacturonan were also observed in suspension-cultured carrot, tobacco and hibiscus cells. However, colchicine was inactive on suspension-cultured monocotyledonous cells, probably because the wall composition varies from that in dicotyledon (Burke *et al*., 1974). This indicates that the compounds and/or receptors binding cells in monocotyledons

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are not the same as those in dicotyledons. In addition, the level of cell aggregation in callus tissue of monocotyledons is usually much higher than that in dicotyledons.

There are various media favorable for suspension cultures include $N_6$ (Lee *et al*., 1989; Wang *et al*., 1989), AA (Abdullah *et al*., 1986; Toriyama *et al*., 1986; Yamada *et al*., 1986), $R_2$ (Fujimura *et al*., 1985; Kyozuka *et al*., 1988), LS (Abdullah *et al*., 1986) and $B_5$ (Fujimura *et al*., 1985). Amino acid medium (AA medium) which is composed of aspartic acid, arginine, glycine, and glutamine as a nitrogen source, has been known to evoke a finely dispersed cell suspension in suspension-cultured rice cells (Abdullah *et al*., 1986; Toriyama *et al*., 1986; Yamada *et al*., 1986). AA medium was effectively established suspension-cultured cells for japonica rice (Toriyama and Hinata, 1985; Yamada *et al*., 1986). For indica rice, a number of suspension-cultured media, such as $N_6$ medium (Lee *et al*., 1989), $R_2$ medium (Kyozuka *et al*., 1988) and AA medium (Datta *et al*., 1990; Sun *et al*., 1990) were used.

Toriyama and Hinata (1985) used AA medium for rice suspension culture to obtain protoplast at the yields of more than 90%; although only a few percent were obtained as protoplasts from an inorganic nitrogen medium such as $B_5$ or $N_6$ medium. Nevertheless, an inorganic medium is essential for the organogenesis and embryogenesis of rice cells (Toriyama and Hinata, 1985; Ozawa and Komamine, 1989) and for increasing cytoplasmic density of cultured cell (Yin *et al*., 1993). Hayashi *et al*. (1994) studied the effects of AA medium on the dissociation of rice callus tissue by examined a finely dispersed cell in suspension-cultured cells. Here we describe a simple procedure for the establishment of homogeneous suspension-cultured of aromatic rice cell by using medium containing arginine in conjunction with inorganic nitrogen or organic nitrogen.

## MATERIALS AND METHODS

### Establishment of suspension-cultured cells

Suspension-cultured cells were initiated from mature seed scutellum of rice [*Oryza sativa* L. cv. Khao Dawk Mali 105 (KDML 105)]. Cultures of friable embryogenic callus obtained in the modified $N_6$ medium containing 2% sucrose, 1.5 mg/l 2,4-D, 10 mM proline, 10 mg/l $AgNO_3$ and 32 mg/l cystein were collected from large cell- aggregated (<2 mm) and transferred to suspension-cultured medium (SM) comprised various nitrogen sources. Three modified SM formulae were applied. $SM_1$ medium contained 2830 mg/l $KNO_3$ and 46.3 mg/l $(NH_4)_2SO_4$ as an inorganic nitrogen source. $SM_2$ medium resembled AA medium, contained 6 mM glutamine, 2 mM aspartic acid, 1 mM arginine and 0.1 mM glycine as an organic nitrogen source. $SM_3$ medium contained 1 mM arginine, 2830 mg/l $KNO_3$ and 46.3 mg/l $(NH_4)_2SO_4$ as a nitrogen source. All media contained 1.5 mg/l 2,4-D and 0.25 mg/l kinetin as a plant hormone, 20 mg/l sucrose as a carbon source plus minerals and vitamins at the same level (Table 1). Suspension-cultured cells were placed on a gyratory shaker at 120 rpm and weekly subcultured at 1:4 dilution with fresh medium. The effect of nitrogen from inorganic or organic source was indicated by the qualities of suspension-cultured cells in terms of growth rate, cell viability, densely cytoplasmic and cell dispersion after inoculation for 2 weeks.

### Electron microscopic observation

Microcalli arising from $SM_1$, $SM_2$ and $SM_3$ were prefixed in 25% butyl alcohol, rinsed 3-4 times in phosphate buffer, dehydrated in an ethanol series (30-100% v/v), and critical-point dried. Dried specimens were coated with gold-palladium alloy and examined in a Joel JSM-35 cf scanning electron microscope at 15 kv.

### Cell aggregate sizing

Suspension-cultured cells were subcultured at 7 days interval. After subculturing for 21 days,

**Table 1** Composition of suspension culture medium (SM) consisted of the $N_6$ basal salt and various nitrogen sources for suspension-cultured rice cells of KDML105.

| Composition | Medium | | |
|---|---|---|---|
| | $SM_1$ | $SM_2$ | $SM_3$ |
| Micronutrients | $N_6$ | $N_6$ | $N_6$ |
| Macronutrients ($KNO_3$ and $(NH_4)_2SO_4$ not included) | $N_6$ | $N_6$ | $N_6$ |
| Vitamins | $N_6$ | $N_6$ | $N_6$ |
| Amino acid (mM) | | | |
| -Proline | 5.0 | 5.0 | 5.0 |
| -Glutamine | - | 6.0 | - |
| -Aspartic acid | - | 2.0 | - |
| -Arginine | - | 1.0 | 1.0 |
| -Glycine | - | 0.1 | - |
| $KNO_3$ (mg $l^{-1}$) | 2830.0 | - | 2830.0 |
| $(NH_4)_2SO_4$ (mg $l^{-1}$) | 46.3 | - | 46.3 |
| Sucrose (g $l^{-1}$) | 20.0 | 20.0 | 20.0 |
| 2,4-D (mg $l^{-1}$) | 1.5 | 1.5 | 1.5 |
| Kinetin (mg $l^{-1}$) | 0.25 | 0.25 | 0.25 |

cell-aggregated were sieved through stainless screen with 1000 μm pore size. They were placed on sterilized paper towels to remove water and then weight was recorded.

**Cell viability**

Cell viability was estimated by staining cells with FDA (Fluorescein diacetate) and Evans blue. The percentage of living cells stained with 0.01% FDA and of dead cells stained with 0.1% Evans blue were recorded.

**Monitoring cell growth**

Growth was monitored at 4 days after subculturing cells on the basis of fresh weight. A 10 ml aliquot of uniformly distributed suspension cells was vacuum filtered through whatman filter paper no. 4. Fresh weight of the retained cells was calculated from the weight of water soaked filter disc minus wet paper weight.

## RESULTS AND DISCUSSION

**Optimization of suspension-cultured medium**

Suspension-cultured cells were established 3-4 months later. Microcalli were transferred to the suspension-cultured medium (SM) for 3 weeks and supplemented with various nitrogen sources designated as $SM_1$ (resembled $N_6$ medium), $SM_2$ (resembled AA medium) and $SM_3$ (resembled $N_6$ medium plus arginine), respectively (Table 1). The differences of cell-aggregated size and cytoplasmic density of cells were visible. 78% of fine suspension cell-aggregated size less than 1 mm while culturing in the $SM_2$ medium was observed (Figure 1A). Culturing in the $SM_1$ medium, approximately 63% of large cell clumps (>1mm) was obtained (Figure 1B), however, the $SM_1$ medium seemed to increase densely cytoplasmic cells (Figure 1C). Small cell-aggregated in the $SM_2$ and $SM_3$ were easily dispersed and finally developed a fine suspension-

cultured cells (Figure 1 D, 1E and 1F). Arginine in the $SM_3$ medium indicated a favorable combination effect of the $SM_1$ and $SM_2$ media. It gave 73% of cell-aggregated developed to more finely and composed of densely cytoplasmic cells (Figure 2A and 2B).

Considering the significance of the nitrogen source in the culture medium, 3 modified media were devised to investigate the effect of nitrogen source on the quality of suspension-cultured cells (Table 2). The estimation of cell viability showed that cell-cultured with inorganic nitrogen ($KNO_3$ and $(NH_4)_2SO_4$) in the $SM_1$ and $SM_3$ media, revealed a higher amount of dead cells than those with amino acids treatment ($SM_2$). This suggested that inorganic nitrogen has a more toxic potential to the cultured cells. The nitrogen in culture medium has been known to play a key role on the qualities of rice cell in tissue culture (Koetje *et al*., 1989; Yin *et al*., 1993; Hayashi *et al*., 1994). High concentration of $NH_4^+$ is harmful to the cultured cells (Chu *et al*., 1975; Yamada *et al*., 1986). In addition, there were several reports suggesting that the difference in nitrogen ratio in medium-cultured causes the

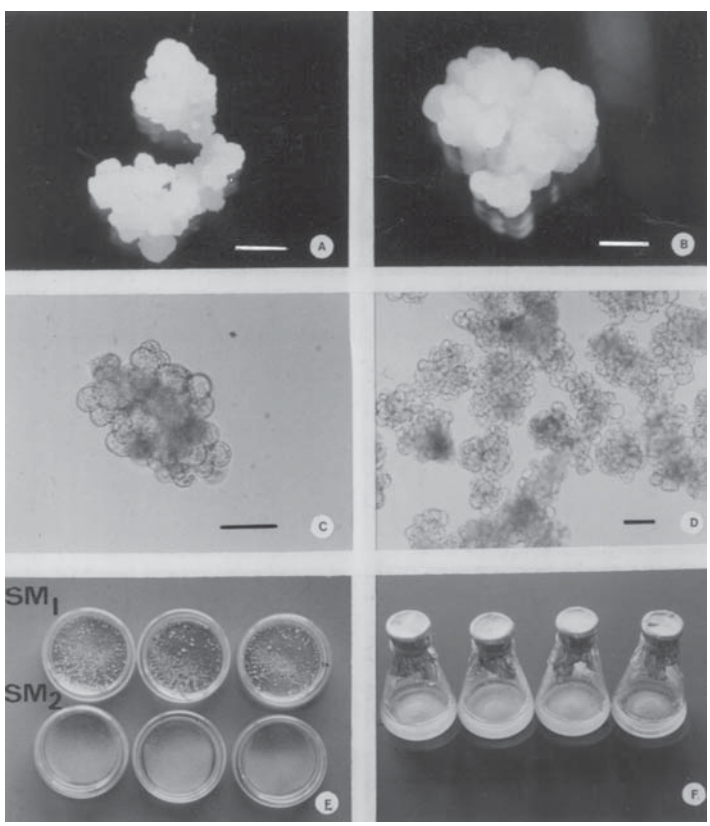


**Figure 1** The microcalli of rice cv. Khao Dawk Mali 105 proliferated in the $SM_2$ and $SM_3$ culture media composed of friable nodular-like structures (bar = 1 mm) (A) which was smaller than those appeared in the $SM_1$ medium (bar = 1 mm) (B). Most of cell aggregations in the $SM_1$ consisted of highly cytoplasmic cells (C). Small cell aggregates in the $SM_2$ and $SM_3$ easily dispersed and finally developed a fine suspension culture (bar = 0.05 mm) (D). Compare suspension-cultured rice cells in the $SM_1$ medium (upper) with the $SM_2$ medium (SM plus arginine, lower) (E). Fine cell suspensions consisted of small and densely cytoplasmic cell colonies in the $SM_3$ medium (F).

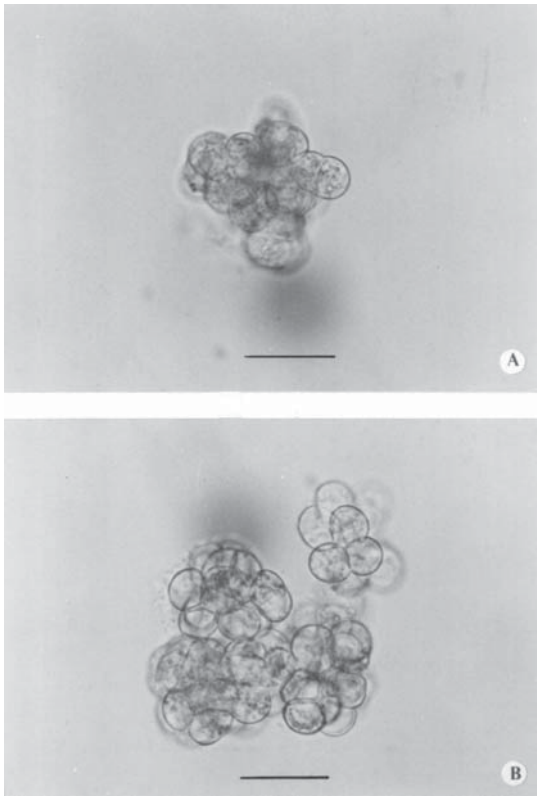


**Figure 2** The cell-aggregated of rice cv. Khao Dawk Mali 105 in the $N_6$ medium containing 1 mM arginine ($SM_3$) composed of more highly cytoplasmic cells (A) than those cultured in the amino acid-containing $N_6$ medium ($SM_2$) (B) (all bar = 0.05 mm).

deviation of nitrogen metabolism especially $NH_3$ induced towards polyamine which is an important source of $H_2O_2$ that provides to the generation and accumulation of high toxic radical OH° (Angelini and Federico, 1989; Le Dily *et al*., 1983). Therefore a dramatic increase in the number of dead cells in the presence of inorganic nitrogen may be due to the adverse effect of $NH_4^+$in the medium.

**Effects of arginine on rice cell-cultured**

From scanning electron micrographs, they indicated the variation in cell-aggregated size among various culture media. Microcalli grown in the $SM_2$ and $SM_3$ media were obtained. They composed of homogeneous nodular-like structures which were smaller than those appeared in the $SM_1$ medium (Figure 3A and 3 B). The appearance of cell-cultured also represented a distinct difference in the intercellular layer of cells. Cells cultured in the $SM_1$ medium (inorganic nitrogen medium) were more deeply embedded (Figure 3D) than those in the $SM_2$ medium (organic nitrogen plus arginine) and the $SM_3$ medium (inorganic nitrogen plus arginine) (Figure 3C). This indicated that cell-cultured in the $SM_1$ medium contained more wall material between cells than those in the $SM_2$ and $SM_3$ media. It also implied that arginine in medium-cultured plays a key role in inhibiting wall material accumulation

**Table 2** Effect of the organic nitrogen source in 3 SM media on the quality of suspension-cultured rice cells of KDML 105.

| Medium | *Quality of suspension-cultured rice cells | | | |
|---|---|---|---|---|
| | Growth rate | Dispersion | Densely cells cytoplasmic | Dead cell percentage |
| $SM_1$ | ++ | ++ | +++ | 17 |
| $SM_2$ | ++ | +++ | + | 5 |
| $SM_3$ | ++ | +++ | +++ | 15 |

* + = low; ++ = medium and +++ = high
$SM_1$ = inorganic nitrogen medium
$SM_2$ = organic nitrogen plus arginine
$SM_3$ = inorganic nitrogen plus arginine

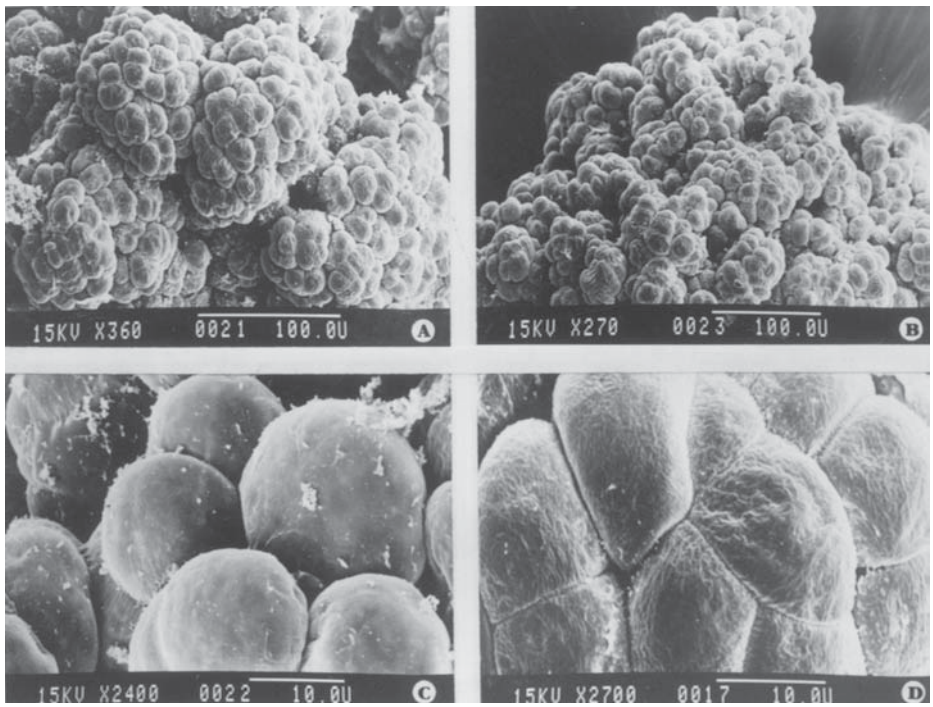


**Figure 3** Scanning electron micrographs of embryogenic calli of rice cv. Khao Dawk Mali 105, cultured in the $SM_1$ medium (A) homogeneously composed of nodular structures which were larger than those appeared after culturing in the $SM_2$ and $SM_3$ media (B). The cultured cells in the $SM_2$ and $SM_3$ media (C) revealed less deeply embedded in intercellular of cells than those in the $SM_1$ medium (D).

which may support the explanation for easily dispersed small aggregated cell in the $SM_2$ and $SM_3$ media. The ability of arginine to interfere with the accumulation of wall material was reported and suggested by Hayashi *et al*. (1994) that arginine in AA medium is metabolized in a rice cell to form urea and that urea solubilizes insoluble proteins of polysaccharides by inhibiting the formation of hydrogen bond.

Cells observed on both media-cultured with inorganic nitrogen ($SM_1$ and $SM_3$) were small, round and densely cytoplasmic which also released round and densely cytoplasmic protoplasts. This suggested that inorganic nitrogens, $KNO_3$ and $(NH_4)_2SO_4$ were needed to increase cytoplasmic density of cell-cultured but they tend to form large cell clumps. Cell-aggregated in the $SM_1$ medium was decreased by several subcultures with the $SM_2$ medium. This implied that the $SM_2$ medium induces the dissociation of cell aggregates. It is likely that the dissociation observed in the $SM_2$ medium localized on the surface of cell-aggregated, where cell division occurs. This result agrees with the observation in AA medium (Hayashi *et al*., 1994). The addition of arginine could evoke cell dispersion the same as a combination of amino acid in the AA medium and appears to be useful for dissociating cell-aggregated. Its effect was also observed in suspension-cultured barley and rice cells (Hayashi *et al*., 1994) and vetiver cells (unpublished data). The established cell suspension could increase the efficiency of electroporation, biolistic and cell selection in monocotyledonous plants. However, we have not succeeded in separating cell-aggregated

into a single cell and the aggregated size is approximately 700-1000 mm, which is similar to that in cell suspension of dicotyledons such as soybeans, carrot and poplar.

In conclusion, arginine addition showed to assist $N_6$ medium in revealing the most prominent appearances to cell-cultured in terms of cell dispersion and increasing densely cytoplasmic cells which lead to a high density of healthy protoplasts. The optimal suspension-cultured medium of rice cv. Khao Dawk Mali 105 is modified $N_6$ medium supplemented with 1 mM arginine. From Table 2, the following points could be summarized:

-The addition of $KNO_3$ and $(NH_4)_2SO_4$ to suspension-cultured medium increased densely cytoplasmic cell-cultured. At the same time, large cell clumps were also formed which made protoplast isolation become more difficult.

-The addition of inorganic nitrogen was harmful to cell-cultured, this may due to an adverse effect of $NH_4^+$.

-The addition of amino acids to suspension-cultured medium evoked cell dispersion.

-The addition of 1 mM arginine played a key role in promoting cell dissociation.

Yin *et al.* (1993) considered the significance of nitrogen source in MS medium for cell-cultured indica rice and found that the ratio of $KNO_3$/ $NH_4NO_3$ plays a critical role in determining the quality of suspension-cultured cells. In their cases, the combination of MS with AA medium was found to be the most effective for maintaining suspension-cultured cells.

Based on this study, $N_6$ basal medium with the presence of 1 mM arginine was suitable for developing rice cv. Khao Dawk Mali 105 cell-cultured used for protoplast isolation and genetic manipulation. The cell line which was established by this system exhibited a rapid growth rate, uniformly small cell-cultured, tiny, round and densely cytoplasmic cells. These cultured procedure should be applied to use with other monocotyledonous plants.

## ACKNOWLEDGEMENT

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## LITERATURE CITED

Abdullah, R., E.C. Cocking, and J.A. Thompson. 1986. Efficient plant regeneration from rice protoplast through somatic embryogenesis. Bio/ Technol 4 : 1087-1090.

Angelini, R. and R. Federico .1989. Histochemical evidence of polyamine oxidation and generation of hydrogen peroxide in the cell wall. J. Plant Physiol. 135 : 212-217.

Bostock, R.M., S. Gerttulal, and R.S. Quatrano. 1999. Superinduction of the Em gene in rice suspension cells in the presence of ABA and cycloheximide. Plant Cell Rep. 18 : 848-852.

Burke, D., P. Kaufman, M. McNeil, and P. Albershein. 1974. The structure of plant cell walls. Plant Physiol. 54 : 109-115.

Chu, C.C., C.S. Wang, C.S. Sun, C. Hsu, K.C. Yin, C.Y. Chu, and F.Y. Bi. 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen source. Sci. Sin. 16 : 659-668.

Datta, S., K. Datta, and I. Potrykus. 1990. Fertile indica rice plants regenerated from protoplasts isolated from microspore derived cell suspension. Plant Cell Rep. 9 : 253-256.

Fujimura, T., M. Sakurai, H. Akagi, T. Negishi, and A. Hirose. 1985. Regeneration of rice plants from protoplasts. Plant Tiss. Cult. Lett. 2 : 74-75.

Hayashi, T., D.R. Polonenko, A. Camirand, and G. Melchers. 1986. Pea xyloglucan and cellulose. Plant Physiol. 82 : 301-306.

Hayashi, T. and K. Yoshida. 1988. Cell expansion and single-cell separation induced by colchicine in suspension-cultured soybean cells. Proc. Natl. Acad. Sci. 85 : 2618-2622.

Hayashi, T., C. Ohsumi, Y. Kato, H. Yamanouchi, K. Toriyama, and K. Hinata. 1994. Effect of amino acid medium on cell aggregation in suspension-cultured rice cells. Biosci. Biotech. Biochem. 58 : 256-260.

Koetje, D.S., H.D. Grimes, W. Yi- Chang, and T.K. Hodges. 1989. Regeneration of indica rice (*Oryza sativa* L.) from primary callus derived from immature embryos. J. Plant Physiol. 135 : 184-190.

Kyozuka, J., E. Otoo, and K. Shimamoto. 1988. Plant regeneration from protoplasts of indica rice : genotypic difference in culture response. Theor. Appl. Genet. 76 : 887-890.

Le Dily, F., C. Huault, T. Gasper, and J.P. Billard. 1983. Does altered nitrogen metabolism and $H_2O_2$ accumulation explain the vitrified status of the fully habituated callus of *Beta vulgaris* (L.) Plant Cell Tiss. Org. Cult. 35 : 69-74.

Lee, L., R.E. Schroll, H.D. Grimes, and T.K. Hodges. 1989. Plant regeneration from indica rice (*Oryza sativa* L.) protoplasts. Planta178 : 325-333.

Ozawa, K. and A. Komamine. 1989. Establishment of a system of high-frequency embryogenesis from long-term cell suspension cultures of rice (*Oryza sativa* L.). Theor. Appl. Genet. 77 : 205-211.

Sun, B.L., X.H. Li, Y.R. Sun, S.M. Zhao, and J. Chen. 1990. High dividing frequency of protoplasts and regeneration of plants in indica rice. Chinese Sci. Bull. 35 : 427-430.

Toriyama, K. and K. Hinata. 1985. Cell suspension and protoplast culture of rice. Plant Sci. 41 : 179-183.

Toriyama, K., K. Hinata, and T. Sasaki. 1986. Haploid and diploid plant regeneration from protoplasts of anther callus in rice. Theor. Appl. Genet. 73 : 16-19.

Yin, Y., S. Li, Y. Chen, H. Guo, W. Tian, Y. Chen, and L. Li. 1993. Fertile plant regeneration from suspension culture derived protoplast of indica type rice (*Oryza sativa* L.). Plant Cell Tiss. Org. Cult. 32 : 61-68.

Wang, D., P.D. Miller, and M.R. Sondah. 1989. Plant regeneration from protoplasts of indica type rice and CMS rice. Plant Cell Rep. 8 : 329-332.

Yamada, Y., Z.Q. Yang, and K.T. Tang. 1986. Plant regeneration from protoplast derived callus or rice (*Oryza sativa* L.) Plant Cell Rep. 4 : 85-88.

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# Function of the Promoter of *PSPAL2*, a Pea Defensive Gene Encoding Phenylalanine Ammonia-lyase

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## ABSTRACT

The phenylalanine ammonia-lyase is a key enzyme in phenylpropanoid synthesis, a pathway for the biosynthesis of a wide range of natural products which play key roles in plant development and protection against environmental stresses including the structural polymer lignin, flavonoids (anthocyanin pigments and UV protectants), isoflavonoids and phytoalexins. In this study, *PSPAL2*, a member of pea *PAL* gene family was determined and structurally characterized. The structure of *PSPAL2* was divided into two exons by the single intron of 90 bp. In *PSPAL2*, some putative *cis*-regulatory elements of box I, box II, and box IV conserved among the promoter of several genes involved in the phenylpropanoid pathway and the retrotransposon-like sequences were found in the 5′-upstream region of *PSPAL2* promoter.

To discriminate the function of *PSPAL2* promoter, the expression of pea *PSPAL2* retrotransposon-like sequence in the region between –406 and –2196 and the three types of sequentially deleted chimeric promoter constructs designated as *PSPAL2-FLd1*, *PSPAL2-FLd2* and *PSPAL2-FLd3* in transgenic tobacco during developmental growth and upon fungal ingression were demonstrated. The histochemical GUS expression in young seedlings and mature plants were found in tissue and specific organs (roots, stems, leaves, flower organs and anthers). Moreover, the levels of GUS activities in tissues of transgenic plants depending on the 5′-upstream region of *PSPAL2* promoter were also determined. Extremely low GUS expression was observed in healthy or undisturbed mature leaves. However, the *PSPAL2* promoter activated in the leaves of transgenic tobacco plants after transferring to the greenhouse was induced upon fungal ingression, especially when the leaves were inoculated with *P. capsici* and incubated at 22-24°C for 48 hr. Marked expression was detected at the HR area surrounding the inoculation site of the transformant of *PSPAL2-FL*. Extremely low GUS expression was observed in the transformant of *PSPAL2-FLd3*. The results demonstrated that the region from –966 to –2196 of *PSPAL2* promoter played a crucial role in the regulation of induction of GUS activities in the mature leaves of transgenic tobacco plants. It was thus clear that the 5′-upstream region between +110 to –594 was insufficient to establish the full capacity of defense gene response under stress even though this region contained important box sequences such as box I, box II and box IV.


**Key words:** promoter, pea, defensive gene, plenylalanine ammonia-lyase


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## INTRODUCTION

Phenylalanine ammonia-lyase (PAL,EC 4.3.1.5) is one of the key enzymes that are essential for defense reactions in plants. PAL catalyzes the first step of the general phenylpropanoid metabolism, the deamination of L-phenylalanine to produce cinnamic acid and regulates subsequent reactions leading to the production of a wide variety of natural products. These essential roles of isoflavonoids in plant development and defense responses against various environmental stresses, including pathogen ingression, and the structural and regulatory function of phenylalanine ammonia-lyase (*PAL*) genes, have been extensively studied in various higher plants such as alfalfa (Gowri *et al*., 1991), *Arabidopsis* (Mauch-Mani and Slusarenko, 1996., Ohl *et al*., 1990 and Wanner *et al*., 1995), carrot (Takeda *et al*., 1997), French bean (Sablowski *et al*., 1995 and Shafflebottom *et al*., 1993), *Lithospermum erythrorhizon* (Yazaki *et al*., 1997), parsley (Lois *et al*., 1989), pea (Yamada *et al*., 1992), pine (Whetten *et. al*., 1992), poplar (Subramanian *et al*., 1993), rice (Minami *et al*., 1989 and Zhu *et al*., 1995), sweet potato (Tanaka *et al*., 1989), tobacco (Fukazawa-Akada *et al*., 1996), and tomato (Lee *et al*., 1992).

In pea, genes encoding phenylalanine ammonia-lyase (*PSPAL*) from a small multigene family comprised of at least three members (Kawamata *et al*., 1997). Two members of a *PSPAL* family, designated as *PSPAL1* and *PSPAL2*, were cloned and characterized (Yamada *et al*., 1994). Both *PSPAL1* and *PSPAL2* were induced by fungal elicitors but suppressed by fungal suppressors produced by a pea pathogen, *Mycosphaerella pinodes* (Berk. Et Blox.) Stone, a fungus pathogenic on pea (Yamada *et al*., 1992)

Recently, the temporal and spatial expression pattern of the *PSPAL1* promoter was characterized in response to pathogen ingreses and wounding in transgenic tobacco plants (Kawamata *et al*., 1997). The promoter dissection of *PSPAL2* has been performed in a transient transforming assay by electroporation of pea protoplasts, then determining their responses to environmental stimuli such as fungal elicitors, supprescin or UV light irradiation (Yamada *et al*., 1992). Functional analysis of 5′-nested deletions showed that an enhancer-like element is located in the TATA-distal region from – 2196 to –406, in which the consensus sequence motifs known as box II and box IV (Lois *et al.*, 1989 and Takeda *et al.*, 1997) were presented in close proximity.

The objective of the present study was to elucidate the function of the promoter of *PSPAL*, a pea defensive gene encoding phenylalanine ammonia-lyase.

## MATERIALS AND METHODS

### 1. Construction of chimeric genes

The pea *PSPAL2* full length promoter (*PSPAL2-FL*, -2196 to +110) and three deleted chimeric promoters designated as *PSPAL2-FLd1* (-1486 to +110), *PSPAL2-FLd2* (-966 to +110) and *PSPAL2-FLd3* (-594 to +110) had been constructed into CAT reporter gene for analyzing the transient expression in electroporated protoplasts (Yamada *et al*., 1994). To investigate the expression of *PSPAL2* promoters in transgenic tobacco plants, the *PSPAL2-FL* promoter and the three selected deletion constructed promoters in the CAT reporter gene were amplified by PCR using specific primers and subcloned into pBluescriptII KS (+) at *Hin*d III and *Bam* HI sites, then ligated with the GUS reporter gene in pBI101.2 (Figure 1). The *PSPAL2 – GUS* chimeric constructed promoters were purified and transformed into *Agrobacterium tumefaciens* LBA4404 using the freeze-thaw method (Holster *et al*., 1978)

### 2. Preparation of sterile tobacco plants

*Nicotiana tabaccum* was used in the transformation experiments. Seeds were surface-sterilized in 5% hypochlorite for 10 min, followed

by soaking in 70% ethanol for 10 min, and rinsed in sterilized water. Sterilized seed were germinated in a sterile petri dish containing the MS medium (Murashige and Skoog, 1962)

**3. Leaf disk transformation**

Leaf disk of the sterilized tobacco was co-cultured in MS medium for 15-30 min with 1-5 × $10^8$ cell/ml of *A. tumefaciens* LBA4404 (McCormick *et al*., 1986) carrying the specified chimeric genes. After drying on a sterilized Whatman 3 MM filter paper to remove excess bacteria, inoculated leaves were placed abaxial surface down on MS medium containing 1.0 mg/l of NAA and 1.0 mg/l of BA. The inoculated leaves were incubated at 22 - 24°C for 2 days, and then, transferred onto MS-selective medium containing the same concentration of NAA, BA, kanamycin (100 mg/l) and claforan (500 mg/l) until shoots formed. After 2-3 weeks of incubation, adventitious shoots were transferred to a new MS medium until roots were formed (Gelvin *et al*., 1990). All plant materials were incubated at 25-28°C under 16-hr light (150 m E/$m^2$/s), 8-hr dark conditions. Young seedlings were transplanted into soil and incubated in a greenhouse.

**4. GUS histochemical assay and PCR analysis**

**GUS histochemical assay**, mature leaves were fixed by soaking in 1% formaldehyde in 50 mM sodiumphosphate buffer (pH 7.0) for 10 min and rinsed three times with 50 mM sodiumphosphate buffer (pH 7.0). Then, they were incubated in X-Gluc solution (1.0 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronide, in 50 mM sodiumphosphate buffer (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA) at 37°C for overnight as described by Jefferson *et al*. (1987). Staining and fixing of X-Gluc solution into tissues was facilitated by vacuum infiltration. Staining reactions were stopped by transferring the tissues into 70% ethanol. GUS-PCR analysis of the genomic DNA extraction for GUS-PCR was performed to confirm the integration of *PSPAL2-GUS* fusion in to the genome of transgenic tobacco plants as described by Hosaka (1994). GUS-PCR was amplified using GUS-specific primers [primer I (upstream) 20 mer : 5′-TAC GTA TCA CCG TTT GTG TG-3′; primer II (down stream) 20 mer : 5′-GTA ATA ACG GTT CAG GCA CA-3′]. DNA manipulation was performed according to the standard methods described by Sambrook *et al*. (1989) or as specified by the manufacturer's protocols.

## RESULTS AND DISCUSSION

**1. GUS expression in transgenic tobacco plants during developmental growth**

Basal GUS expression of *PSPAL2-FL* promoter was observed in tissues of roots, stems and leaves during developmental growth of young seedlings before transplanting to soil. The histochemical GUS expression in young seedlings and mature plants seemed to be conserved in tissue and specific organs as observed in *PSPAL1* (Kawamata *et al*., 1997) and bean *PAL2* (Shufflebottom *et al*., 1993 and Hatton *et al*., 1995). *PSPAL2* promoter showed strong GUS expression in xylem, phloem elements of the vascular and endodermal tissues of lateral roots (Figure 2), stems (Figure 3) and vascular tissues in the veins of leaves, leaf tips, and petrioles (Figure 4). Strong GUS activity was also found in flower organs, especially in the pigment parts of petals, sepal tips, gland cells of trichomes (Figure 5) and anthers (Figure 6). However, the GUS expression could not be observed in root hairs as in *PSPAL1*. Moreover, the level of GUS activities in the organs of transgenic plants significantly declined in corresponding to the deleted 5′-upstream chimeric *PSPAL2* promoters from –2196 to –594, especially in roots, vascular tissues in the veins of leaves and stems as shown in Figure 7.

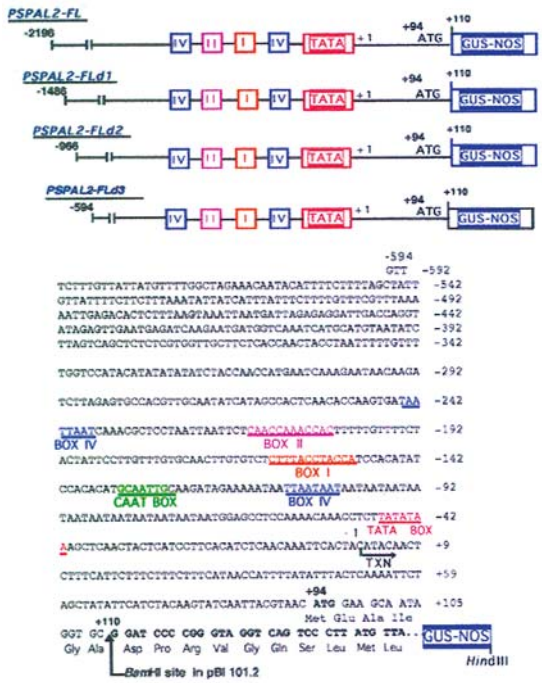


**Figure 1** Schematic representation of *PSPAL2* promoter-*GUS-NOS* fusion. Full length (*PSPAL2-FL*) promoter-sequence and the three deleted chimeric constructed promoter (*PSPAL-FLd1*, *PSPAL-FLd2* and *PSPAL-FLd3*) being fused to GUS in pBI101 (Toyobo Inc, Kyoto, Japan) at *Hin*dIII and *Bam*HI sites. The nucleotide sequences of all chimeric constructed promoter, the positions where *GUS-NOS* cassette being connected, a putative translation initiation codon and transcription start site (TXN) being indicated. Putative TATA box, CAAT box and characteristic sequences motifs such as box I, II and IV in 5/-upstream region relative to the transcriptional start site being denoted by colors with those for *PSPAL2* beneath them. The numbers on top denoing the nucleotide position from the transcriptional start point. *GUS* and *NOS* : b-glucuronidase gene in pBI 101.2 and *Agrobacterium tumefaciens* nopaline synthase gene terminator sequence.

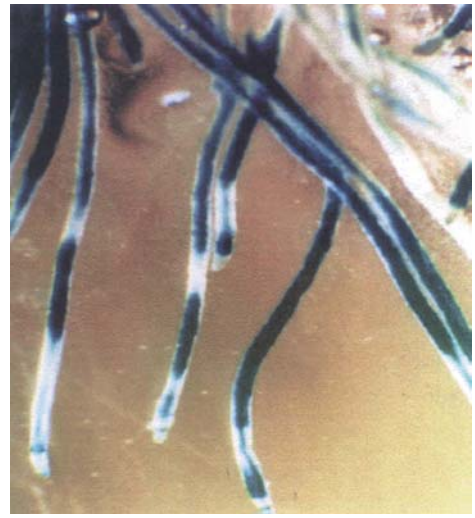

**Figure 2** Histochemical localization of GUS activity in young roots of transgenic tobacco plants containing promoter of *PSPAL2-FL-GUS-NOS* construct.

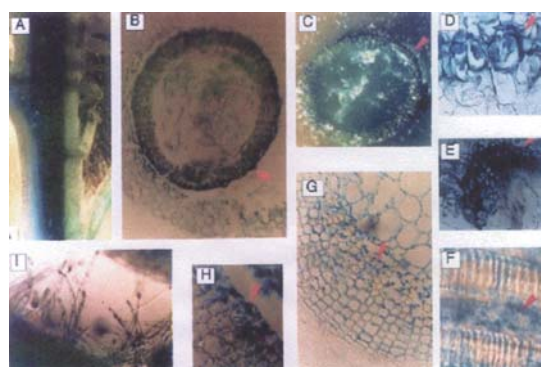


**Figure 3** Histochemical localization of GUS activity in young stems both transverse and cross-sections of transgenic tobacco plants containing promoter of *PSPAL2-FL-GUS -NOS* construct.
A : vascular tissue of stem; B,C : cross-section of stem exhibiting high levels of GUS activity localized in the xylem rays (arrow) and in the internal and external phloem tissue; D,E,G : closer view to the cross-section of stem; F,H: transverse-section of stem; I : stem trichomes

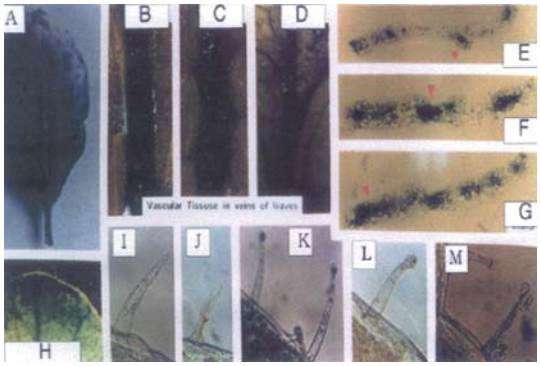


**Figure 4** Histochemical localization of GUS activity in young leaves of transgenic tobacco plants containing promoter of *PSPAL2-FL-GUS-NOS* construct.
A : whole leaf; B-D : vascular in veins of leaves; E-G : transverse-section in leaf veins H : leaf tip; I-M : leaf trichomes

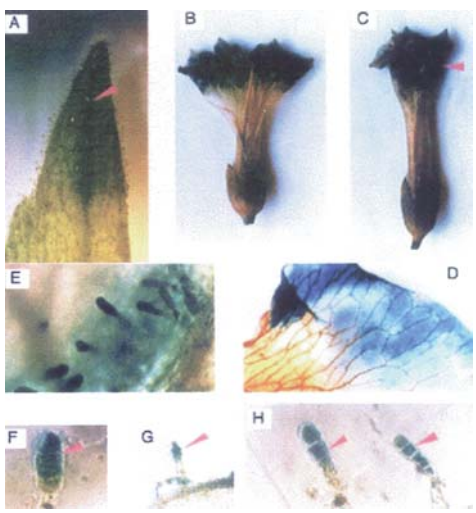


**Figure 5** Histochemical localization of GUS activity in flower organ of transgenic tobacco plants containing promoter of *PSPAL2-FL-GUS-NOS* construct.
A: sepal tip; B,C: whole flower with high levels of GUS activity localized in the petal; D: a portion of a petal, showing GUS activity in the pigmented rim; E-H: gland cells of trichomes

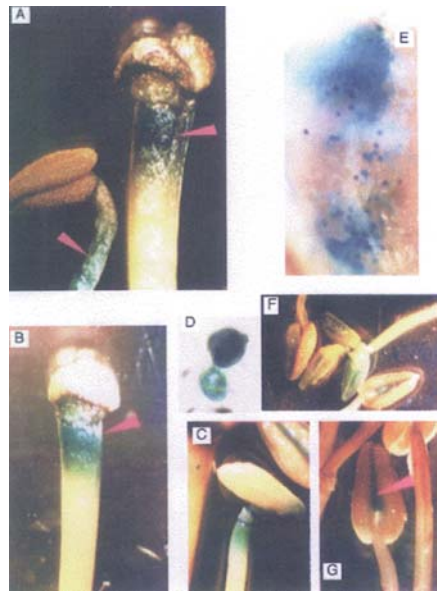


**Figure 6** Histochemical localization of GUS activity anther of transgenic tobacco plants containing promoter of *PSPAL2-FL-GUS-NOS* promoter construct.
A-C :filaments; D-E :pollens and anther wall ; F-G : anthers

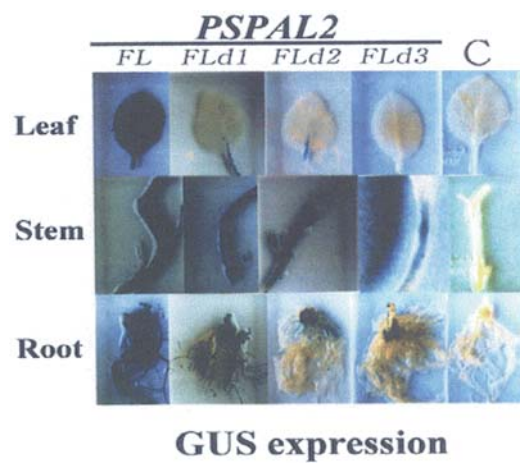


**Figure 7** GUS expression of the pea *PSPAL2* full length promoter (*PSPAL2-FL*, -2196 to +110) and three deleted chimeric promoters designated as *PSPAL2-FLd1* (-1486 to +110), *PSPAL2-FLd2* (-966 to +110) and *PSPAL2-FLd3* (-594 to +110) in leaves, stems and roots of transgenic tobacco seedling during developmental growth.

**2. GUS expression in transgenic tobacco plants upon fungal ingression and injuries**

*Histochemical GUS expression*

The leaves of transgenic tobacco plants carrying *PSPAL2-FL* were inoculated with *Phytophthora nicotianae*, a tobacco pathogenic fungi (P) or with *P. capsici*, a nonpathogen (NP). The results showed that histochemically detected GUS expression in transgenic tobacco plants was highest 48 hr after inoculation with *P. capsici* (Figure 8B) and incubated at 22-24°C. Then GUS expression gradually faded away at the hypersensitive response (HR) area around the inoculation site after 72 hr of incubation (Figure 8C), where a plant defense system had presumably been established for blocking fungal invasion, in a manner similar to the expression of *PSPAL1* promoter (Kawamata *et al*., 1997.) The pattern of GUS expression after inoculation with a pathogen was not so clear as that observed in the necrotic area after inoculation with a nonpathogen and the expression never faded until the whole leaf was colonized.

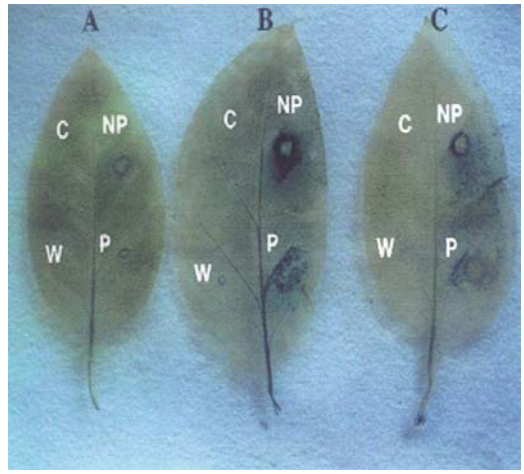


**Figure 8** Histochemical GUS expression of *PSPAL2-FL* promoter upon wounding (W) and fungal ingression with pathogen (P, *P. nicotianae*) and non pathogen (NP,*P. capsici*) in transgenic tobacco leaves. Control treatment is shown as C.
A: 24 hr after inoculation or wounding
B: 48 hr after inoculation or wounding
C: 72 hr after inoculation or wounding

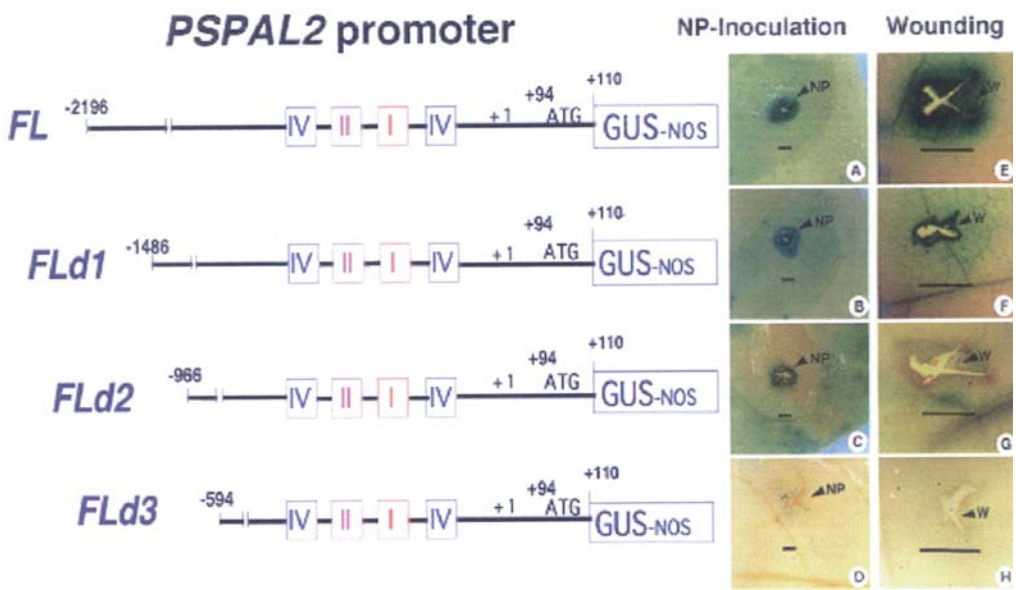


**Figure 9** Expression of full (*PSPAL2-FL* : A,E) and deleted *PSPAL2* promoters (*PSPAL2-FLd1* : B,F) *PSPAL2-FLd2* : C,G; *PSPAL2-FLd3* : D,H) at 48 hr after wounding (W) and inoculation with a nonpathogen (NP,*P. capsici*) in transgenic tobacco leaves. Bars equal 1 mm.

**Expression of deleted *PSPAL2* promoters upon fungal infection**

To discriminate the expression of the deleted *PSPAL2* promoters upon fungal infection, mature leaves of transgenic tobacco plants carrying the *PSPAL2* chimeric constructed promoters were inoculated with *P. capsici*. This nonpathogenic fungus induced a very large, clear GUS expression zone around the site of the hypersensitive response (HR), especially in the transformants of *PSPAL2-FL* (Figure 9A) and *PSPAL-FLd1* (Figure 9B) at 48 hr after the inoculation. However, the responses to fungi infection were not high in the transformants of *PSPAL2-Fld2* (Figure 9C); the expression zone was restricted to the area around the inoculation site. GUS expression in the transformant of *PSPAL-FLd3* did not clearly appear (Figure 9D). The results showed that the pea *PSPAL2* promoter expression in transgenic tobacco was strongly affected by the sequences in 5/-upstream region as previously shown with the transient CAT expression in electroporated pea protoplasts (Yamada *et al*., 1994). The functional analysis of 5/-nested deletions of *PSPAL2* promoter in electroporated protoplasts showed that an enhancer-like element located at the TATA-distal region from –2196 to –406, and this promoter was activated by fungal elicitor from *M. pinodes* and partially suppressed by the suppressor from the same fungus (Yamada *et al*., 1994). Interestingly, the GUS expression of the constructs upon fungal ingression in transgenic tobacco leaves demonstrated the induction of positive defense responses at the sites of infection at different levels depending on the additional sequences of the 5/-upstream region. Because a very low level of the GUS expression was observed in the transformants of *PSPAL2-Fld3* compared to *PSPAL2-FL*, the lower level of GUS expression is not due to a position effect of the integration of the chimeric promoters. This phenomenon might be explained by the lack of some active elements needed for the regulation of the *PSPAL2* promoter. These elements were likely to span from –966 to –2196. Moreover, the GUS expression was observed in mature transgenic tobacco leaves being injured by sterile razor blade. Intense blue colorations were observed at wounding sites (W) and restricted adjacent areas. The GUS expression of the deleted *PSPAL2-FL* promoters after wounding also declined from a high level to an extremely low one as the deletion was extended, in a manner similar to the expression after fungal ingression (Figure 9E-H).

The results demonstrated that the region from –966 to –2196 of *PSPAL2* promoter played a crucial role in the regulation of induction of GUS activities in the mature leaves of transgenic tobacco plants. It was thus clear that the 5/-upstream region between +110 to –594 was insufficient to establish the full capacity of defense gene response under stress even though this region contained important box sequences such as box I, box II and box IV. The additional sequences from –594 to –2196 that include the region of retrotransposon-like sequence are obviously necessary for the functional expression of the gene not only during developmental growth but also in response to fungal ingress and injuries.

## LITERATURE CITED

Fukazawa-Akada, T., S. Kung, and J.C. Watson. 1996. Phenylalanine ammonia-lyase gene structure, expression, and evolution in Nicotiana. Plant Mol. Biol. 30 : 711-722.

Gelvin, S.B., R.A. Schilperoort, and D.P.S. Verma. 1990. Plant Molecular Biology Manual. Kluwer Academic Publishers, NY.

Gowri, G., N.L. Paiva, and R.A. Dixon. 1991. Stress responses in alfalfa (*Medicago sativa*). 12. Sequence analysis of phenylalanine ammonia-lyase (PAL) cDNA clones and appearance of PAL transcripts in elicitor treated cell cultures and developing plants. Plant Mol. Biol. 17 : 415-426.

Hatton, D., R. Sablowski, M.H. Yung, C. Smith, W. Schuch, and M. Bevan. 1995. Two classes of *cis* sequences contribute to tissue-specific

expression of a *PAL2* promoter in transgenic tobacco. Plant J. 7 : 859-876.

Holsters, M., D. de Waele, A. Depicker, E. Messens, N. van Montagu, and J. Schell. 1978. Transfection and transformation of *Agrobacterium tumefaciens.* Mol. Gen. Genet. 163 : 181-187.

Hosaka, K. 1994. Current RAPD Technology. Academic booklet, Kobe University, Kobe.

Jefferson, R A., T.A. Kavanagh, and M.W. Bevan. 1987. GUS fusion: β-glucuronidase as a sensitive and versatile gene marker in higher plants. EMBO J. 6 : 3901-3907.

Kawamata, S., K. Shimoharai, Y. Imura, M. Ozaki, Y. Ichinose, T. Shiraishi, H. Kunoh, and T. Yamada. 1997. Temporal and spatial pattern of expression of the pea phenylalanine ammonia-lyase gene1 promoter in transgenic tobacco. Plant Cell Physiol. 38 : 792-803.

Lee, S.W., J. Robb, and R.N. Nazar. 1992. Truncated phenylalanine ammonia-lyase expression in tomato (*Lycopersicon esculentum*). J. Biol. Chem. 267 : 11824-11830.

Lois, R., A. Dictrich, K. Hahlbrock, and W. Schulz. 1989. A phenylalanine ammonia-lyase gene from pasley : structure, regulation and identification of elicitor and light responsive *cis*-acting elements. EMBO J. 8 : 1641-1648.

McCormick, S., J. Niedermeyer, and J. Fry. 1986. Leaf disc transformation of cultivated tomato (*L .esculentum*) using *Agrobacterium tumefaciens.* Plnat Microbe Interact. 6 : 453-466.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15 : 473-497.

Mauch-Mani, B. and A. Slusarenko. 1996. Production of salicylic acid precursors in a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. Plant Cell 8 : 203-21.

Minami, E., Y. Ozeki, M. Matsuoka, N. Koizuka, and Y. Tanaka. 1989. Structure and some characterization of the gene for phenylalanine ammonia-lyase from rice plants. Eur. J. Biochem. 185 : 19-25.

Ohl, S., S.A Hedrick, J. Chory, and C.J. Lamb. 1990. Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. Plant Cell 2 : 837-848.

Sablowski, R.W.M., D.C. Baulcombe, and M. Bevan. 1995. Expression of a flower-specific Myb protein in leaf cells using a viral vector causes ectopic activation of a target promoter. 92 : 6901-6905.

Sambrook, T., E.F. Fritsch, and J.Manitis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Habor, NY.

Shufflebottom, D., K. Edwards, W. Schuch, and M. Bevan. 1993. Transcription of two members of a gene family encoding phenylalanine ammonia-lyase leads to remarkably different cell specificities and induction patterns. Plant J. 3 : 635-645.

Sriprasertsak, P. 2000. Plant defense responses and control of gene expression: Structure and function of the promoter of *PSPAL2*, a pea defensive gene encoding phenylalanine ammonia-lyase. Ph.D. Dissertation, Okayama University, Japan.

Subramaniam R., S. Reinold, E.K. Molitor, and C.J. Douglas. 1993. Structure, inheritance, and expression of hybrid poplar (*Populus trichocarpa* x *Populus deltoides*) phenylalanine ammonia-lyase genes. Plant Physiol. 102 : 71-83.

Takeda, J., Y. Ozeki, and K. Yoshida. 1997. Action spectrum for induction of promoter activity of phenylalanine ammonia-lyase gene by UV in carrot suspension cells. Phytochem. Photobiol. 66 : 464-470.

Tanaka, Y., M. Matsuoka, N. Yamamoto, Y. Ohashi, Y. Kano-Murakami, and Y. Ozeki. 1989. Structure and characterization of a cDNA for phenylalanine ammonia-lyase from cut-injured

roots of sweet potato. Plant Physiol. 90 : 1403-1407.

Wanner, L.A., G. Li, D. Ware, I.E. Somssich, and K.R. Davis. 1995. The phenylalanine ammonia-lyase gene family in *Arabidopsis thaliana*. Plant Mol. Biol. 27 : 327-338.

Whetten, R.W. and R.R. Sederoff. 1992. Phenylalanine ammonia-lyase from loblolly pine : purification of the enzyme and isolation of complementary DNA clones. Plant Physiol. 98 : 380-386.

Yamada, T., P. Sriprasertsak, H. Kato, T. Hashimoto, H. Shimizu, and T.Shiraishi. 1994. Functional analysis of the promoters of phenylalanine ammonia-lyase genes in pea. Plant Cell Physiol. 35 : 917-926.

Yamada, Y., P. Sriprasertsak, H. Kato, T. Hashimoto, H., Shimizu, and T. Shiraishi. 1994. Functional analysis of the promoters of phenylalanine ammonia-lyase genes in pea. Plant Cell Physiol. 35 : 917-926.

Yamada, Y., Y. Tanaka, P. Sriprasertsak, H. Kato, T. Hashimoto, S. Kawamata, Y. Ichinose, H. Kato, T. Shiraishi, and H. Oku. 1992. Phenylalanine ammonia-lyase genes from *Pisum sativum* : structure, organ-specific expression and regulation by fungal elicitor and suppressor. Plant Cell Physiol. 33 : 715-725.

Yazaki, K., M. Kataoka, G. Honda, K. Severin, and L. Heide. 1997. cDNA cloning and gene expression of phenylalanine ammonia-lyase in *Lithospermum erythrorhizon Biosci.* Biotech. Biochem. 61 : 1995-2003.

Zhu, Q., T. Dabi, A. Beeche, R. Yamamoto, M.A. Lawton, and C.J. Lamb. 1995. Cloning and properties of a rice gene encoding phenylalanine ammonia-lyase. Plant Mol. Biol. 29 : 535-550.

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# Determiation of Genetic Diversity and Relationhips among Thai Litchi Accessions by RAPD and AFLP Markers

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## ABSTRACT

Genetic diversity and relationships within Thai litchi cultivars were investigated using RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism) markers. Fourteen RAPD primers and seven AFLP primers were chosen, resulting in amplification of 52 and 101 reproducible polymorphic fragment products, respectively. The percentages of polymorphic markers for RAPD and AFLP were 34.6% and 36.3% respectively. Each marker system was able to differentiate all accessions. Even each of the AFLP primers could identify all accessions, while the RAPD markers did not show such efficiency. The polymorphism information content (PIC) scores were calculated for each of 52 RAPD and 101 AFLP polymorphic fragments. It ranged between 0.16 to 0.50 for RAPD markers and 0.22 to 0.50 for AFLP markers. Unweighted pair-group method with arithmetic averages (UPGMA) dendrograms using Jaccards coefficients reflected no clear cut grouping based on neither morphology nor climatic adaptation. However, both dendrograms showed that the 47 litchi accessions could be classified into groups between when the similarity coefficients were as low as 0.37 for RAPD marker and 0.25 for AFLP markers. Two accessions (LH80 and LH109) were found to be genetically very far distant from the other accessions using both markers. RAPD and AFLP marker analyses provided a quick and reliable alternative for identification of litchi accessions and determination of genetic diversity among them.


**Key words:** litchi, RAPD, AFLP, genetic, diversity

## INTRODUCTION

Litchi (*Litchi chinensis*) is an economic fruit of Thailand. Although it has originated in Southern China and Northern Vietnam, many varieties are densely distributed and grown in Thailand. The variability among litchi cultivars is still unknown since breeding for new cultivars done by growers based on a low number of parents. Field collection and preservation in gene banks of Plant Genetic Resources (PGR) has been extensively conducted at the international and national level. To identify genetic materials that may contain useful traits for germplasm enhancement, a systematic evaluation of genetic diversity is required to understand relationship among accessions and their corresponding collecting-site environments (Steiner and Greene, 1996). Understanding the genetic diversity within a germplasm collection facilitates their use, provided that information is available from characterizing germplasm collections (Strauss et al., 1988). Comparison of parents using difference in DNA markers may be one of the method by which breeders can increase the probability of


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selecting those parents with different gene sets. This methods will produce progeny with new and more favorable combinations of genes for quality and yield.

Recent reports have focused on using DNA based markers, particularly random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers, to measure genetic diversity in numerous fruit species such as cherry (*Prunus avium*) (Gerlach and Stosser, 1997), lemon (*Citrus lemon* L.) (Machado *et al.*, 1996), mango (*Mangifera indica* L.), peach (*Prunus persica* L.) (Lu *et al*., 1996), grape (*Vitis vinifera*) (Sensi *et al*., 1996), currant (*Ribes grossularia*) (Lanham and Brennan, 1999), and pear (*Pyrus* sp.) (Monte-Corvo *et al*., 2000). Both RAPD and AFLP detected substantial genetic variation within perennial fruit cultivars and generally demonstrate that cultivars can be discriminated on the basis of genetic characteristics (Lopez-Valenzuela *et al*., 1997). Choice of a marker system to use for a particular application depends on its ease of use and the particular objectives of the investigation (Rafalski *et al*., 1996). RAPD and AFLP techniques do not require DNA probes or prior sequence information. These procedures are simple, largely automatable, require only small amounts of DNA, and can be performed without the use of radioactivity (Karp *et al*., 1996). RAPD markers also have limitations such as their dominant character and reproducibility (Williams *et al*., 1990). Reliability may be increased by replicate analyses and PCR performed at different times. Although the AFLP procedure is more labor intensive and expensive than RAPD analysis, but a larger number of loci are detected per reaction in comparison with RAPD-PCR (Powell *et al*., 1996).

The objectives of this study were to : (1) to use RAPD and AFLP analyses to estimate the level of genetic diversity among 47 litchi accessions collected from all over Thailand (2) to determine the relative effectiveness of both markers in revealing variation among closely related cultivars.

## MATERIALS AND METHODS

### Plant materials

Forty-seven litchi accessions were used in this study (Table 1). They were collected from various locations in Thailand. The fresh young leaves were collected from 5 plants of each accession, and used for DNA extraction by the CTAB method (Doyle and Doyle, 1990).

### Random amplified polymorphic DNA (RAPD) analysis

Primers representing 10 random nucleotide sequences, obtained from Operon Technologies (USA) were used in the RAPD assay. PCR reactions were in volumes of 10 μL containing 1 ng/ μL of the extracted genomic DNA, 1 of 10x PCR buffer (100 mM Tris-Cl buffer, 500 mM KCl, 20 mM $MgCl_2$, 0.01% Gelatin ), 0.1 mM dNTPs (Promega, USA), 0.2 mM primer, 0.2 unit/μL Taq DNA polymerase (Promega) and 4.8 μL sterile water. Sterile mineral oil (30 μL) was added to each tube to seal the reaction mixture and to prevent evaporation. The PCR was carried out in a DNA Thermal Cycler (Bio Oven III) programmed to run the following temperature profile; 45 cycles of 91.5°C for 1 min, 36°C. for 1 min, 72°C. for 2 min and the final extension for 5 min at 72°C. All PCR products were fractionated by electrophoresis in 1.6% (w/v) agarose gels and visualized by ethidium bromide staining using 0.5 μg/mL in 1x TAE buffer. The fragments of each gel were recorded with Bioprint-version 96,07 system (France). Polymorphism at all loci was confirmed by three repeated tests.

### Amplified fragment length polymorphism (AFLP) analysis

The same DNA preparation technique used for RAPD analysis was also employed in AFLP analysis. AFLP analysis was carried out according to Vos *et al*. (1995). Total DNA (500ng) was digested twice with EcoRI(recognition sequence 5'-GAATTC3') and Tru9I (recognition sequence

**Table 1** Accessions of litchi used in the variation study.

| Number | Accession/local name | Collection site | Origin |
|---|---|---|---|
| 1 | LH80 Chakra-pad | Tah Ton /Fang/Chiang Mai | |
| 2 | LH18 Samphoa Koew | KU station/Pak Chong/ Nakhon Rachasima | |
| 3 | LH33 Samphoa Koew | Amphawa-1/Samut Songkhram | |
| 4 | LH56 O-Hia | KU station/Pak Chong/ Nakhon Rachasima | |
| 5 | LH91 O-Hia | Mae Ngon/Fang/Chiang Mai | |
| 6 | LH95 O-Hia (heart shape) | Mae Ngon/Fang/Chiang Mai | |
| 7 | LH32 Kra-lok | Amphawa-1/Samut Songkhram | |
| 8 | LH13 Hong Huay | KU station/Pak Chong/ Nakhon Rachasima | |
| 9 | LH83 Hong Huay | Tah Ton /Fang/Chiang Mai | |
| 10 | LH11 Kom (red) | KU station/Pak Chong/ Nakhon Rachasima | |
| 11 | LH19 Kom (green) | KU station/Pak Chong/ Nakhon Rachasima | |
| 12 | LH22 Kom | KU station/Pak Chong/ Nakhon Rachasima | |
| 13 | LH23 Kom | KU station/Pak Chong/ Nakhon Rachasima | |
| 14 | LH25 Kom (lamjiak) | Amphawa-1/Samut Songkhram | |
| 15 | LH35 Kom (200 years) | Amphawa-2/Samut Songkhram | |
| 16 | LH37 Kom | Amphawa-2/Samut Songkhram | |
| 17 | LH43 Kom | Amphawa-3/Samut Songkhram | |
| 18 | LH69 Kom | Thep Raksa/Pak Chong | |
| 19 | LH74 Kom (krathouy) | Thep Raksa/ Pak Chong | |
| 20 | LH86 Kom | Mae Ngon/Farng/Chiang Mai | |
| 21 | LH29 Jean | Umpawa-1/Samut Sakorn | |
| 22 | LH41 Jean | Amphawa-2/Samut Songkhram | |
| 23 | LH30 Tai Ohia | Umpawa-1/Samut Sakorn | |
| 24 | LH36 Tai Yai | Amphawa-2/Samut Songkhram | |
| 25 | LH42 Tai | Amphawa-2/Samut Songkhram | |
| 26 | LH34 Kra-thone Thong Phra-rong | Umpawa-1/Samut Sakorn | |
| 27 | LH59 Kawaini | KU station/Pak Chong/ Nakhon Rachasima | |
| 28 | LH64 Brewster | KU station/Pak Chong/ Nakhon Rachasima | |
| 29 | LH67 Golf | KU station/Pak Chong/ Nakhon Rachasima | |
| 30 | LH87 Kim-cheng | Mae Ngon/Fang/Chiang Mai | |
| 31 | LH100 Jubee-jee | Tah Ton/Fang/Chiang Mai | |
| 32 | LH101 Sweet cliff | Horticulture Research Station, Chiang Rai | |
| 33 | LH102 Mauritius | Horticulture Research Station, Chiang Rai | |
| 34 | LH103 Brewster | Horticulture Research Station, Chiang Rai | |
| 35 | LH104 Kom (long leaf) | Horticulture Research Station, Chiang Rai | |
| 36 | LH105 Chow Rakam | Horticulture Research Station, Chiang Rai | |
| 37 | LH106 Jean -dang | Horticulture Research Station, Chiang Rai | |
| 38 | LH107 Nai-Saard | Horticulture Research Station, Chiang Rai | |
| 39 | LH108 Tip | Horticulture Research Station, Chiang Rai | |
| 40 | LH109 Jean hom | Horticulture Research Station, Chiang Rai | |
| 41 | LH112 Jean -lek | Horticulture Research Station, Chiang Rai | |
| 42 | LH114 Look-laai | Horticulture Research Station, Chiang Rai | |
| 43 | LH116 Jean Kriangsak | Horticulture Research Station, Chiang Rai | |
| 44 | LH117 Kra-lok-Bai-Yoh | Horticulture Research Station, Chiang Rai | |
| 45 | LH118 Kim Chi | Horticulture Research Station, Chiang Rai | |
| 46 | LH119 Sa-laek Tong | Horticulture Research Station, Chiang Rai | |
| 47 | LH120 Hak-ip | Horticulture Research Station, Chiang Rai | |

5'-GACGATGAGTCCTGAG and TACTCAGGACTCAT-3'). After ligating ER adatptors and MS adaptor to the digested DNA, pre-amplification was conducted with an E R-A- primer (EcoRI adaptor sequence) and Ms-C primer (Ms-C adaptor sequence as a selective nucleotide). The pre-amplification product was used as template DNA for selective amplification. Selective amplification was conducted using an ER-A primer containing two selective nucleotides and Ms-C primer containing three selective nucleotides. 12 combination of the selective primers were used. PCR condition was set for 20 cycles in PCR I and 30 cycles in PCR II. The condition in each cycle was the same as RAPD. The products of selective amplification were denatured at 90 C for 3 min. Electrophoresis was performed on 4.5% polyacrylamide gel in 5xTBE Buffer with a sequencer using 50 watt per gel. AFLP gel was stained by silver staining as described by Bassam *et al*.,1991.

**Data analysis**

Bands position for each litchi accession and primer combination were scored as present (1) or absent (0) from photographic prints of gels. Only bright, clearly distinguishable polymorphic bands were used in the statistical analysis. The discrimination power of each RAPD and AFLP marker was evaluated by the polymorphism information content (PIC) as described by Anderson *et al*. (1993). Jaccard's (1908) coefficient of similarity was calculated, and the accessions were grouped by cluster analysis using the unweighted pair-group method (UPGMA). NTSYS-pc, Version 2.01d (Rohlf, 1997) program was used for statistical analyses.

## RESULTS

**RAPD**

Fifty primers were screened for their RAPD products generated against DNA samples extracted from litchi accessions. Fourteen primers were selected for the molecular diversity analysis of accessions and evaluation of their relationships based on the total number of bands, the number of polymorphisms, and their reproducibility in three independent applications (Table 2). These selected primers yielded a total of 153 fragments among the 47 litchi accessions of which 52 fragments were polymorphic (34.6%). The size of fragment scored ranged from approximately 500 to 2000 base pairs. The average of two to six polymorphic bands per primer were scored with a mean ± SD of 3.71 ± 1.1 bands per primer. The range of PIC or genetic diversity scores in this study was 0.16 to 0.50 with the mean of 0.31 ± 0.11.

**AFLP**

The number of bands per primer pair ranged from 31 to 57 with an average of 39.7. A total of 278 AFLP bands were scored with seven primer pair combinations. The total polymorphic bands were 101 (36.3%) with the range from 22.8% to 53.1% (Table 3). The polymorphic bands amplified by any AFLP primer were sufficient to discriminate all litchi accessions. An example of the pattern of amplified products obtained with one AFLP primer pair is presented in Figure 1. The discrimination power of each marker was estimated by the PIC (results not shown), Which found ranging between 0.22 and 0.50 (the expected maximum value for a biallelic locus) with an average of 0.37. A large portion of markers ha a high discrimination power of (≥0.30). Six cultivars, namely Jakrapad, Hong Huay, Kimjeng, O-Hia, O-Hia (heart-shape), and Jubee-jee were tested for intra-varietal variation using primers ACC/CAG, ACC/CAT, and ACC/CAA. A considerable polymorphisms were revealed in this study (Figure 2)

**Cluster analysis**

RAPD and AFLP cluster analysis is illustrated in the dendrogram of Figure 3 and 4, respectively. The dendrogram constructed by 52

**Table 2** List of selected Operon primers, their sequences, number of bands, polymorphism (%) of the RAPD analysis results in 47 litchi accessions.

| Sl no. | Operon code | Sequences (5'-3) | RAPD fragment score | | Polymorphism% |
|---|---|---|---|---|---|
| | | | Monomorphic | Polymorphic | |
| 1 | OPE-04 | GTGACATGCC | 6 | 5 | 45.5 |
| 2 | OPE-15 | ACGCACAACC | 8 | 3 | 27.3 |
| 3 | OPE16 | GGTGACTGTG | 6 | 4 | 40 |
| 4 | OPE-18 | GGACTGCAGA | 7 | 3 | 30 |
| 5 | OPE-20 | AACGGTGACC | 7 | 4 | 36.4 |
| 6 | OPF-01 | ACGGATCCTG | 9 | 2 | 18.2 |
| 7 | OPF-05 | CCGAATTCCC | 3 | 4 | 57.1 |
| 8 | OPF-10 | GGAAGCTTGG | 10 | 4 | 28.6 |
| 9 | OPG-04 | AGCGTGTCTG | 7 | 2 | 22.2 |
| 10 | OPH-04 | GGAAGTCGCC | 9 | 4 | 30.8 |
| 11 | OPH-15 | AATGGCGCAG | 6 | 3 | 33.3 |
| 12 | OPI-06 | AAGGCGGCAG | 11 | 5 | 31.3 |
| 13 | OPK-02 | GTCTCCGCAA | 6 | 3 | 33.3 |
| 14 | OPL-12 | AGAGGGCACA | 6 | 6 | 50 |
| Total | | 14 | 101 | 52 | -- |
| Mean±SD | | | 7.2+1.97 | 3.71+1.1 | |

**Table 3** List of AFLP primers, their sequences, number of bands, polymorphism (%) of AFLP analysis results in 47 litchi accessions.

| Sl. No. | Sequences | AFLP fragment score | | Polymorphism% |
|---|---|---|---|---|
| | | Monomorphic | Polymorphic | |
| 1 | ACC/CAG | 19 | 12 | 38.7 |
| 2 | ACC/CAA | 44 | 13 | 22.8 |
| 3 | ACC/CTC | 29 | 15 | 34.1 |
| 4 | ACC/CAT | 30 | 12 | 28.6 |
| 5 | ACC/CTG | 15 | 17 | 53.1 |
| 6 | ACC/CAC | 21 | 19 | 47.5 |
| 7 | ACC/CTA | 19 | 13 | 40.6 |
| Total | 7 | 177 | 101 | -- |
| Mean (±SD) | | 25.28 ± 9.18 | 14.43 ± 2.5 | 36.3 |

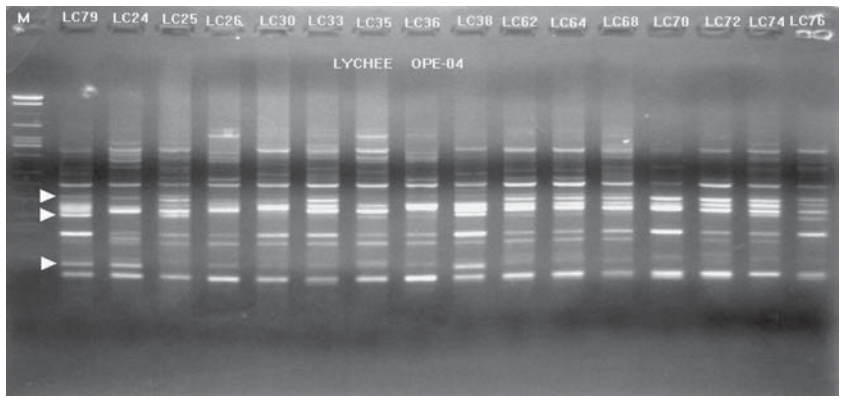


**Figure 1** RAPD bands produced by primer OPE-04 with the genomic DNA from each of 16 litchi cultivars. The cultivars are numered as in Table 1.

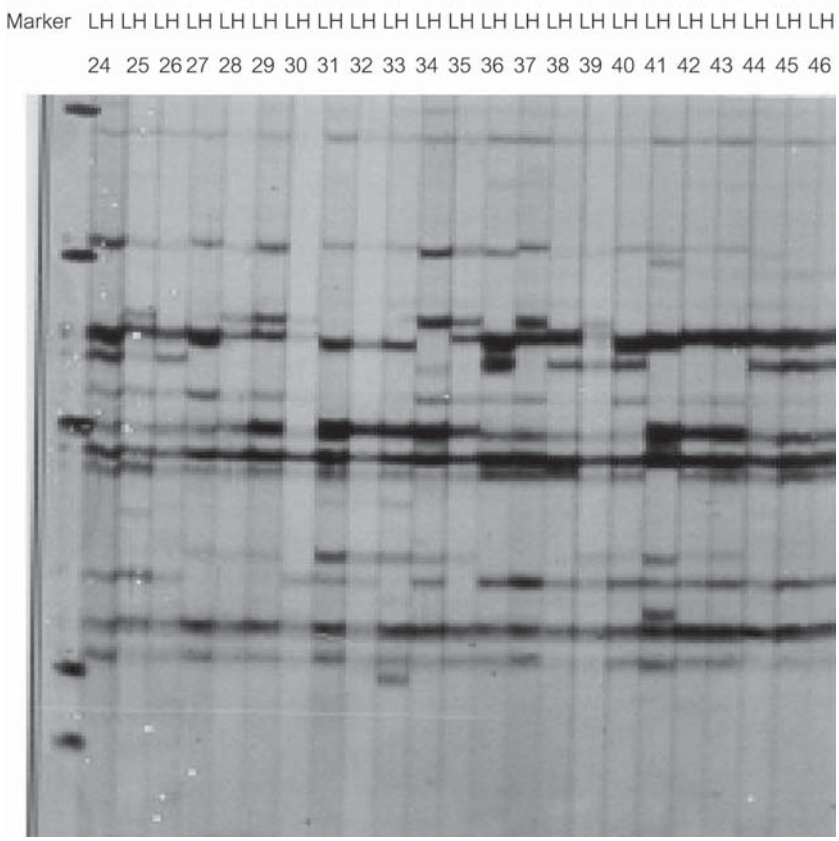


**Figure 2** AFLP bands produced by primer ACC/CAG with the genomic DNA from each of 23 litchi cultivars. The cultivars are numered as in Table 1.

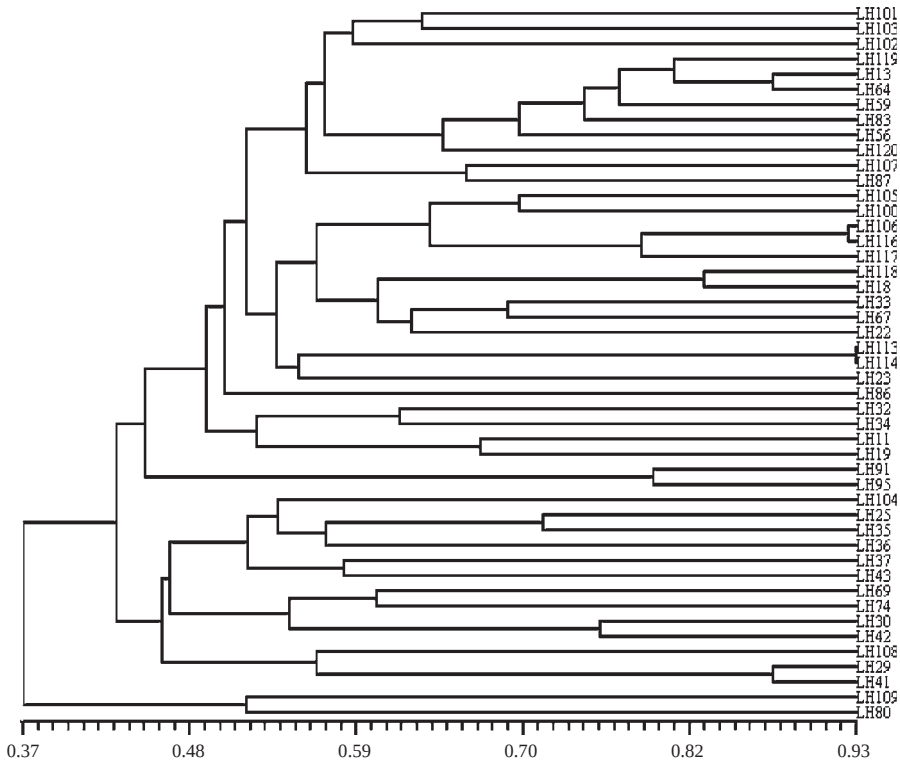


**Figure 3** Dendrogram showing genetic relationship among 47 Thai litchi accessions generated by UPGMA cluster analysis calculated from 52 RAPD markers amplified by 14 10-mer primers.

RAPD markers indicated that the Thai litchi accessions were clearly separated into two main groups. One group, which contains the only two red color fruited accessions named Jakra-pad (LH80) and Jean Hom (LH109). The other group could be furtherly divided into six sub-groups at the 0.50 similarity scale, containing 3, 4, 6, 2, 4, and 26 accessions, respectively (Figure 3). Each of these sub-groups could be furtherly divided into several well defined clusters showing a close association among local basis distinct or same accessions. Similarly using the binary data from 101 AFLP markers for UPGMA cluster revealed 47 accessions to be separated into three main groups. It also indicated that the litchi accessions were rather distinctly separated to RAPD dandrogram. Some accessions showed high tendency to be close among themselves the same as RAPD based dendrogram. Range of similarity values in AFLP dendrogram was higher than that of RAPD dendrogram. Accession LH80 and LH109 revealed a high genetic distant in the both marker system analyses.

## DISCUSSION

Genetic diversity among 47 selected accessions of Thai litchi was assessed with 52 RAPD and 101 AFLP polymorphic bands. A low number of RAPD polymorphisms per primer was detected among accessions. RAPD marker revealed that its specificity for variety discrimination was comparatively more limited than that of AFLP marker. Similar results also reported by Paran *et al*. (1998) in *Capsicum annuum*. By using RAPD and AFLP primers, there was very low genetic variation within 47 accessions of litchi. For RAPD only

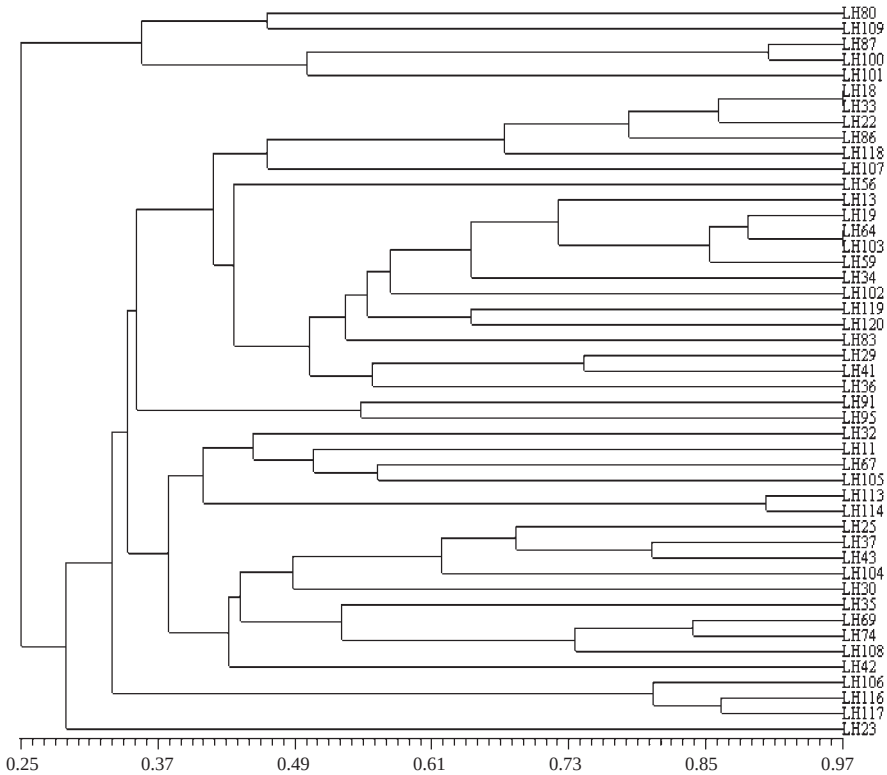


**Figure 4** Dendrogram showing genetic relationship among 47 Thai litchi accessions generated by UPGMA cluster analysis calculated from 101 AFLP markers amplified by 7 pair- primers.

34.6% of bands were polymorphic in all accessions. The percentage of polymorphic bands (PPB) in each accession ranged from 18.2% to 50.0%. For AFLP, the PPB was 36.3%, ranging between 22.8% to 53.1% in each accession. These results more or less agreed with RAPD and AFLP analysis in other species (Chowdhury *et al*., 2001; Bellini *et al*.,1998, Paran *et al*., 1998). Both RAPD and AFLP analyses yield similar results and expressed great potential to identify and establish genetic relationship among litchi accessions. Both methods are highly informative and do not require prior knowledge of the litchi genome. The AFLP assay showed some advantages over RAPD, as it is more reproducible and more informative than that of RAPD. Each AFLP primer is sufficient for the identification of all accessions, but it has license restrictions which is limits its use for commercial fingerprinting (Knorr *et al.*, 2001). Different combinations of the banding patterns provided by different primer, is a clear evidence of the high discrimination capacity of these markers. This capacity is particularly useful for management of a germplasm bank, as it provides an inexpensive and reliable method for identification of a large number of cultivars. Moreover, litchi fruit is cross-pollinated and this evidence is easily detected by AFLP markers using two primersACC/CAG, ACC/CAT, and ACC/CAA. Thus the AFLP method is suitable for detecting intravarietal difference.

For similarity coefficients the ranges were 0.25 to 0.97 for AFLP marker, and 0.37 to 0.93 for RAPD data. The mean value was significantly lower in AFLP (0.34) than that of RAPD analysis (0.47), indicating a higher frequency of polymorphic bands scored as presented among the 47 litchi

accessions when using AFLP versus RAPD. Two pairs of accessions (LH18 vs LH33 and LH64 vs LH103) were genetically similar (97% similarity) on the basis of AFLP analysis however, they showed low similarity (65% and 61%) when using RAPD analysis. A possible explanation for the difference in the resolution was the two techniques targeting on different portions of the genome (Karp *et al*., 1996), although, some studies suggested that both RAPD and AFLP markers represented the specific DNA fragments distributing throughout the genome (Becker *et al*., 1995; Williams *et al*., 1990). Higher level of similarity observed among the cultivars originating from the same or nearby geographic origins agreed with both the hypothesis of authoctonal origin as well as the limited diffusion of litchi cultivars from their zones of cultivation (Barranco, 1994). This result suggested that both markers were useful for molecular diversity estimation but had no allelic relationship between the absence or presence of a given band due to the different primer sequences (Baril *et al*.,1997).

Subhadrabandhu (1990) reported that there were two groups of litchi varieties grown in Thailand. One group mainly cultivated in the central part while the other in the northern part of the country. In central part, existing litchi varieties require no or little cool period for inflorescense induction. These varieties are some times classified as low land litchi or tropical litchi, whereas the varieties that require cooler period for flowering are mainly found in the northern part of the country. Both groups also exhibit difference in flowering and harverting time, fruit size and color at maturity. In these respects, both DNA markers did not reveal any clear pattern of grouping based on morphology or putative climatic or geographic origin, as detected in some other crops (Paul *et al*., 1997, Spooner *et al*.,1996). Belaj *et al* (2001) reported similar results in olive germplasm using RAPD markers. Large portions of this litchi germplasm share common ancestry, since Chinese varieties have been highly utilized as parental materials in chance seedling selection by farmers in Thailand. Hence, the gene pool comprising cultivated litchi may be very restricted. It is also possible that some litchi accessions were introduced from Myanmar or Vietnam.

Although both DNA markers were effective in detecting genetic diversity levels in Thai litchi cultivars, AFLP was more efficient than RAPD in number of polymorphic bands detected per primer and the reproducibility involved. Dendrograms constructed based on AFLP and RAPD polymorphisms indicated that these two marker techniques provided no identical phylogenetic information. This observation may be related to larger number of AFLP bands used in the analyses. Estimates of genetic diversity are highly influenced by the genome selected for evaluation and by the number of markers assayed. Since fruit tree cultivars are maintained by vegetative propagation, accurate identification of vegetative materials is crucial for nurserymen and growers, and is required for the plant breeder's rights. Therefore, these DNA marker techniques can be used to identify genetic variation and detect the find relationship between DNA markers and horticultural traits of interest.

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## LITERATURE CITED

Anderson, J.A., G.A. Churchill, J.E. Autrique, S.D. Tanksley, and M.E. Sorrells. 1993. Optimizing parental selection for genetic linkage maps. Genome 36 : 181-186.

Baril, C. P., D. Verhaegen, P. Vigneron, J. M. Boubet, and A. Kremer. 1997. Structure of the specific combining ability between two species of Eucalyptus. I. RAPD data. Theor. Appl. Genet. 94 : 796-803.

Barranco, D. 1994. Estructura varietal del olivoen Espana Agricultura. 63 : 731-732.

Bassam, B.J., G. Caetano-Anolles, and P.M. Gresshoff. 1991. Fast and sensitive silver staining of DNA in poly acrylamiide gels. Anal, Biochem. 196 : 80-88.

Becker, J., P. Vos, M. Kuiper, F. Salamini, and M. Heun. 1995. Combined mapping of AFLP and RFLP markers in barley. Mol. Gen. Genet. 249 : 65-73.

Belaj, A., I. Trujillo, R. dela Rosa, and L. Rallo. 2001. Polymorphism and discrimination capacity of randomly amplified polymorphic markers in an olive germplasm bank. J. Amer. Soc. Hort. Sci. 126 (1) : 64-71.

Bellini, E., E. Giordani, V. Nencetti, and D. Paffetti. 1998. Genetic relationship in Japanese plum cultivars by molecular markers. Acta Hort. 478 : 53-61.

Chowdhury, A. K., P. Srinives, P. Tongpamnak, and P. Saksoong. 2001. Genetic diversity based on morphology and RAPD analysis in vegetable soybean. Korean J. Crop Sci. 46(2) : 112-120.

Doyle, L.J. and J.J. Doyle, 1990. Isolation of plant DNA from fresh tissue. Focus 12 : 13-14.

Gerlach, H.K. and R. Stosser. 1997. Patterns of random amplified polymorphic DNAs for sweet cherry (*Prunus avium* L.) cultivar identification. J. Appl. Bot - Angewandle. Bot. 71 : 212-218.

Jaccard, P. 1908. Nouvellers recherches sur la distribution florale. Bull Soc Vaud Sci Nat 44 : 223-270.

Karp, A., O. Seberg, and M. Buiatti. 1996. Molecular techniques in the assessment of botanical diversity. Ann. Bot. (London) 78 : 143-149.

Knorr, C., H. H. Cheng and J.B. Dodgson. 2001. DNA cloning and sequence analysis of chicken AFLP. Animal Genetics 32 : 156-159.

Lanham, P.G. and R.M. Brennan. 1999. Genetic characterization of goose-berry (*Ribes grossularia* subgenus Grossularia) germplasm using RAPD, SSR and AFLP markers. J. Hort. Sci. Biotechnol. 74 : 361-366.

Lopez-Valenzuela, J.A., O. Martinez, and O. Paredes-Lopez. 1997. Geographic differentiation and embryo type identification in *Mangifera indica* L. HortScience 32 : 1105-1108.

Lu, Z-X., G.L. Reighard, W.V. Baird, A. G. Abbott, and S. Rajapakse. 1996. Identification of peach rootstock cultivars by RAPD markers. Hort. Science 31 : 127-129.

Machado, M.A., F.H. Coletta, M.N. Targon, and J.J. Pompeu. 1996. Genetic relationship of Mediterranean mandarins (*Citrus deliciosa* Tenore) using RAPD markers. Euphytica 92 : 321-326.

Monte-Corvo, L., L. Cabrita, C.M. Oliveira, and J.M. Leitao. 2000. Assessment of genetic relationships among *Pyrus* species and cultivars using AFLP and RAPD markers. Gen. Resources Crop Evol. 47 : 257-265.

Paran, I., E. Aftergoot, and C. Shifriss. 1998. Variation in *Capsicum annuum* revealed by RAPD and AFLP markers. Euphytica. 99 : 167-173.

Paul, S., F. N. Wachira, W. Powell, and R. Waugh. 1997. Diversity and genetic differentiation among populations of Indian and Kennyan tea (*Camellia sinensis* (L.) O. Kuntze) revealed by AFLP markers. Theor. Appl. Genet. 94 : 255-263.

Powell, W., M. Morgantee, C. Andre, M. Hanafey, J. Vogel, S. Tingey, and A. Rafalski. 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol. Breed. 2 : 225-238.

Rafalski, J.A., J.M. Vogel, M. Morgante, W. Powell, C. Andre, and S.V. Tingey. 1996. Generating and using DNA markers in plants, pp. 75-134.

*In* B. Birren and E. Lai (eds.). Nonmammalian genomic analysis. Academic Press, San Diego.

Rohlf, F.J. 1997. NTSYS-pc. Numerical analysis and multivariate analysis system, Version 2.01d. Applied Biostatistics, New York.

Sensi, E., R. Vignani, W. Rohde, and S. Biricolti. 1996. Characterization of genetic biodiversity with *Vitis vinifera* L. Sangiovese and Colorino genotypes by AFLP and ISTR DNA marker technology. Vitis. 35 : 183-188.

Spooner, D.M., J. Tivang, J. Neinhuis, J.T. Miller, D.S. Douches, and A. Contreras-M. 1996. Comparison of four molecular markers in measuring relationships among wild potato relatives *Solunum* section tuberosum (Subgenus *Potatoe* ). Theor. Appl. Genet. 92 : 532-540.

Steiner, J.J. and S.L. Greene. 1996. Proposed ecological descriptors and their utility for plant germplasm collections. Crop Sci. 36 : 439-451.

Strauss, M.L., J.A. Pino, and J.I. Cohen. 1998. Quanlification of ex situ plant collections. Diversity 16 : 30-32.

Subhadrabandhu, S. 1990. Lychee and Longan Cultivation in Thailand. Rumthai Publication, Bangkok, Thailand. 40 p.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman. M. Kuiper, and M. Zabeau. 1995. AFLP a new technique for DNA fingerprinting. Nucl. Acid. Res. 23 : 4407-4414.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S.V. Tingey. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucl. Acid. Res. 18(22) : 6531- 6535.

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# Effects of Arbuscular Mycorrhiza and Phosphate Fertilizer on Phosphorus Uptake of Vetiver Using Nuclear Technique

**Sombun Techapinyawat[1] , Pannee Pakkong[2] , Poonpilai Suwanarit[3] and Pattarawadee Sumthong[4]**


## ABSTRACT

This experiment was done in 4×4 factorial treatment combinations with 4 replications in completely randomized design to evaluate the effects of 3 species of arbuscular mycorrhizal fungi : *Acaulospora scrobiculata*, *Glomus aggregatum* and *Glomus* sp. combined with phosphate fertilizer on phosphorus uptake of vetiver (*Vetiveria zizanioides* L. Nash). Four levels of superphosphate 0, 30, 60 and 90 kg $P_2O_5$ / ha (labels with P-32)were used. Vetiver height was increased when phosphate fertilizer was supplied, whereas the tiller number and biomass did not show any responses when P-fertilizer was applied. Comparing the different types of mycorrhizal treatments, *A. scrobiculata* significantly increased vetiver growth in terms of height and biomass, while the number of tillers per plant were not affected. The highest number of tiller per plant was found in *G. aggregatum* treated plant.

Arbuscular mycorrhizal fungi significantly increased P-concentration, P-uptake, % P derived from fertilizer and P-availability in vetiver. At 60 kg $P_2O_5$ / ha level of application, the highest percentage of P derived from fertilizer(0.545%), P-availability(0.037%FPU ), and total P-uptake(6.25mg/clump) were obtained from *A.scrobiculata* , whereas *G. aggregatum* treatments with no P-supplied gave the greatest P-concentration (0.104%). Increasing phosphate fertilizer up to 90 kg $P_2O_5$ / ha, resulted in non-significant changes of P-uptake and P-availibility in VA mycorrhizal plants.


**Key words:** vetiver (*Vetiveria zizanioides* L. Nash), arbuscular mycorrhiza , phosphorus uptake, P-32, *Acaulospora scrobiculata, Glomus aggregatum,Glomus sp.*

## INTRODUCTION

It is now widely known that application of arbuscular mycorrhizal fungi could increase the growth of several plant species (Mosse, 1973). The growth improvement has been mainly attributed to the enhanced uptake of phosphorus by the host plant . Phosphorus is an essential element for plant growth development and crop production as well as mycorrhiza fungal growth. Many types of soil in Thailand are defined as having high phosphorus fixation capacity whereby plants growth will be limited due to deficient phosphorus supply to the plant. Mycorrhiza is capable of absorbing and translocating nutrients as well as exploring more soil volume than the non-mycorrhizal roots (Joner and Jacobson,1995), and provide a direct link between soil and roots, thus increase the supply of

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slowly diffusing ions, such as phosphate to the plant. Menge *et al*. (1978) have shown that the control of mycorrhizal colonization is related to phosphorus concentration in the host plant. The growth stimulus by mycorrhizal symbiont is most frequently observed in soil low in phosphate. Addition of high rate phosphate fertilizer to the soil generally decrease the colonization of plant roots by mycorrhizal fungi (Abbott and Robson, 1984). The effect of phosphate supply on mycorrhiza colonization and plant growth may be dependent on the plant species (Valentine *et al*., 2000). However, at extremely low phosphorus available in soil it is expected that the benefit of the symbiosis to the host would be small. Under such condition the development of mycorrhizal symbiosis may not be advantageous to the host plant as compare with the non-mycorrhizal plant, since phosphorus uptake is not facilitated by infection (Hayman, 1983). In most case the addition of more readily available P into the soil eliminates differences in growth and phosphate uptake of the test plant. Many researchers concluded that root colonization of VA mycorrhiza, P- concentration and the host plant are postulated to be the important factors for the determination of mycorrhiza efficacy (Smith and Gianinazzi-Pearson, 1988). Under these circumstances there may be a complex relationships among host performance, mycorrhizal infection and amount of phosphorus in the soil to improve plant growth.

Radioisotope tracer techniques have been used extensively to measure P-uptake in rock phosphate (Fried, 1954). Tracer techniques provided better indexes of availability than the non-tracer method and highly correlation with chemical extraction measurements (Kucey and Bole, 1984). The objective of this study was to evaluate the effects of 3 species of arbuscular mycorrhizal fungi : *Acaulospora scrobiculata*, *Glomus* sp., *Glomus aggregatum* in combinations with phosphate fertilizer on phosphorus uptake of vetiver grass using nuclear technique.

## MATERIALS AND METHODS

### Soil

Pakchong series soil, Reddish Brown Lateritic Group were collected from the National Corn and Sorghum Research Center, Nakhon Ratchasima Province. The soil contained 1.31% organic matter, 12 ppm available P, 62 ppm extractable K, 0.173 mmhos EC at 25°C, and the pH of 7.3,

### Arbuscular mycorrhiza fungi

The arbuscular mycorrhiza fungal species used in this study were obtained from rhizosphere of natural vetiver grass which have been isolated and selected for the highly effective species to improve vetiver growth in the previous experiment (Techapinyawat *et al*., 2001). Spores of arbuscular mycorrhiza fungi were produced using *Zea may* as host plant in pot culture containing sterile soil and were extracted by wet sieving and decanting (Gerdemann and Nicolson,1963). These arbuscular mycorrhiza spores were used as inoculum.

### Host plant

Vetiver grass (*Vetiveria zizanoide*s L. nash) plantlets from tissue culture were propagated in 3 inches diameter plastic bag using sterile soil for one month and then transplanted into 6 inches diameter pot containing 1.5 kg fumigated Pakchong soil per pot. Approximately 200 arbuscular mycorrhizal spores were added to each pot prior to transplanting. After growing for one month the plants were uniformly thinned into 3 clumps / plant and the shoots were cut to the height of 20 cm. Then phosphate fertilizer were applied at this stage of growth.

### The experimental design

This experiment was performed at Botany Department, Kasetsart University, Bangkok, Thailand. The experiment was laid out in 4×4

factorial treatment combination with 4 replications in completely randomized design. The inoculation treatments consisted of 4 inocular types : *Glomus* sp., *Glomus aggregatum*, *Acaulospora scrobiculata*, and a control. The fertilizer treatments consisted of 4 phosphorus levels : 0, 30, 60 and 90 kg $P_2O_5$ / ha (0, 4.8, 9.6 and 14.4 kg / rai). Superphosphate used as phosphorus source was applied into the soil. About 0.048 gm Urea (46%) and 0.038 gm of $K_2O_5$ were also incorporated into each pot as basal fertilizer. Two hundred microliter of P-32 solution with the activity of 4.38 μCi per pot was mixed with 10 gm sterilized sand and used in each treatment except those without phosphate fertilizer application. The plants were grown in the greenhouse and water was given regularly to maintain the field capacity of the soil. Harvest was done at 2 weeks after fertilizer application.

Plant height and the number of tiller were measured at 1 and 2 weeks after phosphate fertilizer application. At harvest time (2 weeks after fertilizer application), the total dry weight of vetiver was determined after drying the plant samples at 70°C for 3 days in a hot air oven .

The whole dry ground plant was ashed at 500°C for 5 hours . The ash was dissolved in 20 ml of 2 N HCl. Ten ml of this ashing solution was pipetted into grass scintillation vials and P-32 activity was measured by Cerenkov technique using liquid scintillation counter.

The percentage of P-31 concentration in the filtrate was determined using Vanadomolybdate method (Tassanee *et al*., 1989). The percentage of P in the plant derived from the fertilizers (% P dff) and P-uptake by the plant were calculated by the isotopic dilution concept as described by Hardarson (1990).

All datas were subjected to analysis of variance and the mean values were compared using Duncan's new multiple range test at 95% confidence.

## RESULTS AND DISCUSSION

### Plant growth

Vetiver height as affected by arbuscular mycorrhizal (AM) inoculation combined with phosphate fertilizer were significantly ($P< 0.05$) different at 1 and 2 weeks after fertilizer applications. Similarly, the effects of AM inoculations on plant height for both stages of growth were significantly ($P< 0.05$) different (Table1). *Acaulospora scrobiculata* treated plant was found to reach the maximum height. However, the average height of the plants inoculated with *Glomus* sp. and G. *aggregatum* were not differed from the control (Table 2 and 3). Phosphate fertiliizer gave highly significant ($P< 0.01$) effect on plant height (Table 1). Applications of phosphate fertilizer the average plant height were increased. The effect of *A. scrobiculata* combined with 30 kg $P_2O_5$ /ha on plant height was clearly shown in both stages of growth. However, the tallest plant at harvesting stage was observed in *A. scrobiculata* treated plant (21.13 inch.) combined with 60 kg $P_2O_5$ / ha, while the height of the control plant was only 18.63 inch. Without phosphate fertilizer application, non-significant responses of AM inoculation on plant height at 1 week stage were attained. However, at 2 weeks the height of *A. scrobiculata* treated plant tend to be taller than other treatments (Table 2 and 3). Since the soil used in this experiment contained low available P (12 ppm), this phenomenon may due to the depletion of nutrient (phosphorus) by plant uptake.

Hayman (1983) indicated that at extremely low phosphorus availability, the benefit of arbuscular mycorrhiza symbiosis would be small, despite the host being limited by the supply of phosphorus. Under such condition, the development of the mycorrhiza symbiosis may represent no advantage to the host plant over the non-mycorrhizal state. Increasing phosphate fertilizer up to 90 kg $P_2O_5$ / ha, the height of mycorrhiza treated plant and the

control were not significantly different at both stages of growth (Table 2 and 3).

Phosphorus had non-significant (P< 0.05) effects on the number of vetiver tiller whereas AM inoculations resulted in significant (P< 0.05) effects on the averge tiller number at 1 week and at harvesting stage (Table 1). *G. aggregatum* inoculum gave the maximum tiller number (Table 4 and 5). Since mycorrhizal fungi can synthesize extracellular hormones such as cytokinin and auxin required by the host plant (Mayer, 1974), the ratio of cytokinin and auxin in the plant also affects plant height and the number of tillers. Cytokinin is known to stimulate shoot growth or tiller in plant. The result indicated

**Table 1** Mean square value from ANOVA of plant height, the number of tillers per clump and biomass of vetiver grass (*Vetiveria Zizaniodes* L. Nash), Surat Thani ecotype inoculated with different types of mycorrhiza and different levels of phosphate fertilizer at 1 and 2 weeks after fertilizer application.

| SV | DF | Height | | Number of tiller | | Biomass |
|---|---|---|---|---|---|---|
| | | 1 week | 2 week | 1 week | 2 week | 2 week |
| Treatment | 15 | 6.86* | 6.23* | 6.77** | 7.63 ns | 0.503 ns |
| Mycorrhiza (V) | 3 | 9.78* | 6.55* | 14.64** | 20.52* | 0.645 ns |
| Phosphate (P) | 3 | 13.34** | 14.43** | 2.93 ns | 3.14 ns | 0.546 ns |
| V × P | 9 | 3.89 ns | 3.39 ns | 5.42 * | 3.84ns | 0.441 ns |
| Error | 48 | 2.852 | 2.662 | 2.359 | 8.380 | 0.406 |
| C.V. (%) | | 11.0 | 8.4 | 23.1 | 34.6 | 12.4 |

**Note :** ** Significant at P = 0.01, * Significant at P = 0.05

**Table 2** Plant height of vetiver grass (*Vetiveria zizanioides* L. Nash, Surat Thani ecotype) inoculated with different types of mycorrhiza and different levels of phosphate fertilizer at 1 week after phosphate fertilizers were applied.

| Treatments | Height (inch) /1<br>Phosphate fertilizer levels ($P_2O_5$ kg/ha) | | | | Mean |
|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | |
| Non AM | 14.38 a /2 | 14.25 b | 15.13 ab | 17.13 a | 15.22 b |
| *Glomus* sp. | 13.88 a | 15.38 ab | 15.38 ab | 14.75 a | 14.84 b |
| *G. aggregatum* | 14.75 a | 12.88 b | 14.25 b | 16.88 a | 14.69 b |
| *A. scrobiculata* | 16.00 a | 16.88 a | 17.00 a | 16.88 a | 16.69 a |
| Mean | 14.75 b /3 | 14.84 b | 15.44 ab | 16.41a | 15.36 |

C.V. (%) = 11.0 LSD .05 V-means = 1.20, P-means = 1.20, V*P means = 3.20

**Note :** /1 means value from 4 replications

/2 Identical characters in each column are not significant in confidential to 95% by DMRT

/3 Identical characters in row are not significant in confidential to 95% by DMRT

that plant height was stimulated by *A. scrobiculata* whereas the maximum tiller number was obtained from *G. aggregatum* inoculated plant. This may due to the ability of some arbuscular mycorrhiza such as *G. aggregatum* in inducing the production of cytokinin in vetiver and altering the ratio of cytokinin and auxin in plant. Therefore, the protrusion of vetiver tiller in *G. aggregatum* inoculum was increased. The control plant at 1 week and at harvesting stage had 5.5 and 7.5 tillers/clump respectively.

The maximum tiller number at 1 week stage

**Table 3** Plant height of vetiver grass (*Vetiveria zizanioides* L. Nash, Surat Thani ecotype) inoculated with different types of mycorrhiza and different levels of phosphate fertilizer at 2 weeks after phosphate fertilizers were applied.

| Treatments | Height (inch) /1<br>Phosphate fertilizer levels ($P_2O_5$ kg/ha)<br>0 | 30 | 60 | 90 | Mean |
|---|---|---|---|---|---|
| Non AM | 18.63 ab /2 | 18.50 bc | 19.00 a | 19.75 a | 18.97 b |
| *Glomus* sp. | 17.50 b | 19.75 ab | 20.50 a | 18.88 a | 19.16 b |
| *G. aggregatum* | 18.63 ab | 16.75 cd | 19.13 a | 20.13 a | 18.66 b |
| *A. scrobiculata* | 20.13 a | 20.88 a | 21.13 a | 21.00 a | 20.78 a |
| Mean | 18.72 b /3 | 18.97 ab | 19.94 a | 19.94 a | 19.39 |

C.V. (%) =8.4 LSD .05 V-means = 1.16, P-means = 1.16, V*P means = 2.32

**Note :** /1 means value from 4 replications

/2 Identical characters in each column are not significant in confidential to 95% by DMRT

/3 Identical characters in row are not significant in confidential to 95% by DMRT

**Table 4** Number of tiller per clump of vetiver grass (*Vetiveria zizanioides* L. Nash, Surat Thani ecotype) inoculated with different types of mycorrhiza and different levels of phosphate fertilizer at 1 week after phosphate fertilizers were applied.

| Treatments | Number of tiller per clump /1<br>Phosphate fertilizer levels ($P_2O_5$ kg/hectare)<br>0 | 30 | 60 | 90 | Mean |
|---|---|---|---|---|---|
| Non AM | 5.50 ab /2 | 7.25 a | 5.75 b | 5.75 b | 6.06 bc |
| *Glomus* sp. | 6.75 ab | 6.00 a | 7.00 b | 8.25 a | 7.00 ab |
| *G. aggregatum* | 7.75 a | 7.00 a | 10.25 a | 6.25 ab | 7.81 a |
| *A. scrobiculata* (big) | 5.25 b | 5.25 a | 6.00 b | 6.25 ab | 5.69 c |
| Mean | 6.31 a /3 | 6.38 a | 7.25 a | 6.63 a | 6.64 |

C.V. (%) = 23.1 LSD $_{.05}$ V-means = 1.09, P-means = 1.09, V*P means = 2.18

**Note :** /1 means value from 4 replications

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was found in *G. aggregatum* treated plant (10.25 tillers/clump) when vetiver grass was fertilized with 60 kg $P_2O_5$ / ha (Table 4 ). On the other hand, at 2 weeks stage, there was no significant ($P< 0.05$) difference affected by AM inocculation combined with phosphate fertilizer application (Table 1 and 5).

The enhancement of AM inoculum and phosphate fertilizer on total biomass or vetiver dry weight at harvesting stage were not significantly ($P< 0.05$) different (Table 1). When no phosphate was applied , those inoculated with AM in the plant receiving *A. scrobiculata* (5.48 g/clump), the highest dry matter was obtained which was not significantly different from the dry matter of *Glomus* sp., and *G. aggregatum* treatments (4.95 and 4.78 g/clmp, respectively) . The dry matter of the control plant tended to be the lowest (4.32 g/clump), however it also shown non significant different from *Glomus* sp. and *G aggregatum* treated plants (Table 6). Generally, the yield and growth of plants enhanced by mycorrhiza were higher than those in non-inoculated plants as reported by Ortus *et al.* (1996). However, the growth of host plant in this experiment in terms of tiller number and dry weight in some AM inoculums were not differed from the control. This may due to the limited time of growth since the plants were still small. On the other hand, if the growth period is extended, one might perceive the degradation of P-32 activity used in the experiment. Heijne *et al*. (1996) also reported that dry matter of herbs was higher in the control than in the AM inoculated plant. Plant fertilized with phosphate usually results in the increasing of plant dry matter yield. However, the yield of vetiver fertilized with various phosphate levels did not show significant ($P< 0.05$) difference among various treatments. Furlan and Berrier-Cardou (1989) reported that the presence of VA mycorrhiza inoculum has a stronger effect on the yield in the absence of phosphate than when phosphate was present.

**Phosphorus in plant**

The effects of AM inoculations and phosphate fertilizer on phosphorus (P) concentration and P-uptake of the plants were presented in Table 7 and 8, respectively. The phosphorus concentration and P-uptake of the control plant were 0.059% and 2.78 mg / clump, respectively. AM inoculation

**Table 5** Number of tiller per clump of vetiver grass (*Vetiveria zizanioides* L. Nash, Surat Thani ecotype) inoculated with different types of mycorrhiza and different levels of phosphate fertilizer at 2 weeks after phosphate fertilizers were applied.

| Treatments | Number of tiller per clump /1<br>Phosphate fertilizer levels ($P_2O_5$ kg/hectare)<br>0 | 30 | 60 | 90 | Mean |
|---|---|---|---|---|---|
| Non AM | 7.50 a /2 | 8.50 a | 7.75 a | 6.25 a | 7.50 b |
| *Glomus* sp. | 7.75 a | 9.25 a | 8.50 a | 9.75 a | 8.81 ab |
| *G. aggregatum* | 10.00 a | 10.50 a | 11.00 a | 7.50 a | 9.75 a |
| *A. scrobiculata* (big) | 7.50 a | 6.50 a | 7.75 a | 7.75 a | 7.38 b |
| Mean | 8.19 a /3 | 8.69 a | 8.75 a | 7.81 a | 8.36 |

C.V. (%) = 34.6 $LSD_{.05}$ V-means = 2.06, P-means = 2.06, V*P means = 4.12

**Note :** /1 means value from 4 replications

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gave highly significant (P< 0.01) increased P-concentration of the vetiver shoot .Likewise, the uptake of P in the shoot of AM inoculated plant was also highly significant (P< 0.01) increased ( Table 7). Among the 3 types of mycorrhizal treatments ,*A. scrobiculata* was the best inoculum to increase P-uptake (4.97 mg/clump) of vetiver On the other hand, the average concentration of P in *A. scrobiculata* plant (0.91%) was not differed from the other AM inocculations. However, when no P-fertilizer was applied, *G.aggregatum* inoculum tend to be the most superior in enhancing P concentration (0.104%) in the vetiver shoot. At 60 kg $P_2O_5$ / ha level of application, the highest P-uptake (6.25 g/ clump) was obtained in *A. scrobiculata* treated plant. Increasing the phosphate fertilizer up to 90 kg

**Table 6** Plant biomass of vetiver grass (*Vetiveria zizanioides* L. Nash, Surat Thani ecotype) inoculated with different types of mycorrhiza and different levels of phosphate fertilizer at 2 weeks after phosphate fertilizers were applied.

| | Plant biomass (gm./clump) /1 Phosphate fertilizer levels ($P_2O_5$ kg/hectare) | | | | |
|---|---|---|---|---|---|
| Treatments | 0 | 30 | 60 | 90 | Mean |
| Non AM | 4.32 b /2 | 5.25 a | 5.10 a | 4.83 a | 4.88 a |
| *Glomus* sp. | 4.95 ab | 5.45 a | 5.33 a | 5.35 a | 5.27 a |
| *G. aggregatum* | 4.78 ab | 5.05 a | 5.48 a | 5.20 a | 5.13 a |
| *A. scrobiculata* | 5.48 a | 5.30 a | 4.77 a | 5.75 a | 5.32 a |
| Mean | 4.88 a /3 | 5.26 a | 5.17 a | 5.28 a | 5.15 |

C.V. (%) = 12.4 LSD $_{.05}$ V-means = 0.91, P-means = 0.91, V*P means = 0.91

**Note :** /1 means value from 4 replications

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**Table 7** Mean square value from ANOVA of percentage of phosphorus (%P), total phosphorus (TP), percentage of phosphorus derived from fertilizer (% Pdff) and Fertilizer P Utilization (%FPU) in vetiver grass (*Vetiveria Zizaniodes* L. Nash, Surat Thani ecotype) inoculated with different types of mycorrhiza and different levels of phosphate fertilizer at 2 weeks after fertilizer application

| SV | DF | % P | TP | % Pdff | % FPU |
|---|---|---|---|---|---|
| Treatment | 15 | 0.000878** | 3.915** | 0.1119** | 0.00164** |
| Mycorrhiza (V) | 3 | 0.002984** | 12.490** | 0.2969** | 0.00368** |
| Phosphate (P) | 3 | 0.000585* | 3.114* | 0.0043 ns | 0.00140** |
| V x P | 9 | 0.000274 ns | 1.323 ns | 0.0552** | 0.00070** |
| Error | 48 | 0.000261 | 0.740 | 0.0094 | 0.00011 |
| C.V. (%) | | 20.7 | 21.1 | 45.5 | 55.0 |

**Note :** ** Significant at P = 0.01, * Significant at P = 0.05

$P_2O_5$ / ha no significant effects of AM inocculums were observed (Table 8 and 9).

The percentage of P-32 derived from fertilizer and the P-availability in vetiver inoculated with various mycorrhizal species and AM inocculations combined with phosphate fertilizer showed significant ($P< 0.05$) difference among treatments (Table 7). Similarly phosphate fertilizer significantly increased P availability but did not significantly ($P< 0.05$) enhance the percentage of P derived from fertilizer . AM inoculation resulted in the increased percentage of P-32 derived from

**Table 8** Percentage of phosphorus in vetiver grass (*Vetiveria zizanioides* L. Nash, Surat Thani ecotype) inoculated with different types of mycorrhiza and different levels of phosphate fertilizer at 2 weeks after phosphate fertilizers were applied.

| Treatments | Phosphorus (%) /1<br>Phosphate fertilizer levels ($P_2O_5$ kg/hectare)<br>0 | 30 | 60 | 90 | Mean |
|---|---|---|---|---|---|
| Non AM | 0.059 c /2 | 0.055 b | 0.058 b | 0.064 b | 0.059 c |
| *Glomus* sp. | 0.083 ab | 0.066 ab | 0.090 a | 0.072 ab | 0.078 ab |
| *G. aggregatum* | 0.104 a | 0.081 a | 0.080 ab | 0.075 ab | 0.085 ab |
| *A. scrobiculata* | 0.091 a | 0.082 a | 0.099 a | 0.090 a | 0.091 a |
| Mean | 0.084 a /3 | 0.071 b | 0.082 ab | 0.075 ab | 0.078 |

C.V. (%) = 20.7 LSD $_{.05}$ V-means = 0.012, P-means = 0.012, V*P means = 0.023

**Note :** /1 means value from 4 replications

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**Table 9** Total phosphorus uptake (mg./clump) in vetiver grass (*Vetiveria zizanioides* L. Nash, Surat Thani ecotype) inoculated with different types of mycorrhiza and different levels of phosphate fertilizer at 2 weeks after phosphate fertilizers were applied.

| Treatments | Total Phosphorus uptake (mg./clump) /1<br>Phosphate fertilizer levels ($P_2O_5$ kg/hectare)<br>0 | 30 | 60 | 90 | Mean |
|---|---|---|---|---|---|
| Non AM | 2.78 b /2 | 2.96 b | 2.64 c | 3.06 a | 2.86 c |
| *Glomus* sp. | 4.06 a | 3.56 ab | 5.50 ab | 3.81 a | 4.23 b |
| *G. aggregatum* | 4.83 a | 4.03 ab | 4.33 b | 3.86 a | 4.26 b |
| *A. scrobiculata* | 4.92 a | 4.34 a | 6.25 a | 4.38 a | 4.97 a |
| Mean | 4.15 ab /3 | 3.72 b | 4.68 a | 3.78 b | 4.08 |

C.V. (%) = 21.1 LSD $_{.05}$ V-means = 0.61, P-means = 0.61, V*P means = 1.22

**Note :** /1 means value from 4 replications

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fertilizer and P availability. *A. scrobiculata* was the best inoculum to increase the percentage of P-32 derive from fertilizer (0.429% Pdff) , P availability (0.044% FPU*), and the effect of this mycorrhiza was clearly shown when low amount of P-fertilizer was applied to the soil (table 10 and 11).

Generally, mycorrhizal plant has greater P-content than non-mycorrhizal plant. The increased uptake of P may result from the increased absorbing area contributed by hyphae (Pearson and Tinker,

**Table 10** Percentage of phosphorus derived from fertilizer (% P dff) in vetiver grass (*Vetiveria zizanioides* L. Nash, Surat Thani ecotype) inoculated with different types of mycorrhiza and different levels of phosphate fertilizer at 2 weeks after phosphate fertilizers were applied.

| Treatments | Phosphorus derived from fertilizer (% P dff *) /1<br>Phosphate fertilizer levels ($P_2O_5$ kg/hectare)<br>30 | 60 | 90 | Mean |
|---|---|---|---|---|
| Non AM | 0.074 b /2 | 0.051b | 0.105 b | 0.077 c |
| *Glomus* sp. | 0.097 b | 0.144 b | 0.114 b | 0.118 c |
| *G. aggregatum* | 0.128 b | 0.173 b | 0.382 a | 0.228 b |
| *A. scrobiculata* | 0.483 a | 0.545 a | 0.257 a | 0.429 a |
| Mean | 0.196 a /3 | 0.228 a | 0.214 a | 0.213 |

C.V. (%) = 45.5 LSD $_{.05}$ V-means = 0.080, P-means = 0.069, V*P means = 0.139

**Note :** /1 means value from 4 replications

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**Table 11** Fertilizer P- utilization (% FPU or A-value) in vetiver grass (*Vetiveria zizanioides* L. Nash, Surat Thani ecotype) inoculated with different types of mycorrhiza and different levels of phosphate fertilizer at 2 weeks after phosphate fertilizers were applied.

| Treatments | Fertilizer P Utilization (% FPU *) /1<br>Phosphate fertilizer levels ($P_2O_5$ kg/hectare)<br>30 | 60 | 90 | Mean |
|---|---|---|---|---|
| Non AM | 0.006 c /2 | 0.002 b | 0.004 a | 0.004 c |
| *Glomus* sp. | 0.012 bc | 0.012 b | 0.005 a | 0.010 bc |
| *G. aggregatum* | 0.023 b | 0.013 b | 0.016 a | 0.017 b |
| *A. scrobiculata* | 0.075 a | 0.037 a | 0.019 a | 0.044 a |
| Mean | 0.029 a /3 | 0.016 b | 0.011 b | 0.019 |

C.V. (%) = 55.0 LSD $_{.05}$ V-means = 0.008, P-means = 0.007, V*P means = 0.0147

**Note :** /1 means value from 4 replications

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* = This data derived from P-32 analysis.

1975) and root growth with greater affinity of site transporting phosphate (Cress *et al*., 1979). An enhanced P-sink was caused by higher photosynthetic rate accompanying mycorrhizal infection, and increased phosphate activity (Allen *et al*., 1981). Gianinazzi and Gianinazzi (1986) reported that in P-deficient soil, mycorrhizal was shown to enable exploitation of non-labile organic phosphate to inorganic available from the soil by excretion of phosphatase enzyme, therefore plant growth was improved.

Increasing phosphate fertilizer upto 90 kg $P_2O_5$ / ha resulted in non-significant ($P< 0.05$) effects of AM inoculum on P-uptake and P-availability. It was clear that at high P-level, the effect of mycorrhiza on plant growth was not observed. This was due to the reduction of the effectiveness of mycorrhizal fungi by phosphate (Smith, 1980), and may change from mutualism to parasitism to endophyte-host relationship as phosphorus availability increased (Crush, 1976). Under such condition mycorrhizal may not contribute to the plant growth. The result showed that radioisotope tracer technique provides better indexes of phosphorus availability and P- uptake of the vetiver plant and also is a good method for selecting effective mycorrhiza species to improve the growth and P- uptake of the vetiver.

## CONCLUSION

**From the results of this study :**

1. Arbuscular mycorrhizal inoculum significantly affects height, tiller number, and vetiver Biomass. *A. scrobiculata* inoculum gave the greatest height and vetiver biomass whereas the maximum tiller number was obtained from *G. aggregatum* treated plant.

2. Plant biomass was not significantly effected by AM inoculation, phosphate fertilizer and AM inocculation combined with P-fertilizer supplied. Howerver, when no P-fertilizer was applied *A. scrobiculata* tended to give greater plant dry matter than other treatments.

3. AM inocculation significantly increased P-concentration, P-uptake,% P derived from fertilizer and P- availability of vetiver. At 60 kg $P_2O_5$/ha level of application, the highest P-uptake,% P derived from fertilizer and P- availability were obtained from *A. scrobiculata* treated plant ,while the highest P-concentration of vetiver was obtained from *G. aggregatum* treatment with no P-fertilizer supplied.

4. Increasing P- fertilizer upto 90kg/ha, non significant effects of AM inocculation on P-concentration, P-uptake,% P derived from fertilizer and P- availability of vetiver were observed.

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## LITURATURE CITED

Abbott, L.K. and A.D. Robson. 1984. The effect of VA mycorrhizal on plant growth, pp. 113-130. *In* C.L. Powell and D.J. Bagyaraj (eds.). VA Mycorrhiza. CRC press, Inc., USA.

Allen, M.F., J.C. Sexton, T.S. Moore, and M. Christensen. 1981. Influence of phosphate source on vesicular-arbuscular mycorrhizae of *Bouteloua gracilis*. New Phytol. 87 : 687.

Cress, W.A., E.D. Throneberry, and D.L. Lindsey. 1979. Kinetics of phosphorus absorption by mycorrhizal and non mycorrhizal tomato root. Plant Physiol. 64 : 484-487.

Crush, J.R. 1976. Endomycorrhizae and legume growth in some soil of Mackenzie Basin, N.Z. J. Agri. Res. 19 : 473-476.

Fried, M. 1954. Quantitative evaluation of proceased and natural phosphate. Agri. Food Chem. 2 : 241-244.

Furlan, V. and M. Bernier-Cardou. 1989. Effect of N, P and K on formation of vesicular- arbuscular mycorrhizae, growth and mineral content of onion. Plant and Soil 113 : 167-174.

Gerdemann J.W. and T.H> Nicolson 1963. Spore of Mycorrhizal Endogone species extracted from soil by wet sieving and decanting. Trans. Br. Mycol.Soc. 46 : 235-244. *In* Workshop on VA Mycorrhiza and its Application in Agriculture and Environment. Kasetsart University, Bangkok, Thailand.

Gianinazzi–Pearson, V. and S. Gianinazzi. 1986. The Physiology improved phosphate nutrition in mycorrhizal plants, pp. 101- 106. *In* V. Gianinazzi-Pearson and S. Gianinazzi (eds.). Physiology and Genetical Aspects of Mycorrhizae. INRA, Paris.

Hardarson, G. 1990. Use of Nuclear Technique in Studies of Soil-Plant Relationship. International Atomic Energy Agency, Vienna. 223 p.

Hayman, D. S. 1983. The Physiology of vesicular arbuscular mycorrhiza formation. New Phytol. 9 : 683-690.

Heijne, B. D., D. Van Dam, G.W. Heil, and R. Bubbink. 1996. Acidification effects on vesicular arbuscular mycorrhizal (VAM) infection, growth and nutrient uptake of established healthland herb species. Plant and Soil. 179 : 197-206.

Joner E.J. and I. Jacobson. 1995. Growth and extracellular phosphatase activity of arbuscular mycorrhizal inoculation on the growth and phosphorus nutrition of maize. J. of Plant Nutri. 20 : 1607-1616.

Kucey, R.M. N. and J.B Bole. 1984. Availability of phosphorus from rock phosphate in moderately and weakly acidic soils as determinated by P-32 dilution, A value and total P uptake methods. Soil Sci. 138 : 180-188.

Mayer, F.H. 1974. Physiology of mycorrhiza. Ann. Rev. Plant Physiol. 25 : 564-568.

Menge, J.A., B. Steirle, D.J. Bagyaraj, E.L.V. Johnson, and R.T. Leonard. 1978. Phosphorus concentrations in plants responsible for inhibition of mycorrhizal infection. New Phytol. 80 : 575-578.

Mosse, B. 1973. Advances in the study of Vesicular-arbuscular mycorrhiza. Ann. Rev. Phytol. Pathol. 11 : 171-198.

Ortas, I., P.J.Harris, and D.L. Powell. 1996. Enhanced uptake of phosphorus by mycorrhizal sorghum plants influenced by forms of nitrogen. Plant and Soil 184 : 255-264.

Pearson, V. and P.B. Tinker. 1975. Measurement of phosphorus fluxes in the external hyphae of endomycorrhizae, pp. 277-287. *In* F.E. Sanders, B. Mosse and P.B. Tinker. (eds.). Endomycorrhizas. Academic Press, New York.

Smith, S.E. 1980. Mycorrhizal of autotrophic higher plants. Bio. Rev. 55 : 475-510.

Smith, S.E. and V. Gianinazzi-Pearson. 1988. Physiological interactions between symbionts in vesicular-arbuscular mycorrhizal plants. Annu. Rev. Plant Physiol. Plant Molecul. Biol. 39 : 221-244.

Tassanee, A., C. Chanjaroensuk, and S. Jintakanon. 1989. Soil and Plant Analysis : Practice and Laboratory Manual. Department of Soil Science, Faculty of Agriculture, Kasetsart University. Bangkok. 171 p.

Techapinyawat,S , P. Suwannarit, P. Pakkong, N. Sinbuathong ,and P. Sumthong. 2001. Selection of Effective Vesicular-Arbuscular Mycorrhizal Fungi on Growth and Nutrient Uptake of Vetiver. Thai J. Agric. Sci. 34(1-2) : 91-99.

Valentine, A.J., B.A. Osborne, and D.T. Mitchell. 2000. Interactions between phosphorus supply and total nutrient availability on mycorrhizal colonization, growth and photosynthesis of cucumber. Scientia Hort. 88 : 177-189.

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# Influence of Feeding Management and Seasons on Yield and Composition of Milk Produced from Friesian Crossbred Cows Raised Under Hot and Humid Environment in Central Thailand

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## ABSTRACT

A twelve-month on-farm experiment was carried out to investigate the effects of feeding management and seasons on yield and composition of milk from a total of 825 dairy cows with different Friesian crossbred levels raised under the hot and humid environment in central Thailand. Forty pre-selected farms were classified into two groups of twenty farms each (standard and substandard) according to feed and feeding management practices. The cows in first group received feeds that met the NRC's energy and protein daily requirments, whereas those in the second group were provided with substandard feeding practices. The % Friesian crossbred cows were classified into < 75, 75, 87.5, and >87.5 %. All cows were raised indoors throughout the three seasons (summer, rainy, and winter). It was observed that milk yield from cows fed the standard diet, averaging 15.90 kg/day, was 2.02 kg/day higher (P<.01) than those in the substandard group. The standard fed cows produced 0.25 % more (P<.01) milk fat (4.37 versus 4.12%), 0.16% milk protein (3.43 versus 3.27%), and 0.23 % SNF (8.81 versus 8.58 %) than the substandard ones respectively. Milk yield increased proportionally with increasing % Friesian crossbred levels, whereas milk components gradually decreased. The decline in milk composition was higher in the substandard fed group when compared to the group receiving the standard feed. In addition, the cows during the hot season produced milk with lower (P<0.01) milk protein and SNF than those in the remaining rainy and winter seasons.

**Key words:** milk yield, milk composition, Friesian crossbred, hot and humid environment

## INTRODUCTION

One of the problems in dairy production in Thailand is the decline of milk composition, especially solid not fat (SNF). Swamiphak (1996) reported that SNF of raw milk from northern, north-eastern, southern and central Thailand averaged at 8.67, 8.43, 8.17 and 8.13 % respectively. According to Kaewkamcharn (2000) and Swamiphak(1996) a gradual decline of SNF from 1993 to 1999 in raw milk from collecting centers under the Dairy Farming Promotion Organization of Thailand was observed. Since SNF is one of the parameters in the milk price payment scheme for most dairy processors in the country, this economic loss has caused a major concern among the dairy farmers and the authorities.

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A number of factors affecting milk composition have been reported. The factors include feed and feeding practices, breed, seasons, lactation period, lactation number, milking technique and health conditions (Philpot, 1984; Collier, 1985; Sutton, 1989; Nickerson, 1995; Davison *et al*., 1996). However, the effects of feeding and the environment on yield and milk components in milking cows with various genetic potential under tropical conditions are limited. Therefore, the objective of this study is to investigate the situation.

## MATERIALS AND METHODS

### Animals and dairy farms

A one year on-farm experiment with a total number of 825 milking cows from 40 preselected dairy farms located around Saraburi province in central Thailand was carried out. The cows in studied area were Holstein Friesian cross breed with varying genetic levels of *Bos taurus*. The farms were equally divided into two groups (standard versus substandard) according to feed and feeding management practices. The cows in first group received feeds that met the NRC's (1988) energy and protein daily requirments, whereas those in the second group were provided with substandard feeding practices. All cows were normally housed in freestall barns all year round. Depending on accessibility, labours and seasons (summer, rainy and winter seasons), the cows were usually provided with fresh cut grasses and / or crop residues *ad libitum*. The animals also received commercially available 16 to 18 % crude protein concentrates with or without grains or agro- industrial by-products twice daily at milking time (about 06.00 and 16.00 h).

### Data collection and statistical analysis

Farm visits were carried out at about 30 day intervals for a period of one year (March 2001 to February 2002). Daily milk yield as well as body score of individual cow was recorded during each farm visit. A composite of potassium dichromate preserved morning (30 ml) and afternoon (20 ml) milk from each milking cow was analyzed for milk composition (fat, protein and SNF) using Foss Electric MilkoScan 104. At the same time, roughages and concentrates provided to the cows were recorded and sampled for composition analysis following the procedures outlined by AOAC (1984). Feed analysis results were used to verify the consistency of the two feeding groups during the trial.

A multifactor factorial model was used in analyzing the data. The fixed effects were two feeding management (standard and substandard feedings), three seasons (summer, rainy, and winter seasons) and four genetic levels of crossbred Friesian (< 75, 75, 87.5, and >87.5 %). Daily milk yield and milk components were taken as random variables. Complex analysis using day in milk (DIM), lactation number and body condition scores as covariates was performed and variations among cows were also included in the following model.

$$Y_{ijkl} = \mu + F_i + S_j + (FS)_{ij} + HF_k + (FHF)_{ik} + (SHF)_{jk} + (FSHF)_{ijk} + ID(FSHF)_{ijk} + \beta(DIM_{ijkl} - \overline{DIM}) + \beta(LNO_{ijkl} - \overline{LNO}) + \beta(BS_{ijkl} - \overline{BS}) + \varepsilon_{ijkl}$$

where $Y_{ijkl}$ = trait of cow associated with all covariates; m = mean intercept; $F_i$ = fixed effect of i $^{th}$ feeding management; $S_j$ = fixed effect of j $^{th}$ season; $HF_k$ = fixed effect of k $^{th}$ % crossbred Friesian cows; $(FS)_{ij}$ = interaction between i $^{th}$ feeding management and j $^{th}$ season; $(FHF)_{ik}$ = interaction between i $^{th}$ feeding management and k $^{th}$ % crossbred Friesian; $(SHF)_{jk}$ = interaction between j $^{th}$ season and k $^{th}$ % crossbred Holstein; $(FSHF)_{ijk}$ = ijk fixed factor interaction; $C(FSHF)_{ijk}$ = random effect of ijk $^{th}$ cow subjected to all fixed factors; $b(DIM_{ijkl} - \overline{DIM})$ = regression effect of days in milk; $b(LNO_{ijkl} - \overline{LNO})$ = regression effect of lactation number; $b(BS_{ijkl} - \overline{BS})$ = regression effect of body condition score; $e_{ijkl}$ = random residual error.

## RESULTS AND DISCUSSION

### Effect of feeding management and levels of Friesian crossbred

#### 1. Milk yield

The effects of nutrition and levels of Friesian crossbred on milk yield of the dairy cows are illustrated in Table 1. The cows under proper feeding program produced an average of 15.90 kg/day milk as compared to 13.88 kg/day from those receiving the substandard feed. In addition, dairy cows for the <75, 75, 87.5, and >87.5 % crossbred groups yielded an average of 12.85, 12.92, 15.17, and 17.76 kg/day of milk respectively. Within the same crossbred group, dairy cows subjected to under feeding management consistently gave less ($P<0.01$) milk than those receiving proper feeding. However, the decline of milk yield was more ($P<0.01$) pronounced in the high Friesian crossbred groups. These findings agree with the reports of Gibson (1989) and Intharatul (1996) that cows with high genetic merit tended to provide more milk and that poor nutrition could alter the situation. Maximum energy intake and utilization had been reported to be crucial for optimal health and production of high yielding dairy cows (Heuer *et al*., 2000). In addition, Collier (1985) also reported that nutrients required by cows were directly related to the changes in yield and composition of milk.

#### 2. Milk composition

Table 2-4 illustrate the influence of feeding management and levels of Friesian crossbred respectively on fat, protein and SNF content in milk. The cows receiving optimal feeding when compared to those fed the substandard feed produced milk with higher ($P<0.01$) contents of fat (4.37 versus 4.12 %), protein (3.43 versus 3.27 %), and SNF (8.81 versus 8.58 %) respectively. In addition, dairy cows for the <75, 75, 87.5, and >87.5 % crossbred groups provided milk with lower ($P<0.01$) fat (4.43, 4.19, 4.18, and 4.07 %), protein (3.61, 3.43, 3.34, and 3.26 %) and SNF (8.93, 8.81, 8.68, and 8.64 %) composition, respectively. Genetics is believed to partially contribute to the declining trends for the three milk components in high Friesian crossbred . On the same token, dilution effects from high milk secretion in these cows also play a role. However, it is evident from this study that proper feeding, to a certain extent, can alleviate the situation. Similar results were evident in the studies of Akerlind *et al*. (1999) and Sandoval-Castro *et al*. (2000) who observed that proper feed supplementation resulted in more fat and protein contents in milk from dairy cows.

**Table 1** Least square means (±SE) of milk yield (kg/day) from Friesian crossbreds receiving different feeding management.

| Feeding management | Friesian crossbred , % | | | | Main effect |
|---|---|---|---|---|---|
| | < 75 | 75 | 87.5 | > 87.5 | |
| Standard | 13.08±0.21 $^{f}$ | 14.09±0.09 $^{e}$ | 16.27±0.10 $^{c}$ | 19.08±0.18 $^{a}$ | 15.90±0.09 $^{x}$ |
| Under standard | 12.32±0.26 $^{g}$ | 11.89±0.12 $^{h}$ | 14.49±0.09 $^{d}$ | 16.92±0.19 $^{b}$ | 13.88±0.10 $^{y}$ |
| Main effect | 12.85±0.24 $^{o}$ | 12.92±0.12 $^{o}$ | 15.17±0.11 $^{n}$ | 17.76±0.21 $^{m}$ | |

$^{abcdefgh}$ Means with different superscripts within feed and % crossbred interactive effects are different ($P<0.01$).

$^{mno}$ Means with different superscripts in the same row for main effects are different ($P<0.01$).

$^{xy}$ Means with different superscripts in the same column for main effects are different ($P<0.01$).

**Table 2** Least square means (±SE) of milk fat (%) from Friesian crossbreds receiving different feeding management.

| Feeding management | Friesian crossbred , % | | | | Main effect |
|---|---|---|---|---|---|
| | < 75 | 75 | 87.5 | > 87.5 | |
| Standard | 4.47±0.02 $^{a}$ | 4.36±0.02 $^{b}$ | 4.32±0.02 $^{b}$ | 4.08±0.04 $^{c}$ | 4.37±0.02 $^{x}$ |
| Under standard | 4.39±0.06 $^{ab}$ | 4.01±0.03 $^{c}$ | 4.06±0.03 $^{c}$ | 4.05±0.04 $^{c}$ | 4.12±0.03 $^{y}$ |
| Main effect | 4.43±0.05 $^{m}$ | 4.19±0.02 $^{n}$ | 4.18±0.02 $^{n}$ | 4.07±0.05 $^{o}$ | |

$^{abc}$ Means with different superscripts within feed and % crossbred interactive effects are different (P<0.01).
$^{mno}$ Means with different superscripts in the same row for main effects are different (P<0.01).
$^{xy}$ Means with different superscripts in the same column for main effects are different (P<0.01).

**Table 3** Least square means (±SE) of milk protein (%) from Friesian crossbreds receiving different feeding management.

| Feeding management | Friesian crossbred , % | | | | Main effect |
|---|---|---|---|---|---|
| | < 75 | 75 | 87.5 | > 87.5 | |
| Standard | 3.68±0.02 $^{a}$ | 3.53±0.01 $^{b}$ | 3.39±0.01 $^{c}$ | 3.31±0.02 $^{d}$ | 3.43±0.01 $^{x}$ |
| Under standard | 3.52±0.02 $^{b}$ | 3.34±0.01 $^{d}$ | 3.27±0.01 $^{e}$ | 3.26±0.02 $^{e}$ | 3.27±0.01 $^{y}$ |
| Main effect | 3.61±0.02 $^{m}$ | 3.43±0.03 $^{n}$ | 3.34±0.01 $^{o}$ | 3.26±0.05 $^{p}$ | |

$^{abcde}$ Means with different superscripts within feed and % crossbred interactive effects are different (P<0.01).
$^{mnop}$ Means with different superscripts in the same row for main effects are different (P<0.01).
$^{xy}$ Means with different superscripts in the same column for main effects are different (P<0.01).

**Table 4** Least square means (±SE) of milk SNF (%) from various Friesian crossbreds receiving different feeding management.

| Feeding management | Friesian crossbred , % | | | | Main effect |
|---|---|---|---|---|---|
| | < 75 | 75 | 87.5 | > 87.5 | |
| Standard | 9.03±0.02 $^{a}$ | 8.95±0.01 $^{b}$ | 8.79±0.01 $^{c}$ | 8.76±0.02 $^{c}$ | 8.81±0.01 $^{x}$ |
| Under standard | 8.80±0.02 $^{c}$ | 8.69±0.01 $^{d}$ | 8.58±0.01 $^{e}$ | 8.57±0.02 $^{e}$ | 8.58±0.01 $^{y}$ |
| Main effect | 8.93±0.02 $^{m}$ | 8.81±0.01 $^{n}$ | 8.68±0.01 $^{o}$ | 8.64±0.02 $^{p}$ | |

$^{abcde}$ Means with different superscripts within feed and % crossbred interactive effects are different (P<0.01).
$^{mnop}$ Means with different superscripts in the same row for main effects are different (P<0.01).
$^{xy}$ Means with different superscripts in the same column for main effects are different (P<0.01).

**Effect of feeding management and seasons**

**1. Milk yield**

Seasonal changes had a significant effect (P<0.01) on milk production. As shown in Table 5, cows during the rainy season gave lower (P<0.01) milk yield than those during the summer and winter (14.52, 15.07, and 15.27 kg/day, respectively). Since green forages are normally avilable during the rainy season, low average milk yield is not expected. This reflect the accessibility problems to green forages by the dairy farmers in the area either from water lodging situation and/or limited pasture area However, milk production from dairy cows under the three climatic conditions varied dependently with the feeding practices. Collier (1985) and Sandoval-Castro *et al*. (2000) reported that the availability and quality of roughage feed under a tropical dairy system were normally variable and consequently could influence the level of milk output as well as its components.This situation could be somewhat alleviated with proper supplementation. It is evident from this study as shown in Table 5 that the cows receiving proper nutrition produced 13.3, 14.2, and 16.3 % more (P<0.01) milk when compared to those receiving substandard feed during the summer, rainy, and winter seasons,respectively.

**2. Milk composition**

Seasonal and feeding effects on milk fat, protein, and SNF are shown in Table 6, 7 and 8, respectively. Average milk fat (4.22, 4.30, and 4.23 %), protein (3.29, 3.41 and 3.36 %), and SNF (8.60, 8.77, and 8.73 %) for the summer, rainy and winter seasons were significantly different (P<0.01). The highest milk fat, protein, and SNF were observed in milk during the rainy season when roughages were supposed to be available in both quantity and quality. However, the low milk yield during this season indicated the influence rather from dilution effect not from roughages. In addition, during the rainy season, the cows recieving standard feeding when compared to those fed the substandard one produced milk with 6.39 % more fat (4.43 versus 4.17 %), 3.58 % more protein (3.47 versus 3.35 %), and 2.07 % more SNF ( 8.86 versus 8.68 %), respectively. The variation of milk components by seasons is in part related to the effect from climatic environment (Nickerson, 1995; Davison *et al*., 1996). In addition, Sutton (1989) and Davison *et al*. (1996) indicated that proper feeding management could improve not only milk components but also milk yield under adverse environment.

**Table 5** Least square means (±SE) of milk yield (kg/day) from cows receiving different feeding management at different seasons.

| Feeding management | Seasons | | | Main effect |
|---|---|---|---|---|
| | Summer | Rainy | Winter | |
| Standard | 16.01±0.11$^{a}$ | 15.48±0.15$^{b}$ | 16.21±0.08$^{a}$ | 15.90±0.09$^{x}$ |
| Under standard | 14.13±0.11$^{c}$ | 13.56±0.13$^{d}$ | 13.94±0.11$^{cd}$ | 13.88±0.10$^{y}$ |
| Main effect | 15.07±0.10$^{m}$ | 14.52±0.12$^{n}$ | 15.07±0.07$^{m}$ | |

$^{abcd}$ Means with different superscripts within feed and % crossbred interactive effects are different (P<0.01).

$^{mn}$ Means with different superscripts in the same row for main effects are different (P<0.01).

$^{xy}$ Means with different superscripts in the same column for main effects are different (P<0.01).

**Table 6** Least square means (±SE) of milk fat (%) from cows receiving different feeding management at different seasons.

| Feeding management | Seasons | | | Main effect |
|---|---|---|---|---|
| | Summer | Rainy | Winter | |
| Standard | 4.36±0.03 $^{b}$ | 4.43±0.04 $^{a}$ | 4.33±0.02 $^{b}$ | 4.37±0.02 $^{x}$ |
| Under standard | 4.07±0.03 $^{d}$ | 4.17±0.03 $^{c}$ | 4.13±0.03 $^{c}$ | 4.12±0.03 $^{y}$ |
| Main effect | 4.22±0.03 $^{n}$ | 4.30±0.03 $^{m}$ | 4.23±0.02 $^{n}$ | |

$^{abcd}$ Means with different superscripts within feed and % crossbred interactive effects are different (P<0.01).
$^{mn}$ Means with different superscripts in the same row for main effects are different (P<0.01).
$^{xy}$ Means with different superscripts in the same column for main effects are different (P<0.01).

**Table 7** Least square means (±SE) of milk protein (%) from cows receiving different feeding management at different seasons.

| Feeding management | Seasons | | | Main effect |
|---|---|---|---|---|
| | Summer | Rainy | Winter | |
| Standard | 3.38±0.01 $^{b}$ | 3.47±0.01 $^{a}$ | 3.45±0.01 $^{a}$ | 3.43±0.01 $^{x}$ |
| Under standard | 3.21±0.01 $^{e}$ | 3.35±0.01 $^{c}$ | 3.27±0.01 $^{d}$ | 3.27±0.01 $^{y}$ |
| Main effect | 3.29±0.01 $^{o}$ | 3.41±0.01 $^{m}$ | 3.36±0.01 $^{n}$ | |

$^{abcde}$ Means with different superscripts within feed and % crossbred interactive effects are different (P<0.01).
$^{mno}$ Means with different superscripts in the same row for main effects are different (P<0.01).
$^{xy}$ Means with different superscripts in the same column for main effects are different (P<0.01).

**Table 8** Least square means (±SE) of milk SNF (%) from cows receiving different feeding management at different seasons.

| Feeding management | Seasons | | | Main effect |
|---|---|---|---|---|
| | Summer | Rainy | Winter | |
| Standard | 8.72±0.01 $^{b}$ | 8.86±0.02 $^{a}$ | 8.87±0.01 $^{a}$ | 8.81±0.01 $^{x}$ |
| Under standard | 8.48±0.01 $^{e}$ | 8.68±0.01 $^{c}$ | 8.60±0.01 $^{d}$ | 8.58±0.01 $^{y}$ |
| Main effect | 8.60±0.01 $^{o}$ | 8.77±0.01 $^{m}$ | 8.73±0.01 $^{n}$ | |

$^{abcde}$ Means with different superscripts within feed and % crossbred interactive effects are different (P<0.01).
$^{mno}$ Means with different superscripts in the same row for main effects are different (P<0.01).
$^{xy}$ Means with different superscripts in the same column for main effects are different (P<0.01).

## CONCLUSION

Feeding management, seasons, and % Friesian crossbred as well as their interactions were found to have significant effect on yield and composition of milk. As the levels of Friesian blood in the dairy cows increased, milk yield also increased. Contrary to this, a decline of milk compositions was evident. Milk protein and SNF were higher during rainy season than the summer and winter seasons. Proper feeding significantly increased milk yield and, to a certain extent, could alleviate the decline in milk components. Hence, it is evident that under hot and humid environments, proper feeding management is important in dairy farming especially for those raising the high genetic merit cows.

## LITERATURE CITED

Akerlind, M., K. Holtenius, J. Bertilsson, and M. Emanuelson. 1999. Milk composition and feed intake in dairy cows selected for high or low milk fat percentage. Livest. Prod. Sci. 59 :1-11.

AOAC. 1984. Official Methods of Analysis. 14th ed. Association of Official Analytical Chemists, Washington, D.C. 1141 p.

Collier, R.J. 1985. Nutritional, metabolic and environmental aspect of lactation, pp. 80-128. *In* B.L. Larson (ed.). Lactation. The Iowa State University Press, Iowa.

Davison, T., M. McGowan, D. Mayer, B. Young, N. Johnson, A. Hall, A. Matschoss, P. Goodwin, J. Goughan, and M. Lake. 1996. Managing Hot Cows in Australia. Department of Primary Industries, Queensland. 58 p.

Gibson, J.P. 1989. Altering milk composition through genetic selection. J. Dairy Sci. 72 : 2815-2825.

Heuer,C., W.M. Van Straalen, Y.H. Schukken, A. Dirkzwager, and J.P.T.M. Noordhuizen. 2000. Prediction of energy balance in a high yielding dairy herd in early lactation: model development and precision. Livest. Prod. Sci. 65 : 91-105.

Intharatul, T. 1996. Productivity and Manangement of High Producing cows. KU Publishing and Trading Ltd., Bangkok. 73 p.

Kaewkamchan, S., P. Vijchulata, P. Chirattanayuth, V. Santisopasri, and S. Panichying. 2000. Raw milk quality from milk collecting centers of Dairy Farming Promotion Organization of Thailand during 1996-1999, pp. 217-223. *In* The 38th Kasetsart University Annual Conference. 2000. Subject: Animals and Veterinary Medicine. Physic Center Pub., Bangkok.

Nickerson, S.C. 1995. Milk production: factors affecting milk composition, pp. 3-33. *In* F. Harding (ed.). Milk Quality. Blackie Academic and Professional, Glasgow.

National Research Council. 1988. Nutrient Requirement of Dairy Cattle. 6th ed., National Academy Press, Washington, D.C. 157 p.

Philpot, N.W. 1984. Mastitis Management. Babson Bros. CO., Illinois. 84 p.

Sandoval-Castro, C.A., S. Anderson, and J.D. Leaver. 2000. Production responses of tropical crossbred cattle to supplementary feeding and to different milking and restricted suckling management. Livest. Prod. Sci. 66 : 13-23.

Swamiphak, S. 1996. Tri-party agreement on the evaluation and pricing system of raw milk. Dairy Cattle Magazine 15 : 37-53.

Sutton, J.D. 1989. Altering milk composition by feeding. J. Dairy Sci. 72 : 2801-2814.

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# Activity Profiles at Different pH and Temperature of Cellulases and Lipases in Freshwater Pearl Mussel: *Hyriopsis* (*Hyriopsis*) *bialatus*, Simpson 1900

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## ABSTRACT

The enzymatic properties of two digestive enzymes, cellulases and lipases, from stomach and intestine of adult freshwater pearl mussel, *Hyriopsis* (*Hyriopsis*) *bialatus*, Simpson 1900, were studied at various pH (1.0-11.0) and temperatures (20-80°C). Activity profiles of both enzymes in male and female mussels at various pH and temperature were similar. Optimum pH of cellulases in stomach and intestine was found to be 6.0 while optimum temperature was 35-55°C in stomach and 30-60°C in intestine. Lipase activity was low in intestine but its optimum pH in both organs was found at pH 8.0 and optimum temperature at 35-55°C. Both cellulase and lipase showed higher activities in the stomach than in intestine. At habitat temperature (28-30°C) of Thai freshwater pearl mussel species, cellulase and lipase specific activities dominated in stomach.

**Key words:** cellulase, freshwater pearl mussels, *Hyriopsis bialatus*, lipase

## INTRODUCTION

*Hyriopsis* (*Hyriopsis*) *bialatus*, Simpson 1900 is a freshwater pearl mussel widely distributed at the bottoms of reservoir and river in the central, northern and northeastern parts of Thailand (Brandt, 1974). Mussels have been used as feed for different animals as well as being used as decorative parts of tools, pearl button, tempering pottery, utensils and costume. Mussels are filter feeders by siphoning nutrients from water column. These filtering activities contribute to maintaining clean river and stream ecosystem. Moreover, mussels have antioxidant enzymes and biotransformation enzymes in the digestive glands to detoxify substances in the water (Birmelin *et al.*, 1999). They are also used as bioindicator of the ecological system (Hudson and Isom, 1984; Biggins *et al.*, 1997).

The population of freshwater mussels has been declining as a result of water quality problems caused by dredging, damming, pollution, and also from the reduction of their fish hosts. To increase the population of freshwater mussels, efforts have been made to use culture techniques for mass production and conservation (Isom and Hudson, 1982, 1984; Hudson and Isom, 1984; Keller and Zam, 1990; Uthaiwan *et al.*, 2001). The most

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important factors for culturing are feeding and feed digestion, but the information on the relationship between feeding physiology and feed availability is limited in mussels (Hawkins *et al.*, 1998; Wong and Cheung, 2001). Recent study on feeding of freshwater pearl mussel *Hyriopsis* (*Limnoscapha*) *myersiana* revealed 99.99% phytoplankton and 0.01% zooplankton in the gastrointestinal tract content (Kovitvadhi *et al*., 2000). Phytoplankton consists of protein, carbohydrate and lipid in cells while its cell wall is composed of cellulose (IFRPD, 1973). However, the ability of mussels to digest cell walls of phytoplankton in order to dissolve phytoplankton cytoplasm is not clear although *H. bialatus* is known to have proteinases and amylases in its stomach and intestine which could digest carbohydrate and protein. The aim of this study was to determine the activities of cellulase and lipase collected from stomach and intestine of adult male and female freshwater mussels *H. bialatus* under various pH and temperature conditions. Data obtained from this study may lead to future development of artificial feed formulation for mussel culture.

## MATERIALS AND METHODS

### Animal and rearing

Samples of adult *H. bialatus* were collected from the Mun River Basin in the northeast of Thailand. The mussels were sexually identified by microscopic observation of sperms and eggs in fluid suctioned from gonad. Male mussels were selected to obtain the average size of 112.00±5.89 cm in length, 42.44±3.12 cm in height and 24.67±2.36 cm in width, while female mussels were 107.78±6.07 cm in length, 40.44±2.50 cm in height and 23.89±2.23 cm in width. They were cultured in circle net from January to August 2001 in a pond (pH ranging from 7.0-7.2 and temperature ranging from 28-30°C) at the Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. The mussels were allowed to feed freely on natural plankton in the pond.

### Preparation of enzyme extracts

Nine each of male and female mussels were cleaned with dechlorinated tap water to remove adhering detritus. Shells of the mussels were opened by cutting off anterior and posterior adductor muscles. Their stomach and intestine were dissected. The organs were weighed and pooled separately. Three stomach or intestine was used as one sample. The sample was homogenized on ice without addition of any buffer solution. The homogenate was centrifuged at 13,000 ×*g* for 15 min at 4°C and the upper lipid layer was discarded. Supernatant was collected, divided into aliquots and assayed for enzyme specific activities.

### Protein determination

Protein content of stomach and intestinal extracts was determined using the method of Lowry *et al.* (1951). Bovine serum albumin (BSA) was used as protein standard. The assays were performed in duplicates.

### Cellulase specific activity determination

Cellulase activity was measured using the method of Mendels et al. (1969). Carboxymethyl cellulose (CMC) was prepared by boiling 1% soluble CMC in various buffers (HCl-KCl buffer for pH 1.0 and 2.0 (Fasman, 1984), citrate phosphate buffer for the pH range of 3.0-5.0, phosphate buffer for the pH range of 6.0-8.0 and $NaHCO_3$-$Na_2CO_3$ buffers for the pH range of 9.0-11.0). To determine the optimum pH, the substrate and crude enzymes (dilution 1:50 in various buffers) were separately preincubated for 10 min at 50°C. Enzyme reactions were allowed at 50°C for 30 min. To determine the optimum temperature, the enzyme was diluted in phosphate buffer (pH 6.0) and preincubated at various temperatures (20-80°C) for 10 min. After preincubation, reaction mixture containing 250 µl of the diluted enzymes and 250 µl of the substrate

were incubated for 30 min at the designed temperatures (20-80°C). The reaction was stopped by adding 500 µl of 3,5-dinitrosalicylic acid (DNS) to the solution. It was then heated in boiling water for 5 min, cooled down in ice and finally added with 2.5 ml distilled water. The amount of reducing sugar liberated was determined by measuring the absorbance at 540 nm. Blank was the reaction mixture without the enzyme and the control was prepared by adding the crude enzyme after the DNS reagent. Calibration curve was made using 0.2-1.0 mg/ml glucose. Cellulase specific activity was defined as the amount of enzyme liberating 1 mg of glucose $min^{-1}$ mg $protein^{-1}$ under the specified reaction conditions.

**Lipase specific activity determination**

Activity of lipase (EC 3.1.1.3) was measured using the method modified from Versaw *et al.* (1989). To determine the optimum pH, the assay mixtures containing 50 µl of 200 mM sodium taurocholate, 940 µl of various pH buffers as described above and 10 µl of the crude enzyme extract were used. All reagents were preincubated separately for 5 min at 45°C. For optimum temperature study, the reaction was performed at pH 8.0 and preincubated at various temperatures (20-80°C) for 5 min. After preincubation, the reaction was added with 10 µl of 200 mM β-naphthyl caprylate in dimethyl sulfoxide [DMSO] and incubated at 45°C for 10 min. The reaction was stopped with 10 µl of 100 mM fast blue BB solution in DMSO and further incubated for 5 min at the same temperature before 200 µl of 0.72 N TCA was added. Then the reaction mixtures were clarified with 1,355 µl of (1:1,v/v) ethyl acetate:ethanol solution. Blank was prepared by replacing the enzyme solution with the same buffer of specific pH in the reaction mixture. The control was prepared by mixing the crude enzyme, TCA and the substrate in that order. Product liberated by lipase activity was determined by measuring the changes in absorbance at 540 nm. One unit (U) of lipase specific activity was defined as the amount of enzyme giving an increase of 0.01 absorbance unit at 540 nm $min^{-1}$ mg $protein^{-1}$ under the specified reaction conditions.

**Statistical analysis**

Mean and standard deviation of the means of each enzyme specific activity were calculated. Statistical analysis at 95% significance level was determined using analysis of variance (ANOVA), and multiple comparisons were analyzed by least-significant difference (LSD).

## RESULTS AND DISCUSSION

**Cellulase activity in stomach and intestine of mussels**

Cellulase specific activity in female stomach and intestine of both sex exhibited optimum pH at pH 6.0 while in male stomach the optimum pH was found at pH 7.0 (Figure 1A). Optimum temperature of cellulase specific activity in both stomach and intestine was at 50°C, in both male and female mussels (Figure 1B). The enzymes showed distinct active temperature range of 35-55°C in the stomach and 30-60°C in the intestine at pH 6.0 in both sex. However, the total cellulase specific activity in the stomach was significantly higher than that in the intestine in both profiles ($P<0.05$) (Table 1 and Table 2).

**Lipase activity in stomach and intestine of mussels**

Optimum pH of lipase in both stomach and intestine of both sex was 8.0. However, the activity in stomach was approximately 75-90% at pH 4.0-9.0, while in intestine, the lipase was 75% active at pH 7.0 and 9.0 (Figure 2A). By varying temperature at pH 8.0 assay condition, lipase in the stomach showed optimum temperature at 45°C while at 35, 40, 50 and 55°C, the activities were relatively high. The activity was, however, very low in intestine of both sex (Figure 2B). In the stomach, lipase specific

activities were significantly higher in male than in female in both profiles ($P<0.05$) (Table 3 and Table 4).

In *H. bialatus*, stomach and intestine contributed about 2.9% and 3.5% of the mussel body weight, respectively. pH of the homogenized crude extracts of these organs without addition of any buffer solution was found to be neutral (pH 7.0). In the current experiment, the optimum pH for cellulase activity in both stomach and intestine of *H. bialatus* was 6.0 but the temperature for cellulase activity in stomach was in the range of 35-55°C and in intestine was 30-60°C (Figure 1A and 1B). These meant that a certain amount of cellulase could be actively present at neutral pH in the digestive organs but only 50% active at habitat temperature of 28-

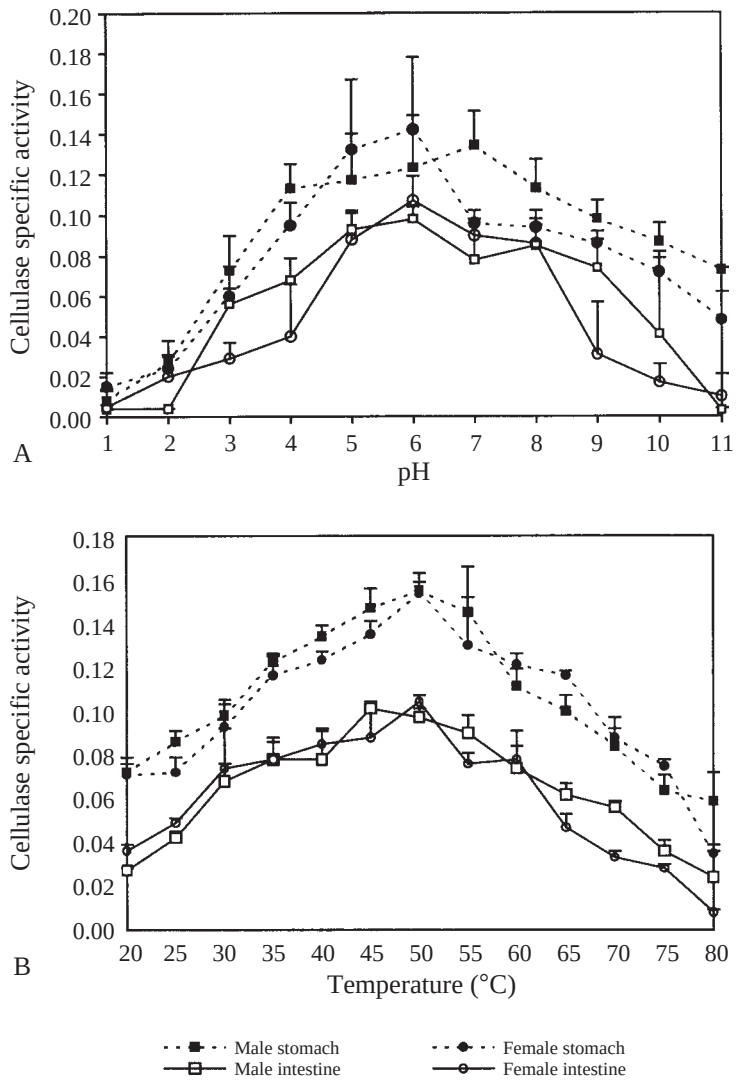


**Figure 1** Cellulase specific activity (mg of glucose $min^{-1}$ mg $protein^{-1}$) in the stomach and intestinal extracts of adult male and female *Hyriopsis bialatus*. Enzymatic reaction was performed at 50°C showing at various pH (A). At pH 6.0, the enzyme activity was done at different temperatures (B).

**Table 1** Cellulase specific activity (mg of glucose $min^{-1}$ mg $protein^{-1}$) in the stomach and intestinal extracts of adult male and female *Hyriopsis bialatus*. Enzymatic reaction was performed at 50°C at various pH.

| pH | Stomach | | Intestine | | Means±SD |
|---|---|---|---|---|---|
| | Male | Female | Male | Female | |
| 1 | 0.008 ± 0.01 | 0.015 ± 0.01 | 0.004 ± 0.002 | 0.005 ± 0.01 | 0.008 $^{a}$ ± 0.01 |
| 2 | 0.027 ± 0.003 | 0.024 ± 0.01 | 0.004 ± 0.001 | 0.020 ± 0.02 | 0.019 $^{ab}$ ± 0.01 |
| 3 | 0.073 ± 0.02 | 0.060 ± 0.02 | 0.056 ± 0.01 | 0.029 ± 0.01 | 0.055 $^{c}$ ± 0.02 |
| 4 | 0.113 ± 0.01 | 0.095 ± 0.01 | 0.068 ± 0.01 | 0.040 ± 0.02 | 0.079 $^{de}$ ± 0.03 |
| 5 | 0.117 ± 0.06 | 0.132 ± 0.01 | 0.093 ± 0.01 | 0.088 ± 0.02 | 0.108 $^{fg}$ ± 0.03 |
| 6 | 0.123 ± 0.06 | 0.141 ± 0.01 | 0.098 ± 0.01 | 0.110 ± 0.01 | 0.117 $^{g}$ ± 0.03 |
| 7 | 0.134 ± 0.02 | 0.096 ± 0.007 | 0.078 ± 0.02 | 0.090 ± 0.01 | 0.090 $^{fg}$ ± 0.03 |
| 8 | 0.113 ± 0.01 | 0.094 ± 0.01 | 0.085 ± 0.02 | 0.086 ± 0.01 | 0.095 $^{ef}$ ± 0.02 |
| 9 | 0.098 ± 0.01 | 0.086 ± 0.01 | 0.074 ± 0.02 | 0.031 ± 0.02 | 0.072 $^{cd}$ ± 0.03 |
| 10 | 0.087 ± 0.01 | 0.072 ± 0.01 | 0.041 ± 0.02 | 0.02 ± 0.01 | 0.054 $^{c}$ ± 0.04 |
| 11 | 0.073 ± 0.01 | 0.048 ± 0.02 | 0.003 ± 0.001 | 0.010 ± 0.01 | 0.034 $^{b}$ ± 0.03 |
| Means ± SD | 0.088 a ± 0.05 | 0.079 a ± 0.04 | 0.055 b ± 0.04 | 0.048 b ± 0.04 | |

The values in the same row and column with different letters are significantly different ($P< 0.05$)

**Table 2** Cellulase specific activity (mg of glucose $min^{-1}$ mg $protein^{-1}$) in the stomach and intestinal extracts of adult male and female *Hyriopsis bialatus*. Enzymatic reaction was performed at pH 6.0 at different temperatures.

| Temperature | Stomach | | Intestine | | Means±SD |
|---|---|---|---|---|---|
| (°C) | Male | Female | Male | Female | |
| 20 | 0.072 ± 0.01 | 0.071 ± 0.01 | 0.027 ± 0.001 | 0.035 ± 0.004 | 0.052 $^{b}$ ± 0.02 |
| 25 | 0.086 ± 0.01 | 0.072 ± 0.01 | 0.042 ± 0.003 | 0.049 ± 0.002 | 0.063 $^{c}$ ± 0.02 |
| 30 | 0.100 ± 0.01 | 0.093 ± 0.01 | 0.068 ± 0.01 | 0.074 ± 0.02 | 0.083 $^{d}$ ± 0.02 |
| 35 | 0.122 ± 0.004 | 0.116 ± 0.01 | 0.078 ± 0.01 | 0.078 ± 0.01 | 0.099 $^{e}$ ± 0.02 |
| 40 | 0.134 ± 0.01 | 0.123 ± 0.01 | 0.078 ± 0.02 | 0.085 ± 0.01 | 0.105 $^{ef}$ ± 0.03 |
| 45 | 0.150 ± 0.01 | 0.135 ± 0.01 | 0.101 ± 0.004 | 0.088 ± 0.01 | 0.118 $^{g}$ ± 0.03 |
| 50 | 0.155 ± 0.01 | 0.154 ± 0.01 | 0.097 ± 0.01 | 0.104 ± 0.004 | 0.128 $^{h}$ ± 0.03 |
| 55 | 0.145 ± 0.01 | 0.130 ± 0.04 | 0.090 ± 0.01 | 0.076 ± 0.01 | 0.110 $^{fg}$ ± 0.04 |
| 60 | 0.111 ± 0.01 | 0.121 ± 0.01 | 0.074 ± 0.01 | 0.073 ± 0.02 | 0.096 $^{e}$ ± 0.02 |
| 65 | 0.100 ± 0.01 | 0.112 ± 0.003 | 0.062 ± 0.01 | 0.047 ± 0.01 | 0.080 $^{d}$ ± 0.03 |
| 70 | 0.084 ± 0.01 | 0.083 ± 0.01 | 0.056 ± 0.004 | 0.033 ± 0.003 | 0.066 $^{c}$ ± 0.02 |
| 75 | 0.068 ± 0.01 | 0.075 ± 0.004 | 0.036 ± 0.01 | 0.028 ± 0.003 | 0.052 $^{b}$ ± 0.02 |
| 80 | 0.060 ± 0.02 | 0.035 ± 0.01 | 0.024 ± 0.01 | 0.007 ± 0.002 | 0.031 $^{a}$ ± 0.02 |
| Means ± SD | 0.106 a ± 0.03 | 0.102 a ± 0.04 | 0.064 b ± 0.03 | 0.062 b ± 0.03 | |

The values in the same row and column with different letters are significantly different ($P< 0.05$)

30°C. An important role of cellulase in mussels was related to the digestion of phytoplankton cell wall which contains cellulose and other polysaccharides. The results showed that the amount of cellulase activity in the stomach of both sex was relatively higher than that in the intestine at every temperature including the habitat temperature of 28-30°C. This correlated well with the intracellular and extracellular carbohydrate digestion and absorption, which predominantly occurred in stomach (George, 1952; Stone and Morton, 1958; Teo and Sabapathy, 1990; Fernandes-Reiriz *et al*., 2001).

Optimum pH of lipases in both stomach and intestine were at pH 8.0 (Figure 2A). At this pH, both stomach and intestine lipases showed high activity at 35-55°C (Figure 2B). Lipases activities exhibited in the stomach of both sex were higher than that in the intestine. This could be related to the extracellular fat digestion, which occurred in stomach more than in intestine (George, 1952).

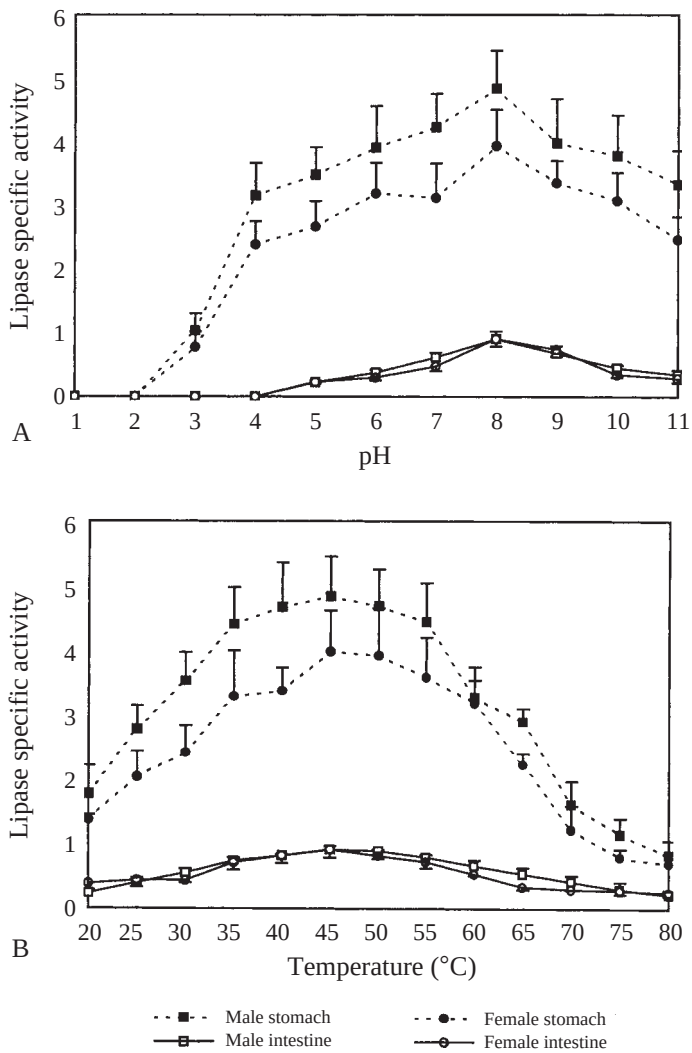


**Figure 2** Lipase specific activity (U $min^{-1}$ mg $protein^{-1}$) in the stomach and intestinal extracts of adult male and female *Hyriopsis bialatus*. Enzymatic reaction was performed at 45°C showing at various pH (A). At pH 8.0, the enzyme activity was done at different temperatures (B).

**Table 3** Lipase specific activity (U $min^{-1}$ mg $protein^{-1}$) in the stomach and intestinal extracts of adult male and female *Hyriopsis bialatus*. Enzymatic reaction was performed at 45°C at various pH.

| pH | Stomach | | Intestine | | Means±SD |
|---|---|---|---|---|---|
| | Male | Female | Male | Female | |
| 1 | nd | nd | nd | nd | $O^{a}$ |
| 2 | nd | nd | nd | nd | $O^{a}$ |
| 3 | 1.030 ± 0.34 | 0.777 ± 0.30 | nd | nd | 0.452 $^{b}$ ± 0.52 |
| 4 | 3.166 ± 0.62 | 2.397 ± 0.45 | nd | nd | 1.391 $^{c}$ ± 1.52 |
| 5 | 3.490 ± 0.52 | 2.683 ± 0.48 | 0.221 ± 0.06 | 0.228 ± 0.03 | 1.656 $^{cd}$ ± 1.55 |
| 6 | 3.907 ± 0.79 | 3.200 ± 0.58 | 0.373 ± 0.10 | 0.300 ± 0.05 | 1.944 $^{de}$ ± 1.75 |
| 7 | 4.223 ± 0.64 | 3.507 ± 0.65 | 0.614 ± 0.11 | 0.470 ± 0.08 | 2.203 $^{e}$ ± 1.80 |
| 8 | 4.827 ± 0.74 | 3.933 ± 0.69 | 0.901 ± 0.06 | 0.906 ± 0.15 | 2.642 $^{f}$ ± 1.90 |
| 9 | 3.977 ± 0.85 | 3.363 ± 0.42 | 0.679 ± 0.14 | 0.740 ± 0.14 | 2.190 $^{e}$ ± 1.60 |
| 10 | 3.780 ± 0.77 | 3.087 ± 0.53 | 0.444 ± 0.12 | 0.343 ± 0.05 | 1.913 $^{de}$ ± 1.66 |
| 11 | 3.337 ± 0.64 | 2.477 ± 0.44 | 0.341 ± 0.04 | 0.287 ± 0.08 | 1.610 $^{cd}$ ± 1.43 |
| Means ± SD | 2.885 a ± 1.73 | 2.311 b ± 1.42 | 0.325 c ± 0.31 | 0.297 c ± 0.31 | |

nd = not detected.

The values in the same row and column with different letters are significantly different ($P< 0.05$)

**Table 4** Lipase specific activity (U $min^{-1}$ mg $protein^{-1}$) in the stomach and intestinal extracts of adult male and female *Hyriopsis bialatus*. Enzymatic reaction was performed at pH 8.0 at different temperatures.

| Temperature (°C) | Stomach | | Intestine | | Means±SD |
|---|---|---|---|---|---|
| | Male | Female | Male | Female | |
| 20 | 1.760 ± 0.54 | 1.363 ± 0.09 | 0.237 ± 0.04 | 0.392 ± 0.13 | 0.938 $^{b}$ ± 0.71 |
| 25 | 2.767 ± 0.44 | 2.020 ± 0.48 | 0.403 ± 0.08 | 0.437 ± 0.13 | 1.407 $^{c}$ ± 1.10 |
| 30 | 3.523 ± 0.54 | 2.400 ± 0.52 | 0.553 ± 0.07 | 0.435 ± 0.03 | 1.728 $^{cd}$ ± 1.40 |
| 35 | 4.407 ± 0.70 | 3.283 ± 0.88 | 0.733 ± 0.06 | 0.709 ± 0.14 | 2.283 $^{e}$ ± 1.75 |
| 40 | 4.677 ± 0.83 | 3.373 ± 0.44 | 0.817 ± 0.02 | 0.813 ± 0.15 | 2.420 $^{ef}$ ± 1.79 |
| 45 | 4.837 ± 0.76 | 3.987 ± 0.78 | 0.903 ± 0.06 | 0.907 ± 0.15 | 2.658 $^{f}$ ± 1.92 |
| 50 | 4.690 ± 0.69 | 3.923 ± 0.91 | 0.883 ± 0.03 | 0.810 ± 0.05 | 2.577 $^{ef}$ ± 1.89 |
| 55 | 4.443 ± 0.74 | 3.593 ± 0.75 | 0.793 ± 0.04 | 0.720 ± 0.12 | 2.388 $^{ef}$ ± 1.79 |
| 60 | 3.270 ± 0.33 | 3.183 ± 0.69 | 0.657 ± 0.11 | 0.533 ± 0.03 | 1.911 $^{d}$ ± 1.41 |
| 65 | 2.887 ± 0.25 | 2.213 ± 0.22 | 0.540 ± 0.11 | 0.337 ± 0.05 | 1.494 $^{c}$ ± 1.14 |
| 70 | 1.603 ± 0.44 | 1.217 ± 0.45 | 0.420 ± 0.12 | 0.303 ± 0.01 | 0.886 $^{b}$ ± 0.63 |
| 75 | 1.140 ± 0.31 | 0.793 ± 0.15 | 0.303 ± 0.14 | 0.283 ± 0.06 | 0.630 $^{ab}$ ± 0.41 |
| 80 | 0.831 ± 0.26 | 0.697 ± 0.19 | 0.220 ± 0.08 | 0.252 ± 0.06 | 0.500 $^{a}$ ± 0.32 |
| Means ± SD | 3.141 a ± 1.48 | 2.465b ± 1.24 | 0.574 c ± 0.25 | 0.533 c ± 0.24 | |

The values in the same row and column with different letters are significantly different ($P< 0.05$)

Interestingly, male mussels showed significantly higher stomach lipase specific activity than that in female. The higher level of lipase found in male might indicate a higher lipid consumption rate in male than in female mussels. Lipase was very important for mussel's feed digestion since freshwater phytoplankton contained high level of lipids that function in accumulation of energy. Some Thai freshwater phytoplankton strains contained 22.30-42.21% lipid (or 33.70-67.00 % essential fatty acids) on dry weight basis (Salaenoi *et al*., 1990).

Temperature and pH played important roles on enzyme activity and stability and can cause changes in enzyme structure as well as its catalytic performance. Range of pH and temperature of the digestive enzyme activities and stability was one of the indicators for *in vivo* digestion conditions of the animal, and would be useful for formulating artificial diets that is suitable for the digestion under different rearing conditions of the freshwater mussel.

At 28-30°C, cellulase and lipase specific activities dominated in the stomach. This suggested that at habitat temperature, primary digestion occurred in stomach. Secondary digestion of large particles which might be partially digested followed later by activities of lower levels of cellulase and lipase in intestine which agreed with Owen (1955).

In addition, the study on cellulase and lipase specific activity in the mussels at different development stages could provide some knowledge for improvement of feed formulation for culturing of *H. bialatus* and other mussel species.

## CONCLUSION

This study showed that cellulase specific activities had optima pH at 6.0 in both stomach and intestine and optimum temperature at 35-55°C in the stomach and 30-60°C in the intestine. Lipase showed its optimum activity at pH 8.0 and optimum temperature at 35-55°C in stomach. Both enzymes showed higher specific activities in stomach than in intestine. At habitat temperature (28-30°C), cellulase and lipase specific activities dominated in the stomach.

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## LITERATURE CITED

Biggins, R.G., R.J. Neves, and C.K. Dohner. 1997. National Strategy for the Conservation of Native Freshwater Mussels. U.S. Fish and Wildlife Service, Asheville, North Carolina. 26 p.

Birmelin, C., R.K. Pine, P.S. Goldfarb, and D.R. Livingstone. 1999. Primary cell-culture of the digestive gland of the marine mussel *Mytilus edulis*: a time-course study of antioxidant-and biotransformation enzyme activity and ultrastructural changes. Mar. Biol. 135 : 65-75.

Brandt, R.A.M. 1974. The non marine aquatic mollusca of Thailand. Archiv für Molluskenkunde 105, 1-423.

Fasman, G.D. 1984. Handbook of Biochemistry and Molecular Biology. CRC Press, Inc., Boca Raton, Florida. 552 p.

Fernandes-Reiriz, M.J., U. Labarta, J.M. Navarro, and A. Velasco. 2001. Enzymatic digestive activity in *Mytilus chilensis* (Hupe, 1854) in response to food regimes and past feeding history. J. Comp. Physiol. B. 171 : 449-456.

George, W.C. 1952. The digestion and absorption of fat in lamellbranchs. Biol. Bull.102 : 118-127.

Hawkins, A.J.S., R.F.M. Smith, S.H. Tan, and Z.B. Yasin. 1998. Suspension-feeding behavior in tropical bivalve molluses: *Perna riluris*, *Crassasastrea beleheri*, *Crassasastrea iradelei*,

*Saccastrea cucculata* and *Pinctala margarifera.* Mar. Ecol. Prog. Ser. 166, 173-185.

Hudson, R.G. and B.G. Isom. 1984. Rearing juveniles of the freshwater mussels (*Unionidae*) in a laboratory setting. The Nautilus 98 : 129-135.

IFRPD. 1973. Annual report of algae project. 1972-1973. 41 p.

Isom, B.G. and R.G. Hudson. 1982. *In vitro* culture of parasitic freshwater mussel glochidia. Naut. 96 : 147-151.

_____ . 1984. Freshwater mussels and their fish hosts; physiological aspects. J. Parasite 70 : 318-319.

Keller, A.E. and S.G. Zam. 1990. Simplification of *in vitro* culture technique for freshwater mussels. Environ. Toxicol. and Chem. 9 : 1291-1296.

Kovitvadhi, U., A. Nagachina, and K. Aungsirirut. 2000. Species composition and abundance of plankton in the gut contents of freshwater pearl mussel *Hyriopsis* (*Limnoscapha*) *myersiana.* J. Med. Appl. Malacol. 10 : 203-209.

Lowry, H.O., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurements with the folin phenol reagent. J. Biol. Chem. 193 : 265-275.

Mendels, M. and J. Weber. 1969. The product of cellulose in cellulase and their application. Adv. Chem. Ser. 95 : 39-443.

Owen, G. 1955. Observations on the stomach and digestive diverticula of the Lamellibranchia. Quartery Journal of Microscopical Science 97 : 514-567.

Salaenoi, J., S. Deetae, C. Chuensri, R. Suvanich, and S. Piyatiratitivorakul. 2000. Compositions of fatty acids in some phytoplankton, pp. 3-9. *In* Proceeding in the 38th Kasetsart University Annual Conference. Bangkok.

Stone, B.A. and J.E. Morton. 1958. The distribution of cellulases and related enzymes in the mollusca. Proc. Malac. Soc. 33 : 127-141.

Teo, L.H. and U. Sabapathy. 1990. Preliminary report on the digestion enzymes present in the digestive gland of *Pera viridis* (L.). Mar. Biol. 106 : 403-407.

Uthaiwan, K., N. Noparatnraporn, and J. Machado. 2001. Culture of glochidia of the freshwater pearl mussel *Hyriopsis myersiana* (Lea, 1856) in artificial media. Aquaculture 195 : 61-69.

Versaw, W.K., S.L. Cuppet, D.D. Winters, and L.E. Williams. 1989. An improved colorimetric assay for bacterial lipase in nonfat dry milk. J. Food Sci. 54 : 1557-1558.

Wong, W.H. and S.G. Cheung. 2001. Feeding rates and scope for growth of green mussels, *Pera uiridis* (L.) and their relationship with food availability in Kat O, Hong Kong. Aquaculture 193 : 123-137.

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# Applications of Paddle Wheel Aerators and Diffused-Air System in Closed Cycle Shrimp Farm System

**Wara Taparhudee**


## ABSTRACT

Shrimp culture practices are changing from high water exchange systems to low water exchange systems to reduce the risk of disease contamination and water pollution. The low water exchange systems require a great number of aerators to prevent water deterioration due to high organic loading associated with limited water exchange. However, high rates of aeration can not only cause excessive water currents and erode pond bottom badly but also increase power cost. In this present study, applications of paddle wheel aerators and diffused-air systems were investigated in grow-out ponds of a closed cycle farm. The results showed that these systems were successful used in shrimp production, and of the systems examined, a combination of paddle wheel and medium bubble size diffused-air system, with a polyethylene pipe network proved to be most effective and convenient. This aeration system provided suitable dissolved oxygen concentrations to the water body and the pond bottom, which could stimulate decomposition at the pond bottom. In addition, this system had low operating costs. It is suitable to be applied in low water exchange culture systems and it can also reduce problems of pond bottom erosion, a typical problem, when using large numbers of paddlewheels.


**Key words:** aeration, paddle wheel aerators, diffused-air system, low water exchange systems, closed cycle shrimp farm system

## INTRODUCTION

The shrimp culture practices in Thailand are presently changing from high water exchange systems to low water exchange systems in order to reduce the risk of disease contamination and water pollution from external water. The low water exchange systems have to apply a great number of aerators to prevent water deterioration, which can occur associated with the limited water exchange. Nevertheless, high rates of aeration can cause excessive water currents, erode pond bottoms badly and increase sediment in the ponds. Funge-Smith and Briggs (1998) indicated that the pond soil erosion was responsible for the bulk of the accumulated sediment (88-93%) and was a major source of organic matter to the pond (40-60%). These are not only increasing the power cost but also the pond preparation cost.

In the low water exchange systems, feed usually represents more than 50% of the total variable costs of shrimp culture, seed around 10-12%, and power 10% of the cost or more, depending on the intensity of the system. However, attention to the quality and regime of feeding can reduce feed cost. Seed cost can be reduced by moderate stocking density. Pumping cost can be reduced associated with the low water exchange management. The cost

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of aeration is dependent on number of aerators, types of aerators, operating times and culture systems but it can be reduced by selection the system providing optimum dissolved oxygen and low maintenance cost without any effect on the shrimp production. Then, using the low water exchange culture system as well as the appropriate aeration management would improve an economic feasibility of the shrimp culture, especially in the closed recycle system.

In this study, applications of paddle wheel aerators and diffused-air systems were studied. The numbers of paddle wheel aerators could be decreased and replaced by diffused-air systems, which provided air to three types of diffusers positioned on the pond bottoms. These were compared to the control ponds, which were only supported by paddle wheel aerators. The study was carried out to evaluate the effect of the four aeration system types on the water and pond bottom soil qualities, survival rate, yield and feed conversion ratio in a closed cycle system shrimp farm and to develop an economic comparison of the options described.

## MATERIALS AND METHODS

The experiment was conducted in a shrimp farm in Ratchaburi province, central area of Thailand during February to June 2000. This farm was chosen as it operated in closed cycle system and was about 2 hours travel from the laboratory station (Kasetsart University, Bangkok). Its size was of 16 ha water area comprising two 1.6 ha of reservoir ponds, eight grow-out ponds of 0.64 ha and two 0.8 ha treatment ponds. The remaining area was for buildings and dikes. The depth of the reservoir was 4 m, with 3 m water depth and the grow-out pond 2 m, with 1 m water depth. Apart from compensating water loss by evaporation and/or seepage, there was no water exchange in the first two months after stocking. In the third month, water exchange was approximately 10 cm every three days (i.e.10 % of total volume) and this was increased to 20 cm in the final month (20% of total volume). The water exchange was approximately 25 mm $day^{-1}$ (2.5% of total volume per day). Effluent water was drained into two 0.8 ha treatment ponds and then to the reservoir.

Eight grow-out ponds, each of 0.64 ha (1 m water depth) were used for this experiment, based on a single-factor completely randomised experimental design with two replication ponds. Each pond was stocked with *Penaeus monodon* postlarvae sized PL 13-15 at a stocking density of 37.5 PL/$m^2$. Four types of aeration systems (Figure 1) were used as follows:

1) Eight long-armed paddle wheel aerators (Type I).

2) Four long-armed paddle wheel aerators combined with a diffused-air system. For this, a blower (11.7 kW $ha^{-1}$ or 15.6 HP $ha^{-1}$) was employed to deliver air through a 5 cm diameter PVC pipe and then through eleven 1.5-cm diameter PE (polyethylene) pipes positioned on the pond bottom each of 60-m length with a total of 80 holes each of 1 mm diameter. The distance between each PE pipe was 5 m) (Type II). A total of 880 holes, or 1,400 holes $ha^{-1}$ (0.0011 $m^2$ pore area per ha) of pond bottom was deployed.

3) Four long-armed paddle wheel aerators combined with a diffused-air system. For this a blower (11.7 kW $ha^{-1}$ or 15.6 HP $ha^{-1}$) delivered air through a 5 cm diameter PVC pipe connected to a 3.5 cm diameter PVC pipe, releasing air through eight 80-cm diameter porous disks with each disk having 8,000 holes of < 1 mm diameter positioned on the pond bottom under the paddle wheel aerators) (Type III). A total of 48,000 holes, or 75,000 holes $ha^{-1}$ (0.4125 $m^2$ pore area per ha) of pond bottom was deployed.

4) Four long-armed paddle wheel aerators combined with a diffused-air system. Here a blower (11.7 kW $ha^{-1}$ or 15.6 HP $ha^{-1}$) delivered air through a 5 cm diameter PVC pipe, releasing air through eleven 3.5-cm diameter PVC (polyvinyl chloride) pipes of 60-m length with each pipe having 40 holes of 5 mm diameter positioned on the pond bottom.

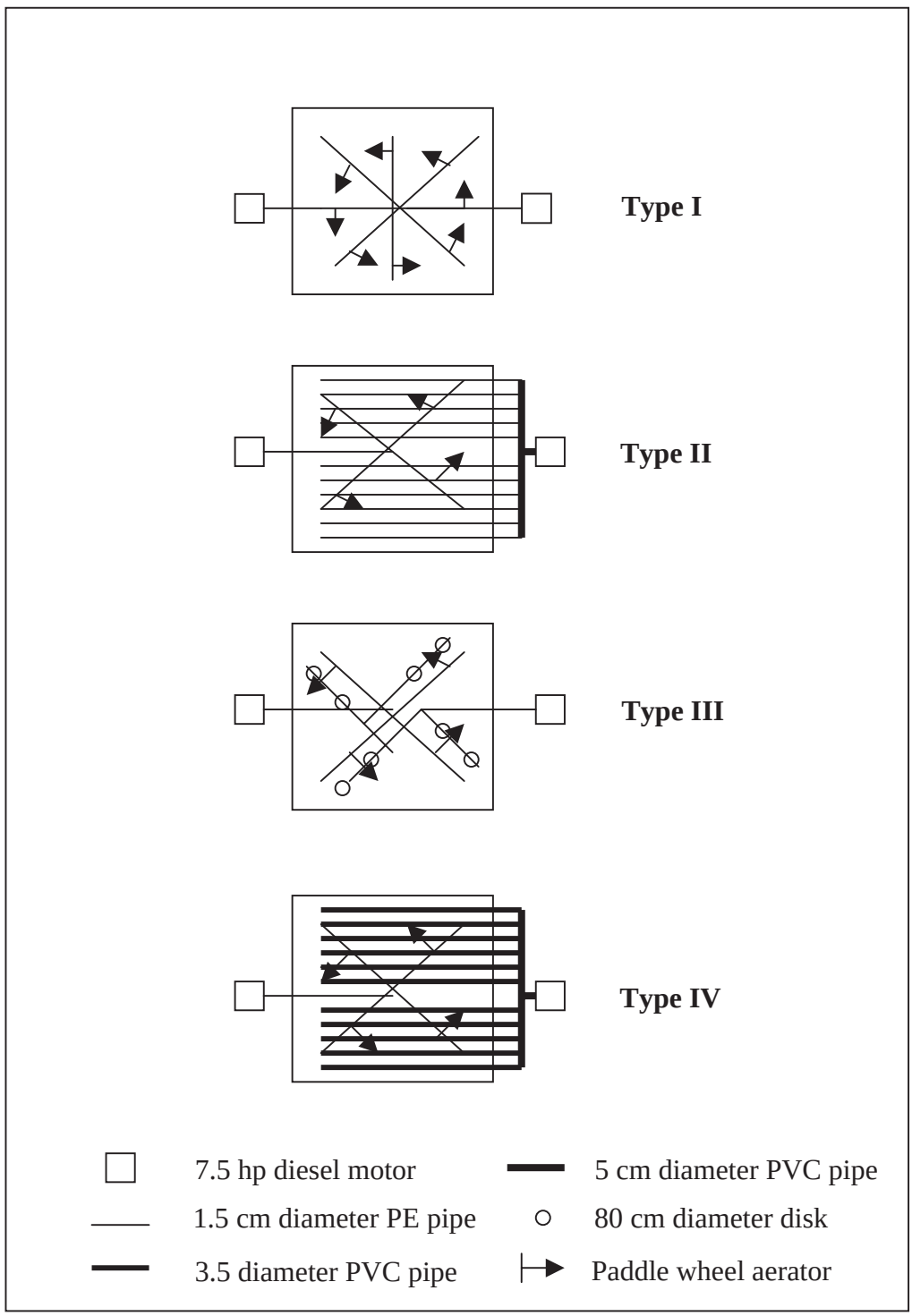


**Figure 1** Aeration systems.

The distance between each PE pipe was 5 m (Type IV). A total of 440 holes $m^{-2}$, or 700 holes $ha^{-1}$ (0.09625 $m^2$ pore area per ha) of pond bottom was deployed.

Each long-armed paddle wheel aerator consisted of a long shaft made of 2.5-3.0 cm diameter steel pipe and fitted with sixteen 60-cm diameter paddle wheels. Each paddle was 0.3 cm thick, 22 cm long and 15 cm wide. Two 10 horsepower (7.5 kW) diesel motors were used to power the paddle wheel aerators and diffused-air system in each experimental pond. The total cost (i.e., two diesel motors, paddle wheel aerators and diffuser system costs) for aeration system type I, II, III and IV was US$ 3,000, US$ 2,575, US$ 2,313 and US$ 2,450 per pond or US$ 4,688, 4,023, 3,613 and 3,828 $ha^{-1}$, respectively.

The schedule for aeration use for all systems was for one hour during daytime, between 13.00 to14.00 h using four paddle wheel aerators in the type I system and only the diffuser in type II, III and IV systems. At night all aerators (i.e., both paddle wheels and diffuser system) were employed from 24.00 to 6.00 h (6 hrs), 21.00 to 6.00 h (9 hrs), 21.00 to 6.00 h (9 hrs) and 18.00 to 6.00 h (12 hrs) during the $1^{st}$, $2^{nd}$, $3^{rd}$ and $4^{th}$ culture months, respectively. The aerators would also be run at short notice in cloudy, or rainy weather, or in the evidence of a plankton crash, but all aerators were stopped during feeding for half an hour.

Water samples were collected fortnightly at 10.00 - 12.00 h. Water parameters including salinity, temperature, Secchi disk visibility, dissolved oxygen and pH were measured in the field using field equipment. A sample of 2 L of water from each pond was taken from 50 cm below the water surface using a 100-cm water column sampler at two stations; one at the centre of the pond and the other one 3 m. from the pond dike for further analyses of alkalinity, total ammonia ($NH_3$-N), nitrite-ammonia ($NO_2$-N), nitrate-ammonia ($NO_3$-N), soluble orthophosphate ($PO_4$-P), total phosphorus (TP), suspended solids (SS), total solids (TS), dissolved solids (DS), Biochemical Oxygen Demand ($BOD_5$) and chlorophyll-a (Chl a) were conducted in the laboratory.

Temperature was measured using a mercury thermometer while water salinity was measured using a Salino refractometer (Atago, Model. S-28). pH was measured by pH meter (Hach Model); transparency was measured with a Secchi disk; Dissolved Oxygen (DO) was measured using a Polarographic DO meter and probe (Yellow Spring Instrument Co., Model 51B). Regarding laboratory parameters, alkalinity, Biochemical Oxygen Demand ($BOD_5$), total solids, dissolved solids, suspended solids, chlorophyll a and soluble reactive phosphorus (Orthophosphates) were analysed using the standard methods (APHA, 1989). Total ammonia-nitrogen was measured by the Indophenol method and nitrite-nitrogen were measured by diazotization (Grasshoff, 1974). Finally, nitrate-nitrogen and total phosphorus were analysed by the methods described by Strickland and Parson (1972).

Top 10-cm sediment samples were collected underwater monthly using a 10-cm diameter, 20-cm penetrating core sampler, at the centre and 3 m from the pond dike at the feeding area of each pond. Sediment samples were analyzed in the laboratory for total nitrogen (TN), available phosphorus ($PO_4^{3-}$), total ammonia nitrogen ($NH_3$-N), organic matter (OM), organic carbon (OC), pH and biochemical oxygen demand ($BOD_5$).

$BOD_5$ was analysed along the Standard method (APHA, 1989; and Musig and Yutharutnukul, 1991). Using this technique, 1-2 gm wet weight of sediment was diluted with sea water. The water sample in BOD bottle was incubated at 20 ° C for 5 days. Dissolved oxygen in the sample was calculated by comparing it to the dissolved oxygen in a blank sample in units of $BOD_5$ per 1 gm of dry sediment. From this figure, $BOD_5$ was calculated as mg/gm sediment. Bottom soil texture was analysed using the Hydrometer method (Kilmer and Alexander, 1949; Day, 1965). Organic matter and organic carbon were analysed

by Wet oxidation (Jackson, 1958; Walkely and Black, 1934). pH was measured by pH meter (dilution of soil : water = 1 : 1). Ammonium-nitrogen was measured by mixing approximately 0.5 g of soil with 200 ml-distilled water and then supernatant was analysed by Koroleff's indophenol blue method (Grasshoff, 1974). Total nitrogen and available phosphorus were analysed by the Kjeldahl method of Murphy and Rilly, respectively (Authanam *et al.*, 1989).

The statistical analyses for the quantity and quality of water and sediment, shrimp yield, survival rate and feed conversion ratio was carried out using one-way ANOVA. In all tests, means were considered different at $P<0.05$. A Turkey's test was employed to compare and rank means. All statistical analyses were performed by SPSS 10.0 for Windows.

## RESULTS

### Water quality

The study showed significant differences in TS and DS concentrations ($P<0.05$) between the four aeration system applications. All other parameters (i.e., salinity, temperature, Secchi disk visibility, DO, pH, alkalinity, $NH_3$-N, $NO_2$-N, $NO_3$-N, $PO_4^{3-}$, TP, SS, $BOD_5$, Chl a) were not significantly different ($P>0.05$) (Table 1).

The type IV aeration system generated lowest levels both of total and dissolved solids, with 3,952 mg $L^{-1}$ of TS and 3,882 mg $L^{-1}$of DS, while the type I aeration system was highest at 5,758 mg $L^{-1}$ of TS and 5,676 mg $L^{-1}$of DS.

Other important water quality parameters were DO, $NH_3$-N, $BOD_5$ and Chl a. Aeration type I provided the highest DO levels in the ponds during

**Table 1** Water quality in the ponds with different types of aeration. (Values are means* and standard deviation.)

| Parameters | Aeration system | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| Salinity (ppt) | 3.9 ± 1.5 | 3.8 ± 1.5 | 3.8 ± 1.5 | 3.7 ± 1.5 |
| Temperature (°C) | 32.6 ± 1.4 | 32.7 ± 1.5 | 32.3 ± 1.5 | 32.4 ± 1.5 |
| Secchi disk visibility (cm) | 23 ± 11 | 19 ± 9 | 24 ± 13 | 22 ± 13 |
| DO (mg $L^{-1}$) | 8.4 ± 1.8 | 8.2 ± 2.5 | 7.5 ± 2.9 | 7.1 ± 1.8 |
| pH | 8.5 ± 0.7 | 8.5 ± 0.6 | 8.8 ± 0.7 | 8.8 ± 0.6 |
| Alkalinity (mg $L^{-1}$) | 105 ± 14 | 108 ± 19 | 108 ± 16 | 103 ± 17 |
| $NH_3$-N (mg $L^{-1}$) | 0.19 ± 0.23 | 0.17 ± 0.18 | 0.18 ± 0.29 | 0.22 ± 0.46 |
| $NO_2$-N (mg $L^{-1}$) | 0.07 ± 0.13 | 0.09 ± 0.12 | 0.07 ± 0.19 | 0.03 ± 0.05 |
| $NO_3$-N (mg $L^{-1}$) | 0.01 ± 0.01 | 0.02 ± 0.04 | 0.00 ± 0.00 | 0.02 ± 0.04 |
| $PO_4^{3-}$ (mg $L^{-1}$) | 0.01 ± 0.01 | 0.01 ± 0.00 | 0.01 ± 0.01 | 0.01 ± 0.01 |
| TP (mg $L^{-1}$) | 0.15 ± 0.06 | 0.15 ± 0.07 | 0.15 ± 0.08 | 0.18 ± 0.13 |
| SS (mg $L^{-1}$) | 82 ± 62 | 65 ± 29 | 58 ± 46 | 70 ± 55 |
| DS (mg $L^{-1}$) | 5,676 ± 1,099(a) | 5,254 ± 1,509(a) | 5,111 ± 1,024(a) | 3,882 ± 776(b) |
| TS (mg $L^{-1}$) | 5,758 ± 1,063(a) | 5,319 ± 1,499(a) | 5,169 ± 1,013(a) | 3,952 ± 758(b) |
| $BOD_5$ (mg $L^{-1}$) | 15.1 ± 3.7 | 15.3 ± 4.7 | 15.7 ± 4.4 | 16.1 ± 4.7 |
| Chl a (mg $m^{-3}$) | 122.6 ± 100.8 | 99.7 ± 76.2 | 102.6 ± 84.6 | 130.8 ± 150.5 |

* Means in the same row with followed by the same letters are not statistically different ($P>0.05$)

the culture cycle, followed by type II, III and IV aerators, respectively. Average DO levels in ponds with type I and II systems were 8.4 and 8.2 mg $L^{-1}$ whilst those in types III and IV were 7.5 and 7.1 mg $L^{-1}$. Ponds using type IV aeration had the highest concentrations of $NH_3$-N, BOD and Chl a, while ponds using aeration type II had the lowest $NH_3$-N and Chl a concentrations.

**Bottom sediment quality**

The bottom sediments in all the ponds were classified as the sandy clay type (sand 47.7%, silt 14.8% and clay 37.5%). There were no significant differences ($P>0.05$) in bottom sediment quantity with aeration type (Table 2).

In most cases, contents of OM, OC, BOD, $NH_3$-N, TN, $PO_4^{3-}$ in the sediment at the centres of the ponds were higher than at the feeding areas. However, for ponds using type III aeration, $BOD_5$ levels in the feeding areas were higher than in the central areas, and for ponds using type IV aeration system, $NH_3$-N levels in the feeding areas were higher than in the central areas. These implied that the efficiency of type III and IV systems in providing DO to the pond bottom was lower than type I and II systems. Meanwhile, pH levels were similar in feeding areas and pond centres in all experiments.

No sediment quality parameters in the pond centres differed significantly with aeration system, but organic carbon contents in feeding areas were significantly different ($P<0.05$) (Table 3; 4). Ponds using type II aeration had the lowest OC levels, which ponds using type I, II and IV systems had 18%, 19%, and 49% OC, respectively, which were higher than those of type II. Other parameters in these areas were not significantly different, though ponds using aeration type II had the lowest levels of OM, $NH_3$-N and $BOD_5$ contents in the feeding areas. Ponds with type IV aeration had the highest contents of OM, OC and $NH_3$-N, while those with type III aeration had the highest BOD levels.

**System performance**

Production levels, food conversion ratio and survival rate were not significantly different at $P<0.05$ with aeration (Table 5). The type II system obtained the highest production at 4,700 kg $ha^{-1}$ followed by type I at 4,534 kg $ha^{-1}$, type IV at 4,288 kg $ha^{-1}$ and type III at 3,883 kg $ha^{-1}$, respectively. Food conversion ratio was the lowest in ponds using aeration system type III at 1.26 and followed by type II, type IV and type I at 1.36, 1.40 and 1.64, respectively. Survival rate was little different between ponds using types I and III, at around 67%, while those for types II and IV were 74.2% and 77.8%, respectively. However, if the survival rate

**Table 2** Bottom soil quality in the ponds with different types of aeration. (Values are means and standard deviation).

| Parameters | Aeration system | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| TN (%) | 0.11 ± 0.05 | 0.10 ± 0.05 | 0.09 ± 0.05 | 0.09 ± 0.05 |
| Available phosphorus (mg $kg^{-1}$) | 72.8 ± 14.7 | 80.3 ± 15.1 | 74.6 ± 21.6 | 77.5 ± 13.5 |
| $NH_3$-N (mg $g^{-1}$) | 0.09 ± 0.07 | 0.09 ± 0.07 | 0.08 ± 0.10 | 0.10 ± 0.07 |
| OM (%) | 2.46 ± 1.20 | 2.27 ± 0.70 | 2.49 ± 0.85 | 2.90 ± 0.73 |
| OC (%) | 1.32 ± 0.45 | 1.34 ± 0.40 | 1.45 ± 0.50 | 1.68 ± 0.42 |
| pH | 7.25 ± 0.16 | 7.30 ± 0.13 | 7.29 ± 0.09 | 7.31 ± 0.10 |
| $BOD_5$ (mg $g^{-1}$) | 2.54 ± 1.21 | 3.31 ± 2.83 | 3.47 ± 2.26 | 4.35 ± 2.60 |

**Table 3** Bottom soil quality in the pond centres in the different types of aeration. (Values are means and standard deviation)

| Parameters | Aeration system | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| TN (%) | 0.12 ± 0.06 | 0.12 ± 0.06 | 0.12 ± 0.05 | 0.11 ± 0.07 |
| Available phosphorus (mg $kg^{-1}$) | 68.81 ± 19.08 | 84.76 ± 20.53 | 76.56 ± 28.08 | 81.12 ± 18.91 |
| $NH_3$-N (mg $g^{-1}$) | 0.10 ± 0.07 | 0.11 ± 0.08 | 0.10 ± 0.14 | 0.08 ± 0.06 |
| OM (%) | 2.31 ± 0.91 | 2.65 ± 0.77 | 2.73 ± 1.11 | 2.97 ± 0.90 |
| OC (%) | 1.34 ± 0.53 | 1.58 ± 0.41 | 1.58 ± 0.64 | 1.72 ± 0.52 |
| pH | 7.25 ± 0.15 | 7.29 ± 0.13 | 7.32 ± 0.10 | 7.29 ± 0.09 |
| $BOD_5$ (mg $g^{-1}$) | 2.85 ± 1.57 | 4.42 ± 3.16 | 2.88 ± 1.40 | 4.70 ± 3.15 |

**Table 4** Bottom soil quality in pond feeding areas in the different types of aeration (Values are means* and standard deviation).

| Parameters | Aeration system | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| TN (%) | 0.09 ± 0.04 | 0.08 ± 0.03 | 0.06 ± 0.03 | 0.07 ± 0.01 |
| Available phosphorus (mg $kg^{-1}$) | 76.8 ±10.0 | 75.9 ± 7.4 | 72.6 ±17.1 | 73.9 ± 5.4 |
| $NH_3$-N (mg $g^{-1}$) | 0.08 ± 0.08 | 0.06 ± 0.03 | 0.06 ± 0.04 | 0.12 ± 0.08 |
| OM (%) | 2.62 ± 1.49 | 1.90 ± 0.37 | 2.26 ± 0.45 | 2.82 ± 0.55 |
| OC (%) | 1.30 ± 0.38(a,b) | 1.10 ± 0.22(a) | 1.31 ± 0.26(a,b) | 1.64 ± 0.32(b) |
| pH | 7.25 ± 0.18 | 7.32 ± 0.13 | 7.27 ± 0.08 | 7.32 ± 0.12 |
| $BOD_5$ (mg $g^{-1}$) | 2.23 ± 0.68 | 2.21 ± 2.08 | 4.07 ± 2.86 | 4.00 ± 2.08 |

* Means in the same row with followed by the same letters are not statistically different (P>0.05)

**Table 5** Results of production, FCR, survival rate, harvested size and income of different aeration system applications (mean ± s.d.).

| Aeration | Type I | Type II | Type III | Type IV |
|---|---|---|---|---|
| Production (kg $ha^{-1}$) | 4,534 ± 1,065 | 4,700 ± 71 | 3,883 ± 55 | 4,288 ± 5 |
| FCR | 1.64 ± 0.13 | 1.36 ± 0.11 | 1.26 ± 0.04 | 1.4 ± 0.13 |
| Survival rate (%) | 67.9 ± 19.4 | 74.2 ± 3.5 | 67.2 ± 0.4 | 77.8 ± 10.3 |
| Size (individual $kg^{-1}$) | 56 ± 5 | 59 ± 5 | 65 ± 4 | 68 ± 2 |
| Total revenue (US$ $ha^{-1}$) | 24,509 ± 4,705 | 25,278 ± 932 | 18,527 ± 358 | 19,991 ± 3,595 |
| Culture period (days) | 105 | 107 | 108 | 110 |

factor was taken out, type I system would obtain the highest production level. Average shrimp harvest size of ponds using type I system was largest at 56 shrimp $kg^{-1}$ followed by those of type II, III and IV at 59, 65, and 68 shrimp $kg^{-1}$, respectively.

**Financial analysis**

Table 6 shows financial analysis of each aeration system. Capital cost was estimated only for the aeration system (i.e. it was assumed that capital costs of other production components were similar and hence marginal capital costs only could be compared). For type I was highest at US$ 4,688 $ha^{-1}$ followed by type II, IV and III at US$ 4,023, 3,828, and 3,613 $ha^{-1}$, respectively.

Total operating costs were highest in type I ponds at US$ 7,924 $ha^{-1}$ $crop^{-1}$ followed by type II, type IV and type III ponds at US$ 7,160, 6,892 and 6,038 $ha^{-1}$, respectively. These were primarily related to greater production with higher feed cost. Labour, chemical, energy and other costs did not differ between aeration systems. Total operating costs – energy cost was highest in type I at US$ 7,265 $ha^{-1}crop^{-1}$ followed by type II, IV, and III at US$ 6,484, 6,190, and 5,354 $ha^{-1}crop^{-1}$, respectively. Production per energy cost of type II was highest at 7 kg US$ followed by type I, IV and III at 6.9, 6.1 and 5.7 kg US$, respectively.

As a result of the larger harvested size in type I ponds and higher survival rate in type II ponds, these obtained more revenue than the others. Type II ponds achieved the highest average total revenue of US$ 25,278 $ha^{-1}$, type I obtained US$ 24,509 $ha^{-1}$, type IV ponds US$ 19,991 $ha^{-1}$, and type III ponds recorded the lowest total revenue of US$ 18,527 $ha^{-1}$. As a consequence, type II ponds obtained highest profit at US$ 18,118 $ha^{-1}$ at 9%, 38%, and 45% higher than type I, IV, and III ponds, respectively.

Shrimp production (kg $crop^{-1}$) per capital

**Table 6** Capital, operating costs and profit of each aeration system. Unit : US$ $ha^{-1}$

| Items | Type I | Type II | Type III | Type IV |
|---|---|---|---|---|
| **Capital cost** | | | | |
| Aeration system | 4,688 | 4,023 | 3,613 | 3,828 |
| **Operating costs** | | | | |
| Feed | 5,577 | 4,794 | 3,669 | 4,503 |
| Seed | 1,219 | 1,219 | 1,219 | 1,219 |
| Energy | 659 | 676 | 684 | 702 |
| Labour | 312 | 312 | 312 | 312 |
| Others (lubricants, ice, etc.) | 134 | 134 | 134 | 134 |
| Chemical | 23 | 25 | 20 | 22 |
| Total operating costs | 7,924 | 7,160 | 6,038 | 6,892 |
| Production (kg $ha^{-1}$) | 4,534 | 4,700 | 3,883 | 4,288 |
| Total revenue | 24,509 | 25,278 | 18,527 | 19,991 |
| Profit (total revenue-total operating costs) | 16,585 | 18,118 | 12,489 | 13,099 |
| Production/capital cost of aeration system (kg US$) | 0.97 | 1.17 | 1.07 | 1.12 |
| Production/ energy cost (kg US$) | 6.88 | 6.95 | 5.68 | 6.11 |
| Total operating costs/production (US$ $kg^{-1}ha^{-1}$) | 1.75 | 1.52 | 1.55 | 1.61 |
| Profit/ capital cost of aeration system | 3.54 | 4.50 | 3.46 | 3.42 |

cost of aeration system (paddle wheels and/or diffused-air system costs) and per energy cost (diesel cost) was highest in type II system at 1.17 kg US$$^{-1}$$ and 6.95 kg US$$^{-1}$$, respectively, while type I system was lowest for production per system cost at 0.97 kg US$$^{-1}$$ and type III lowest for production per energy cost at 5.68 kg US$$^{-1}$$. Total operating costs per production of type II system was lowest at US$ 1.52 kg$$^{-1}$$ followed by type III, IV and I systems at US$ 1.55, 1.61 and 1.75 kg$$^{-1}$$, respectively. There were no relationships between production level / profit and aeration system or energy costs.

## DISCUSSION

All aeration systems showed the benefit of controlling water and sediment quality in the ponds at an acceptable level for shrimp growth, accumulating sediment in the centre of the pond and maintaining low levels of waste on the feeding area. However, most water and sediment quality parameters increased as the culture period progressed, associated with increased feed and shrimp biomass.

Comparing aeration systems, DO, TS, SS and DS concentrations in type I ponds were the highest because this system comprised of eight long-armed paddle wheel aerators, while the other systems had only four paddle wheel aerators combined with a diffused-air system. Pond water was thus overall better oxygenated but had higher solids levels. Boyd and Ahmad (1987) have evaluated aerators in shallow tanks under standard conditions of 20$^{o}$C and clean, fresh water and found that paddle wheel aerators were more efficient than other types of aerators, and diffused-air aerators had the lowest standard aeration efficiency values (SAE). Based on the calculation of average SAE values of paddle wheel aerators at 2.2 kg $O_2$ kW·hr$^{-1}$ cited by Boyd (1998), type I system (operated by two 7.5 kW (10 hp) diesel motors (23 kW ha$^{-1}$), oxygen would be delivered approximately at 51 kg $O_2$ hr$^{-1}$ha$^{-1}$. Furthermore, they could produce highest levels of water circulation, as well as creating turbulence at the pond bottoms, which resulted in the highest of all the solids levels. Boyd (1992) commented that if excessive aeration were used, the bottom material would be eroded from the inside slopes of levees and around the periphery of pond bottom. These then settled to the bottom in the centre of the pond. Ponds using aeration type IV had the lowest DO, TS and DS concentrations, but highest concentrations of $NH_3$-N and $BOD_5$. This system comprised four long-armed paddle wheel aerators and a diffused-air system, with a total of 440 holes of 5 mm diameter, discharging coarse air bubbles, which are less efficient in oxygen exchange because of their smaller exchange surface area per unit of air bubble volume, and their more rapid rise in the water column. By comparison the fine air bubbles distributed by type II and III aeration produced a greater surface area to the surrounding water and allowed oxygen to diffuse into the water more effectively and rose more slowly, facilitating greater oxygen absorption.

Typical standardised aerator efficiencies (SAE) of fine, medium and coarse bubbles provided by diffused-air systems are 1.2-2.0, 1.0-1.6 and 0.6-1.2 kg $O_2$ kW$^{-1}$hr$^{-1}$, respectively (Colt and Orwicz, 1991). As the result of the coarse air bubbles and the smaller numbers of diffuser holes, the type IV aeration created the least pond bottom disturbance but with lower dissolved oxygen concentrations (oxygen transfer at 36 kg $O_2$ hr$^{-1}$ ha$^{-1}$) and highest $NH_3$-N and $BOD_5$ concentrations compared to the other systems with oxygen transfer of type II (produce medium bubbles) and III (produce fine bubbles) at 41 and 44.5 kg $O_2$ hr$^{-1}$ha$^{-1}$, respectively. This accords with the findings of Martinez-Cordova *et al.* (1998), who noted that $NH_3$-N in the water is higher in ponds with lower rates of aeration as the oxidation of ammonia to nitrite, and nitrite to nitrate can occur more easily in more highly aerated conditions.

In the feeding areas of the pond, indicator levels of bottom soil quality, especially OM, OC,

$NH_3$-N and $BOD_5$ contents, were the lowest in ponds using type II aeration, and highest in type IV ponds, except for $BOD_5$ which was highest in type III ponds. This appeared to be because type II aeration provided oxygen directly to the pond bottom, particularly in feeding areas, which enhanced local waste oxidation and decomposition. In this case, the system (type II) was better than type I, in which water was induced to circulate only through the pond bottom surfaces. It was also better than type IV, due to the greater number and smaller diameter of diffuser holes. Though type III aeration could provide more and smaller sized air bubbles when it ran alone in the daytime, oxygen dispersion was very slow as air was produced only from the eight porous disks. These did not cover the total area of pond bottom; and therefore, this system also showed the highest $BOD_5$ levels.

Installation of type I system was relatively simple while types II, III and IV required the diffusion networks to be fixed or loaded on the pond bottom before filling with water, or else they would float if they have air inside the pipes. Placing of pipes on the central area of the pond should also be avoided as sediment was accumulated on this area and when a diffuser system was run, the bubbles produced could disperse accumulated waste and release toxic gases. After harvesting, polyethylene pipes (PE) especially were easy to keep and clean, and could be used in the next crop. The cost of the PE pipe network was lower than the paddlewheel and disk diffuser applications. The polyvinyl chloride (PVC) pipes system was cheapest but as noted, its disadvantage was the coarse air bubbles produced. As a practical issue, the connections also usually came loose and required higher levels of maintenance.

In comparing production, FCR and survival rate, the type II system gave the highest production and survival rate, though FCRs did not differ significantly among systems. The harvested sizes of shrimp in type I and II ponds were larger than those in type III and IV ponds. These were probably due to the higher levels of dissolved oxygen and the lower levels of waste on the pond bottoms, particularly at the feeding areas, as shrimp spend a lot of time at the bottom.

This study indicated that DO levels were not significantly different between all aeration systems. However, aeration system type II (i.e., four paddle wheel aerators + a diffuser system delivering air through eleven 880 holes and 1 mm diameter PE pipes) showed advantages over other types, including higher production and survival rate, lower installation cost, less pond bottom erosion, easier of cleaning the PE pipes for reuse in the next crop, and less waste in feeding areas. Moreover, type II system had highest returns in terms of production per energy cost and production per aeration system cost.

The type III system (i.e., four paddle wheel aerators + a diffuser system providing air through eight 48,000 holes and <1mm diameter disks produced lowest returns in production per energy cost and the type I system (i.e., eight paddle wheel aerators) had the lowest production per aeration system cost.

In the study, fixed cost was estimated only for aeration system (other fixed costs were assumed similar in each system). An interest rate was 10% annually and life span of each aeration system was four years. Thus total cost (fixed + operating costs) of type II system was US$ 8,568 $ha^{-1}$, 10.4% lower than type I, and 17.3% and 4.1% higher than type III and IV, respectively. Total revenue of type II was US$ 25,278 $ha^{-1}$ which was 3.1%, 36.4% and 26.4% higher than this of type I, III and IV, respectively. Moreover, total operating costs per production of type II system was lowest at US$ 1.52 $kg^{-1}$ $ha^{-1}$ followed by type III, IV and I systems at US$ 1.55, 1.61 and 1.75 $kg^{-1}$ $ha^{-1}$, respectively.

Thus, type II system was an alternative aeration system, which could be applied in the shrimp ponds, especially for the low water exchange systems, which had to use a great number of aerators in the ponds. Diffused-air aeration systems could

release air bubbles near pond bottoms that rised to the surface causing water to move upward. Water from the surface moved downward to replace the rising water in zones where bubbles were released. These could blend supersaturated surface water with bottom waters of lower dissolved oxygen concentration and a uniform dissolved oxygen profile could be established. Nevertheless, using the diffused-air system alone in the shrimp ponds wass impractical due to lower oxygen transfer rate and less water circulation compared to the paddlewheel application. Further study should focus on the proper design of the combination of paddle wheels and a diffused-air system such as the number of paddle wheels and pipes, their positions, and number and size of the holes. A well designed aeration system could reduce the risk of oxygen depletion, mix pond water and kept the feeding areas clean with low pond bottom and walls scouring. If excessive aeration systems were used, they could cause high sediment accumulation resulting from pond bottom and walls erosion and also increased suspended solids in the water. On the other hand, if numbers of aerators used were not enough, they might be not able to maintain DO at suitable level for shrimp growth. As mentioned before, if large holes were used, they produced large air bubbles, which were less oxygen transfer efficiency compared with small holes. Howere, too small holes were also too easy to get clogged.

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## LITERATURE CITED

APHA (American Public Health Association). 1989. Standard Method for the Examination of Water and Waste Water. 17th edition, American Public Health Association, Washington, D.C. 1108 p.

Authanam, T., K. Chanchareonsuk, and S. Jithagunon. 1989. Manual for Soil and Plant Analysis. Soil Science Department, Faculty of Agriculture, Kasetsart University, Bangkok. 88 p.

Boyd, C. E. 1992. Shrimp pond bottom soil and sediment management, pp. 166-181. *In* J. Wyban (ed.). Proceedings of the Special Session on Shrimp Farming. World Aquaculture Society, Baton Rouge, LA.

Boyd, C.E. and T. Ahmad. 1987. Evaluation of Aerators for Channel Catfish Farming, Bulletin 584, Alabama Agricultural Experiment Station, Auburn University, Alabama. 52 p.

Colt, J. and C.Orwicz. 1991. Aeration in intensive culture, pp.198-271. *In* E.B. David and R.T. Joseph. (eds.). Aquaculture and water quality. Advances in World Aquaculture, World Aquacult. Soc. Volume 3.

Day, P.R. 1965. Particle fraction and particle size analysis, pp. 545-567. *In* C.A. Blach. (ed.). Method of soil analysis. Part I, physical and minerabilogical properties including statistics of measurement and sampling. Agronomy, No.9. Amer. Soc. Of Agro. Inc., Madison, Wisconsin.

Funge-Smith, S.J. and M.R.P. Briggs. 1998. Nutrient budgets in intensive shrimp ponds :implications for sustainability. Aquaculture 164 : 117-133.

Grasshoff, K. 1974. Method of Sea Water Analysis. Verlag Chemic, New York. 317 p.

Jackson, M.L. 1958. Soil Chemical Analysis. Pretice-Hall, Inc., New York. 498 p.

Kilmer, V.J. and L.T. Alexander. 1949. Method of making mechanical analysis of soil. Soil Sci. 52 : 8-24.

Martinez-Cordova, L.R., H. Villarreal-Colmenares, and M.A. Porchas-Cornejo. 1998. Response of biota to aeration rate in low water exchange ponds farming white shrimp, *Penaeus vanamei* Boone. Aquacult. Res. 29 : 587-593.

Musig, Y. and P. Yuthrutyaneekul. 1991. Settling, sediment and soil quality in intensive marine shrimp farms at inner Gulf of Thailand. Fisheries Sci. 1 : 47-55.

Strickland, J.D.H. and T.R. Parsons. 1972. A Practical Handbook of Seawater Analysis. Fisheries Research Board of Canada, Ottama. 311 p.

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# The Effect of Steaming Time on Microstructural Changes of Instant Noodles

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## ABSTRACT

Confocal laser scanning microscopy (CLSM) was used for examining microstructural differences resulted from different steaming time in modern instant noodles production. Fresh noodles were subjected to a saturated steam tunnel for 0, 1 or 3 minutes prior to entering a 135°C fryer for 50 seconds. The obtained noodles were then rehydrated and examined by CLSM. Three microstructural parameters: degree of starch swelling, starch-protein association and protein continuity were evaluated. CLSM images showed the 3-minutes steamed samples to have a greater amount of swollen starch, better association between starch granules and protein matrix and better continuity of protein network. Thus, fundamental understanding of microscopic spatial arrangement between starch and protein affected by steaming time in instant noodles processing could be achieved via CLSM.

**Key words:** steaming, microstructure, instant noodles

## INTRODUCTION

The knowledge of food microstructure has become a key in commercial food production, particularly, in the development of new products. The information is necessary for the complete understanding of the changes that occur during food processing.

It was recognized that Confocal Laser Scanning Microscopy (CLSM) has advantage over conventional light microscopy and electron microscopy, and is one of the most powerful techniques available. In addition to better resolution, it can particularly perform optical sectioning, allowing for disturbance-free observations of three-dimensional internal structure without the need for physical sectioning, thereby offering an opportunity to study a microstructure in food systems (Heertje *et al.*, 1987; Blonk and van Aalst, 1993). Moreover, CLSM can identify the spatial location of different components by detecting fluorescence from dyes specific to different chemical species, thus providing views of important phenomena relating to these components, such as component miscibility, physical aggregation and coalescence and phase separation (Alder *et al.*, 1994; Vodovotz *et al.*, 1996). CLSM was employed to study potato and wheat starch granules by some investigators (Alder *et al.*, 1994; Baldwin *et al.*, 1994; Lynn and Cochrane, 1997). Acridine orange was proven to produce a general fluorescence in undamaged potato starch granules, while loss of fluorescence was believed to reflect a reduced structural integrity of the granule (Adler *et al.*, 1994). Heerje *et al.* (1987) analyzed the dynamic processes of rising dough under CLSM using fluorescein isothiocyanate (FITC) as a specific fluorochrome. Bread was investigated employing CLSM by Vodovotz *et al.*

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(1996) using Periodic Acid–Schiff's (PAS) and FluoroLink Cy3 for marking wheat starch and gluten, respectively.

Instant noodles have become the most popular convenience food not only in Asia but also around the world. The most common type, known as modern instant noodles, requires steaming and frying to achieve a unique quality for only 2-3 minutes cooking in boiling water to rehydrate the noodles before eating (Moss, undated, Nagao. 1992).

Wang and Sieb (1996) postulated that at the surfaces of alkaline noodle, the trapping of either large or small starch granules by gluten matrix was primarily depended on the matrix density. The hard and strong elastic gel resulted from the restricted swollen starches at the interior was related to the chewy texture of alkaline noodles. It was also hypothesized that both the surface and the interior of salted noodles were largely depended on swelling ability of starch granules.

In this investigation, CLSM has been employed to examine microstructural changes in terms of starch swelling, starch-protein association and protein continuity in rehydrated instant noodle prepared from different steaming time.

## MATERIALS AND METHODS

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

### Fluorochromes

Triple staining for observing protein, starch and fat simultaneously was modified from 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 oil marking, respectively.

### Instant noodle formula and process

Instant noodle samples were prepared from the formula comprising of 20 kg wheat flour, 4.5-5.5 kg water, 355g salt, 30g carboxymethylcellulose, 18 g sodium hexametaphosphate, 15 g potassium carbonate and 10 g sodium carbonate.

Instant noodle samples were processed by a continuous production line. Wheat flour was mixed with the predissolved salts and texture modifiers in water for 20 min. Compression and reduction stages were operated by 7 pairs of rollers to achieve a dough thickness of 1.0 mm, the sheeted dough was then proceeded to a slitting 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 then shaping into a block of 85.0 g in a mold to convey to a fryer which was 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 vaporized metal polypropylene bags and then stored in cold storage at 4°C. Instant noodle samples were processed 2 replications for each steaming time.

### Instant noodles rehydrating method

A cake of 85 g instant noodles was rehydrated in 450 ml boiling water for 2 min, stirred occasionally and left standing for 3 min. The noodles were then transfered to 600 ml of room temperature water and drained immediately. Excess water was removed with tissue paper. Two cakes of instant noodle samples were randomly sampling from each processing run for rehydration.

**CSLM examination**

The CLSM examination was performed using a Carl Zeiss 410 LSM system attached to a 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, whereas the emission filter 515-565 LP, 510, 525, 575-640, 590, 665, 590-610 were selected, and the filter block 1 containing a VHS-510 DCLP filter was used. A cover slips 22×50 mm, were used for supporting the samples. The magnification of 200X was applied in all image capturing

**Samples preparation**

Freshly rehydrated instant noodle samples were carefully stored in a closed chamber at 20°C. A razor cutter was cautiously employed to section a 2 mm and 5 mm long of noodle strands prior to transferring onto a cover slip either horizontally or vertically for a cross-section and a transverse viewing, respectively. The fluorochromes were immediately applied simultaneously. Five of each cross-section and transverse sections were randomly selected and examined. CLSM capturing was taken at 3 viewing areas around the edge and one viewing area at the center of each individual specimen. Two images were captured at each viewing area by employing an optical sectioning of 10 mm thickness. One hundred and sixty images for cross-section viewing and transverse image capturing for each steaming time were evaluated and the most representing images were illustrated.

**Evaluation of CLSM images for a Microstructure of Starch and Protein**

The CLSM images taken from rehydrated instant noodle samples were evaluated in three attributes: starch swelling, protein continuity and starch-protein association. The evaluation of starch swelling and starch-protein association was performed on the images taken from the cross-section of rehydrated noodle samples while protein continuity was evaluated from those taken from transverse section.

Starch swelling parameter was defined as the amount of starch granules observed to increase in size due to swelling effect accompanied by the decrease in fluorescence caused by reduced granule structural integrity. Starch-protein association parameter was defined as subsequent association between starch and protein component whereby the optimal swelling of starch granules was attained to occupy the cavities of protein network. Poor association was caused by non-swelling starch granules which had migrated from the original sites in the protein network. Protein continuity referred to the area of protein strands which form a continuous network in the absence of disruption. The low continuity of protein network also caused a poor starch-protein association.

## RESULTS AND DISCUSSION

The degree of starch swelling was observed from the images captured from the cross-section of noodle strands (Figure 1.1, 2.1 and 3.1) with the increase in steaming time. Greater number of granules with higher structural integrity displaying fluorescence were observed in the images captured from 0 and 1 steaming time (Figure 1.1 and 2.1) as compared to the image captured from 3 min steaming time (Figure 3.1). Figure 1.1, 2.1 and 3.1 also displayed starch–protein association. A better association was observed in the images captured from a 3 min steaming time sample (Figure 3.1). In contrast, a poor association was obviously detected in the images taken from the samples prepared without steaming (Figure 1.1) in which gluten matrix disruption was noticed. Subsequently, starch granules were discovered migrating from the matrix. It was apparent that the 1 min steaming sample showed a lesser degree of starch swelling than that

of the 3 min steaming. As a result, the degree of association between starch granule and protein matrix was less in the sample prepared from 1 min steaming. Transverse images implied that steaming at 3 min resulted in a more continuity of protein network (Figure 1.2, 2.2 and 3.2). A greater loss of starch granules at the edges of the noodle samples prepared without steaming (Figure 1.2) showed less protein continuity.

Oil droplets were observed only in rehydrate instant noodle prepared without steaming (Figure 1.1 and 1.2). The absence of oil droplets in other noodle samples may be due to the migration of oil into the boiling water during rehydration.

## CONCLUSIONS

It was discovered that by employing the steaming time of 3 min prior to frying at 135 °C for 50 s in instant noodle processing offered a better degree of starch swelling, starch–protein association and protein continuity at the surface of noodle strands. The changes in microstructural arrangement of starch and protein as affected by different steaming time could be illustrated by employing CLSM. Thus, process variables could be optimized by means of further establishing the relation of microscopic information to the targeted macroscopics properties.

## ACKNOWLEDGEMENTS

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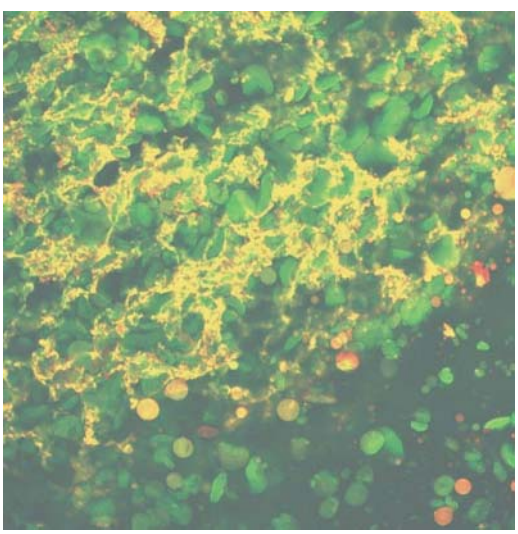

**Figure 1.1** CLSM image of a cross-section rehydrated instant noodles prepared without steaming (X200).

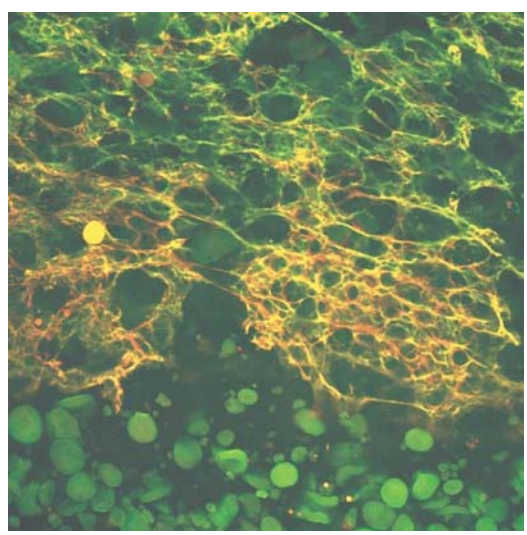

**Figure 1.2** CLSM image of transverse section of rehydrated instant noodles prepared without steaming (X200).

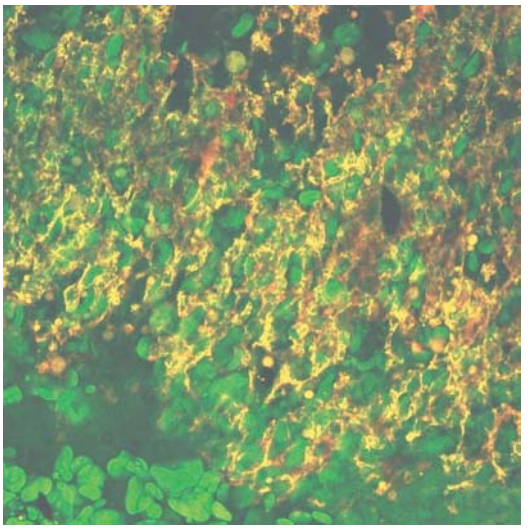

**Figure 2.1** CLSM image of a cross-section of rehydrated instant noodles prepared with 1 min steaming (X200).

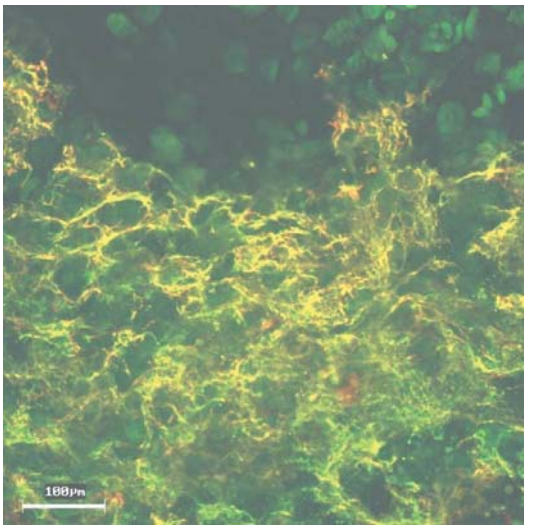


**Figure 2.2** CLSM image of a transverse-section of rehydrated instant noodles prepared with 1 min steaming (X200), bar = 100 mm

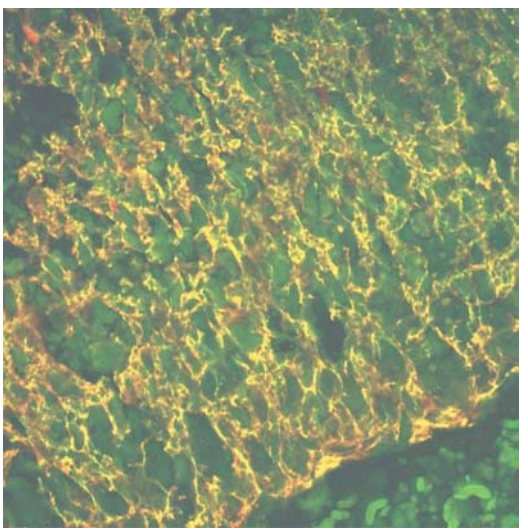

**Figure 3.1** CLSM image of a cross-section of rehydrated instant noodles prepared with 3 min steaming (X200).

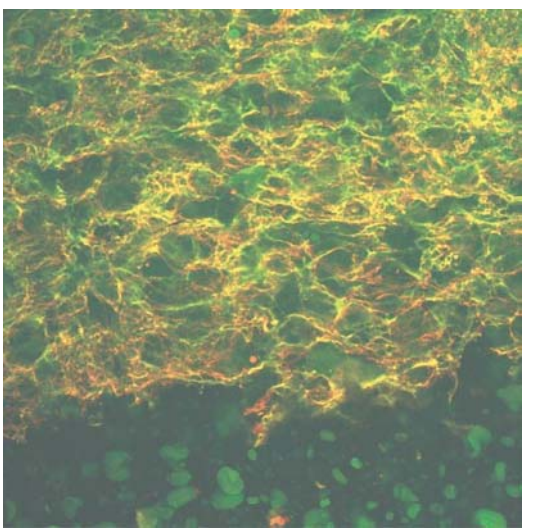

**Figure 3.2** CLSM image of a transverse-section of rehydrated instant noodles prepared with 3 min steaming (X200).

## LITERATURE CITED

Adler, J., P.M. Baldwin, and C.D. Melia. 1994. Starch damage part 2: Types of damage in ball-milled potato starch, upon hydration observed by confocal microscopy. Starch 46 : 252.

Baldwin, P.M., J. Adler, M.C. Davies, and C.D. Melia. 1994. Holes in starch granules: confocal, SEM and light microscopy studies of starch granule structure. Starch 46(9) : 341.

Blonk, J.C.G. and H. Van Aalst. 1993. Confocal scanning light microscopy in food research. Fd. Res. Int. 26 : 297.

Heertje, I., P. Van der Vlist, J.C.G. Block, H.A.C.M. Hendrickx., and G.J. Brakenhoff. 1987. Confocal scanning laser microscopy in food research : some observations. Food Microstruc. 6 :115.

Lynn, A. and M.P. Cochrane. 1997. An evaluation of confocal microscopy for the study of starch granule enzymatic digestion. Starch 46 : 106.

Moss, H.J. Undated. Chinese Noodle Production. Publication of Bread Research Institute of Australia, New South Wales, Australia.

Nagao, S. 1992. Recent advances in traditional non Western cereal foods. 9 th Intl. Cereal and Bread Congress, Paris.

Wang, L. and P. Seib. 1996. Australian salt-noodle flours and their starched compared to US wheat and their starches. Cereal Chem. 73(2) :167.

Vodovotz, Y., E. Vittadini, J.Coupland, D.J. McClements, and P. Chinachoti. 1996. Bridging the gap: Use of confocal microscopy in food research. Food Tech. 50(6) :74.

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# Suitability of Using Herbs as Functional Ingredients in Thai Commercial Snacks

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## ABSTRACT

Forty-three Thai consumers were informed about the physical properties and medicinal uses of selected Thai herbs prior to asked to make a decision on which of 14 functional herbs could be used in 7 different types of Thai commercial snack foods. Thirty-eight New Zealanders evaluated the suitability of 8 Thai herbs together with other 8 herbs grown in New Zealand. The results of both Thai and New Zealand respondents showed that functional herbs could be added to prawn crackers, dried squid and dried fish snacks, puffed snacks and potato chips rather than nuts or popcorn. The suitable Thai herbs selected by Thais were pepper, holy basil and garlic. For New Zealanders, the suitable NZ herbs were chives and parsley, and the more suitable Thai herbs were garlic, lemon grass, pepper, and ginger.

**Key words:** Thai commercial snacks; Thai herbs; New Zealand herbs

## INTRODUCTION

The health-promoting effect of foods is the food industry's big idea at the start of the 21st century. The future of food will increasingly be about how it affects consumer health and well-being and the sorts of products and ingredients that will deliver such health benefits (Heasman and Mellentin, 2001). Simultaneously, traditional meal patterns are being broken down. Lifestyles are becoming more hectic and consumers are less likely to spend time preparing and consuming meals affecting the inexorable rise of snacking patterns (Promar International, 1997). Unfortunately, snack foods have had to battle the junk food image for a long time due to their high fat and salt content. The drive toward a healthier diet has led many producers to look at alternative snack products that avoid high levels of fat, sugar and salts; are fortified with vitamins and minerals or even ingredients described as functional; or present fruit and vegetables in a snack format (Tuley, 2000).

Herbs have been added to foods throughout history for either preservation and/or flavour purposes. Creative use of herbs can make foods far more enjoyable to eat, no less healthy because they add negligible calories, and in many cases make food healthier because using herbs allow salt and fat to be removed because herbs are such effective flavouring agents (Dole Food Company, 2001). Moreover, the pharmacological properties of herbs have been documented recently by Thomas (2000) and Mazza and Oomah (2000).

Given the facts that herbs are such effective flavouring agents for snacks (Williams, 1999; Nordmark, 1999), and some of herbs have been used to possess pharmacological activity to these products (Pszczola, 1999). It might be possible to

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produce a range of snacks that have a healthier image and possibly health improving properties by the judicious inclusion of some herbs that possess the nutraceutical activity along with other spices and flavouring agents.

Thailand's commercial snacks market was worth about Bt10 billion in 2000. Extruded snacks accounted for 35 per cent of this market, potato chips 31 per cent, dried fish snacks 11 per cent, prawn crackers 10 per cent, nuts 9 per cent, dried squid snacks 3 per cent, and popcorn 1 per cent (Thansettakij, 2001). The objective of this research was to ascertain which functional herbs could be incorporated into 7 commercial snack groups.

## MATERIALS AND METHODS

### (1) Respondent selection

A group of 40 consumers who ate snacks on a regular basis in Thailand and also Palmerston North, New Zealand, were asked to participate in this study. These respondents were classified into 4 age groups: 12-18, 18-22, 23-35, and over 35 year old, with the equal numbers of male and female in each age group. Therefore, 5 males and 5 females would be collected as respondents for each age group.

### (2) Questionnaire and procedure

The questionnaire objected to determine which functional herbs could be combined with each of 7 snack categories with a view to improve the health promoting properties of each snack type. These snack types consisted of puffed snacks, potato chips, dried fish snacks, prawn crackers, nuts, dried squid, and popcorn. Sixty-one Thai herbs having primary health care properties (Hehmhongsha, 1998) were screened by using 3 criteria. Firstly, the herbs are used for the pharmacological care by consuming. Secondly, they possess at least 4 out of the 5 following medicinal aspects: minimize risks of cancer; prevent or relieve heart and vascular disease; prevent and relieve diabetes; prevent and relieve abdominal pains from ulcers, burps, etc; and finally improve the immune system. Finally, the herb should not be bitter or have a strong medicinal flavour. Herbs will be selected when meet all 3 criteria. Fourteen selected herbs are asiatic pennywort, cloves, galangal, garlic, ginger, holy basil, Indian laburnum, lalang grass, lemon grass, myrobalan, nut grass, pepper, roselle, and turmeric, and are used in questionnaire for Thai consumer. For New Zealander respondents who were unfamiliar to all Thai herbs, some of them in the list were replaced by pharmacologically active herbs available and known to New Zealanders. Therefore, the selected 8 Thai herbs in their questionnaires were asiatic pennywort, cloves, holy basil, garlic, ginger, lemon grass, pepper, and turmeric, and the selected 8 New Zealand herbs consisted of alfalfa, chives, echinacea, horseradish, parsley, red clover, rosemary, and thyme (Painter, 1995).

First, the respondents were informed about the properties of each selected herb with a photograph and a description of part used, flavour, and medicinal uses. Second, they were asked to taste 3 blind samples of one snack category and then they were requested to state their opinions about the suitability of using each herb in that product by using 9-point scales (1 = extremely unsuitable, 5 = neither suitable nor unsuitable, 9 = extremely suitable). There was 10-minute break before the samples of next category were served, but the break between the fourth and the fifth category was extended to 20 minutes.

### (3) Data analysis

The analysis of variance by Randomized Completely Block Designs (RCBD) was used to indicate a significant difference exists and then the mean comparison was done by Duncan's multiple range test (Resurreccion, 1998) by SPSS version 10 (SPSS Inc., Chicago, IL). In addition, the perceptual maps of products and herbs were created by Principal Component Analysis (Neal, 1988) by Senstools version 2.3.28 (OP&P Product Research BV,

Utrecht, The Netherlands).

## RESULTS AND DISCUSSION

The survey for Thai respondents was conducted in February 2001. In the field works, there were one more of male and female in 12-18 year old group and one more female in 23-35 year old group. Therefore, total amounts of Thai respondents were 43. For New Zealand respondents, the survey was conducted in October 2001. Two females in 19-22 group did not complete all of their questionnaires. Therefore, there were 38 complete responses for New Zealanders.

The results of both Thais and New Zealanders showed that prawn crackers, dried fish snacks, dried squid, puffed snacks, and potato chips were more suitable for herb seasoning than nuts and popcorn as shown in Table 1. The suitable Thai herbs from Thai respondents were pepper, holy basil, and garlic, followed by lemon grass, ginger, cloves and so on as shown in Figure 1. The suitable herbs from New Zealanders' statements were chives, parsley, lemon grass, and garlic, followed by pepper, ginger and so on as shown in Figure 2. Table 1 and Figures 1-2 show that Thai respondents tended to accept the herb in snack products rather than did New Zealanders (see lower suitability scores of New Zealand respondents). These might be reasoned by the more familiarity of using herbs as spices in Asia countries and many herbs are used as ingredients or condiments in Thai cuisine (Uhl, 2000) but only chives are used as a normal condiment for New Zealanders (Painter, 1995).

When the results for each product category of Thai responses were considered (Table 2). It was found that the compatible herbs which should be added in prawn crackers, dried fish snacks, dried squid, puffed snacks, and potato chips were pepper, garlic and holy basil, whilst the suitable herbs for popcorn were laburnum, asiatic pennywort, lemon grass, lalang grass, roselle, and turmeric. These results might be influenced by the flavour of each herb.

Form the literature review on the flavour of herbs, cloves, garlic, galangal, holy basil, pepper and nut grass are hot and pungent. Ginger is sweet, hot and pungent. Turmeric is astringent, sweet and aromatic. Lemon grass is aromatic. Roselle and myrobolan are sour. Asiatic pennywort and lalang grass are mild, and laburnum is sweet (Wuthithummawate, 1997; Phuthiyanun, 1999). It seemed that hot pungent flavoured Thai herbs should be added to prawn crackers, dried fish snacks, dried squid, puffed snacks, and potato chips which Thai people are familiar to their savoury flavoured. In the other hand, sweet, mild, sour or aromatic would be more suitable for popcorn which most products

**Table 1** Mean of suitability scores of herb inclusion in each snack category.

| | Mean of suitability scores | |
|---|---|---|
| | Thais | New Zealanders |
| Prawn crackers | 6.13$^{a}$ | 5.69$^{a}$ |
| Dried fish snacks | 6.07$^{a}$ | 5.18$^{bc}$ |
| Dried squid | 6.08$^{a}$ | 4.98$^{c}$ |
| Puffed snacks | 6.05$^{a}$ | 5.23$^{b}$ |
| Potato chips | 5.97$^{a}$ | 5.69$^{a}$ |
| Nuts | 5.50$^{b}$ | 4.36$^{d}$ |
| Popcorn | 4.96$^{c}$ | 4.08$^{e}$ |

a, b,... are significantly different ($p \leq 0.05$) in each column.

are flavoured by butter, caramel, and chocolate.

For nuts, the compatible herbs were pepper, garlic, holy basil, lemon grass, and ginger but the range of suitability scores of all selected herbs were narrow when they were compared to the other products (see the column for score range of all selected herbs in Table 2). These showed the less difference of Thai consumer views on flavoured nuts with herbs since they could be either sweet or savoury. Especially peanut, the representative of nuts in this study, it is the best example of all-purpose nut that can be and has been treated in innumerable ways. For example, it can be salted and spiced, or sugared and spiced, or salted and sugared with or without spices, or flavoured with various materials such as smoke flavour and so on (Booth, 1990).

All Thai results may be more evidently if they are presented by a perceptual map in Figure 3. The suitability scores of 5 products and 9 herbs highly related to the horizontal axis (dimension 1). These 5 products were dried fish snacks, dried squid, puffed snacks, nuts, and popcorn, and these 9 herbs consisted of holy basil, garlic, pepper, cloves, ginger, galangal, lemon grass, asiatic pennywort, and Indian laburnum. The suitability scores of the rest 2 products and 5 herbs highly related to the vertical axis (dimension 2). The rest 2 products and 5 herbs were potato chips, prawn crackers, lalang grass, roselle, myrobalan, nut grass, and turmeric, respectively. Prawn crackers, dried fish snacks, dried squid, puffed snacks, and potato chips located on the positive side of horizontal axis whilst nuts and popcorn were on the opposite side but nuts seem to be in the middle between the first 5 products and popcorn. Simultaneously, prawn crackers located far from the other products in negative side of vertical axis. These positions showed the differences of compatible herbs to products in each direction of each axis. The

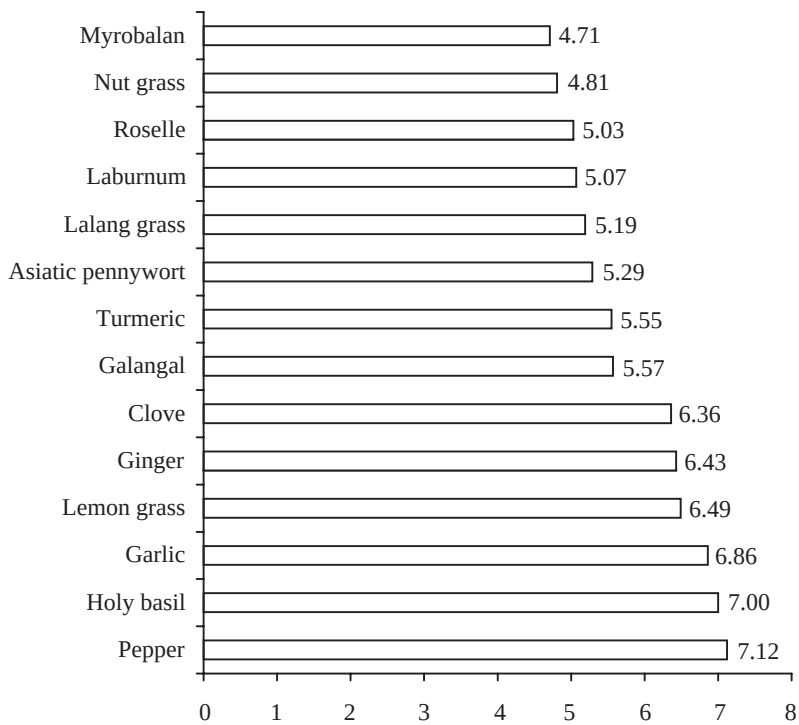


**Figure 1** Mean of suitability scores of each Thai herbs responded by Thai respondents.

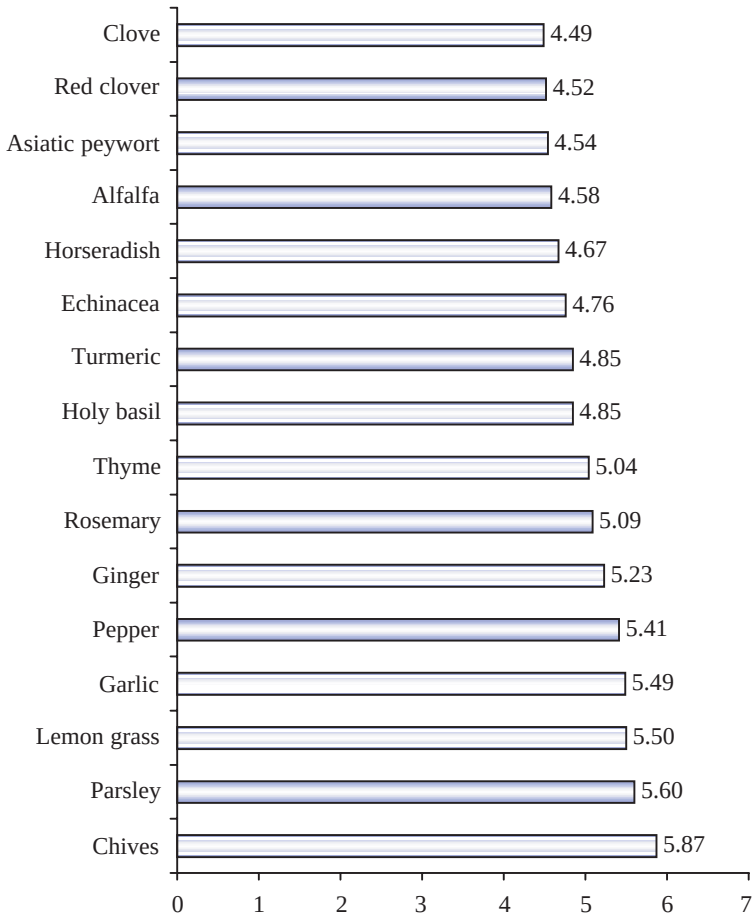


**Figure 2** Mean of suitability scores of each Thai and New Zealand herbs responded by New Zealanders.

**Table 2** The responses of suitability of using herbs in each snack category by Thai consumers.

| | Compatible herbs | Score range of compatible herbs | Score range of all selected herbs |
|---|---|---|---|
| Prawn crackers | Pepper, garlic, holy basil | 7.44-7.73 | 4.80-7.73 |
| Dried fish snacks | Pepper, garlic, holy basil | 7.67-7.84 | 4.63-7.84 |
| Dried squid | Pepper, garlic, holy basil | 7.65-7.81 | 4.58-7.81 |
| Puffed snacks | Pepper, garlic, holy basil | 7.45-7.76 | 4.69-7.76 |
| Potato chips | Pepper, garlic, holy basil | 7.32-7.78 | 4.54-7.78 |
| Nuts | Pepper, garlic, holy basil, lemon grass, ginger | 5.93-6.42 | 4.83-6.42 |
| Popcorn | Laburnum, asiatic pennywort, lemon grass, lalang grass, roselle, turmeric | 5.40-6.02 | 4.02-6.02 |

suitability of using horizontal axis related herbs were different between the group of 5 products, nuts and popcorn. The suitability of using vertical axis related herbs for prawn crackers were not similar to the other products. The suitability of using each herb for each product could be considered by projecting that product position on the vector of the interested herb (Lilien and Rangaswamy, 1998). The length of herb vectors showed the degree of suitability of using each herb as an ingredient in snacks. The vectors of holy basil, garlic and pepper being in the positive direction of the horizontal axis were longer than the other herbs' vectors. These meant they more suited to add in snacks, especially the ones located in the same direction. The vectors of laburnum, asiatic pennywort, and roselle were almost in the popcorn directions. It indicated their suitability to each other. (Figure 3)

For New Zealand respondents, their results for each product category are shown in Table 3.

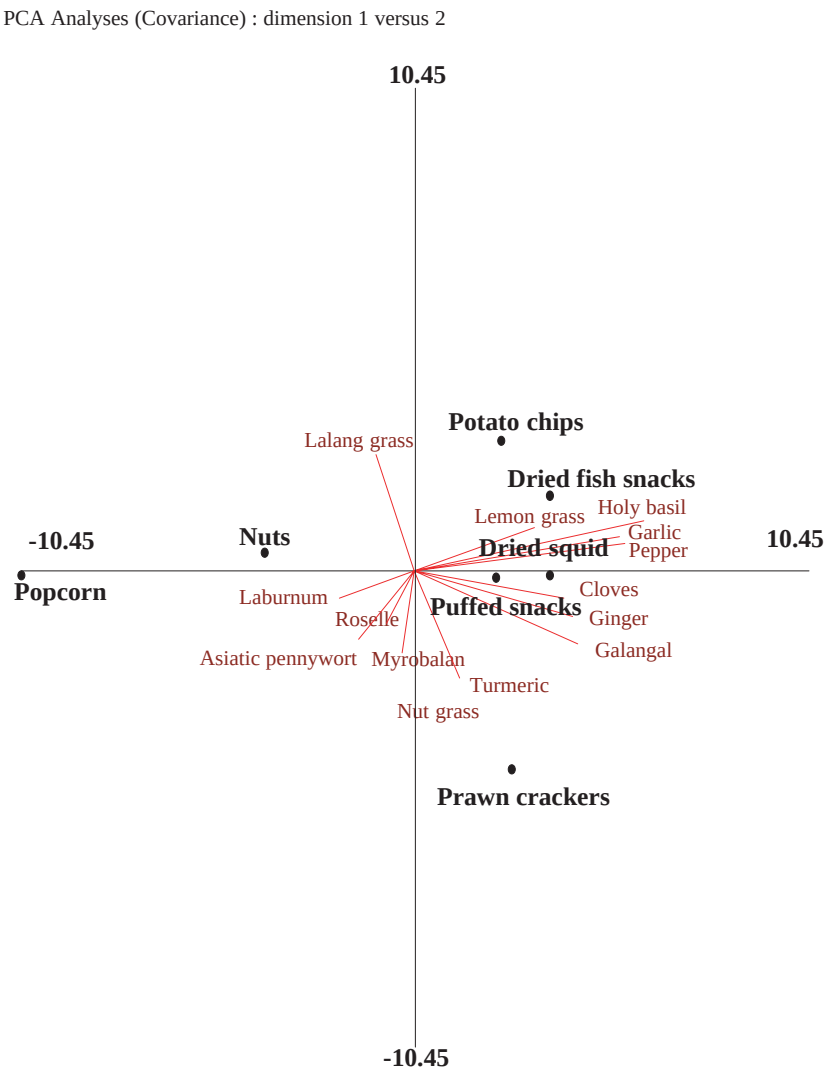


**Figure 3** Perceptual mapping of the suitability scores of using herbs as functional ingredients in Thai commercial snacks by Thai respondents (Explained variance = 96 per cent).

Chives and parsley were more suitable than other NZ herbs for adding in prawn crackers, dried fish snacks, dried squid, and puffed snacks. Only NZ herb being compatible to potato chips was chives. There was no significant difference ($p>0.05$) in suitability scores between NZ herbs for nuts. Chives, parsley, echinacea, and rosemary were more properly to popcorn. For Thai herbs, garlic and lemon grass were more fitting to every snack category. Pepper also more suited for any category except nuts. Ginger was compatible to dried fish snacks, dried squid, puffed snacks, nuts, and popcorn, as well. However, the suitability scores of any herbs for nuts and popcorn were less than 5 (neither suitable nor unsuitable) as shown in Table3. It meant that there was no suitable herb identified for nuts and popcorn in New Zealanders' opinions.

A perceptual map of the suitability of adding herbs in snacks responded by New Zealanders was created as Figure 4. The suitability scores of 5 products and 15 herbs highly related to the horizontal axis (dimension 1). These 5 products were composed of prawn crackers, potato chips, puffed snacks, nuts, and popcorn and these 15 herbs were chives, parsley, thyme, red clover, rosemary, horseradish, echinacia, alfafa, turmeric, pepper, garlic, holy basil, lemon grass, asiatic pennywort, and cloves. The suitability scores of dried fish snacks, dried squid, and ginger highly related to the vertical axis (dimension 2). The positions of 7 products were unable to be grouped. However, all of the herb vectors were in the positive side of the horizontal axis. It meant that the more positive value of product position on the horizontal axis, the more suitability to use herbs as its ingredients. The longer vectors of NZ herbs reflected New Zealanders preferred to have NZ herbs in snack products rather than Thai herbs. The longer projections on the horizontal axis of the vectors for chives and parsley indicated their more compatibility to use in snacks. The similar length of the projections of Thai herb vectors on the horizontal axis indicated New Zealanders rated

**Table 3** The responses of suitability of using herbs in each snack category by New Zealand consumers.

| | Compatible herbs | Score range of compatible herbs | Score range of all selected herbs |
|---|---|---|---|
| Prawn crackers | NZ: chives, parsley | 6.55-6.89 | 5.18-6.89 |
| | TH: garlic, lemon grass, pepper | 6.11-6.21 | 4.92-6.21 |
| Dried fish snacks | NZ: chives, parsley | 5.76-5.97 | 4.36-5.97 |
| | TH: garlic, lemon grass, pepper, ginger | 5.66-5.84 | 4.32-5.84 |
| Dried squid | NZ: chives, parsley | 5.50-5.55 | 4.18-5.55 |
| | TH: garlic, lemon grass, pepper, ginger | 5.37-5.73 | 4.50-5.73 |
| Puffed snacks | NZ: chives, parsley | 6.00-6.45 | 4.53-6.45 |
| | TH: garlic, lemon grass, pepper, ginger | 5.53-5.79 | 4.21-5.79 |
| Potato chips | NZ: chives | 7.34 | 5.24-7.34 |
| | TH: garlic, lemon grass, pepper | 5.94-6.50 | 4.76-6.50 |
| Nuts | NZ: - | - | 4.16-4.53 |
| | TH: ginger, garlic, lemon grass, cloves, asiatic pennywort | 4.37-4.79 | 4.00-4.78 |
| Popcorn | NZ: chives, parsley, echinacea, rosemary | 4.16-4.55 | 3.63-4.55 |
| | TH: garlic, lemon grass, pepper, ginger, turmeric | 4.13-4.74 | 3.74-4.73 |

only a small difference on the suitability of Thai herbs because they might not be much familiar to Thai herbs.

## CONCLUSION

The idea to use herbs as functional ingredient in Thai commercial snack products seems to be possibly, especially for prawn crackers, dried fish snacks, dried squid, puffed snacks and potato chips which are savoury snacks. However, this concept must be further studied in details since the products which are going to be developed have to be designed to meet the combination of the good flavour and the sufficient quantity of herbs for both palatability and pharmacological activity of products.

## ACKNOWLEDGEMENT

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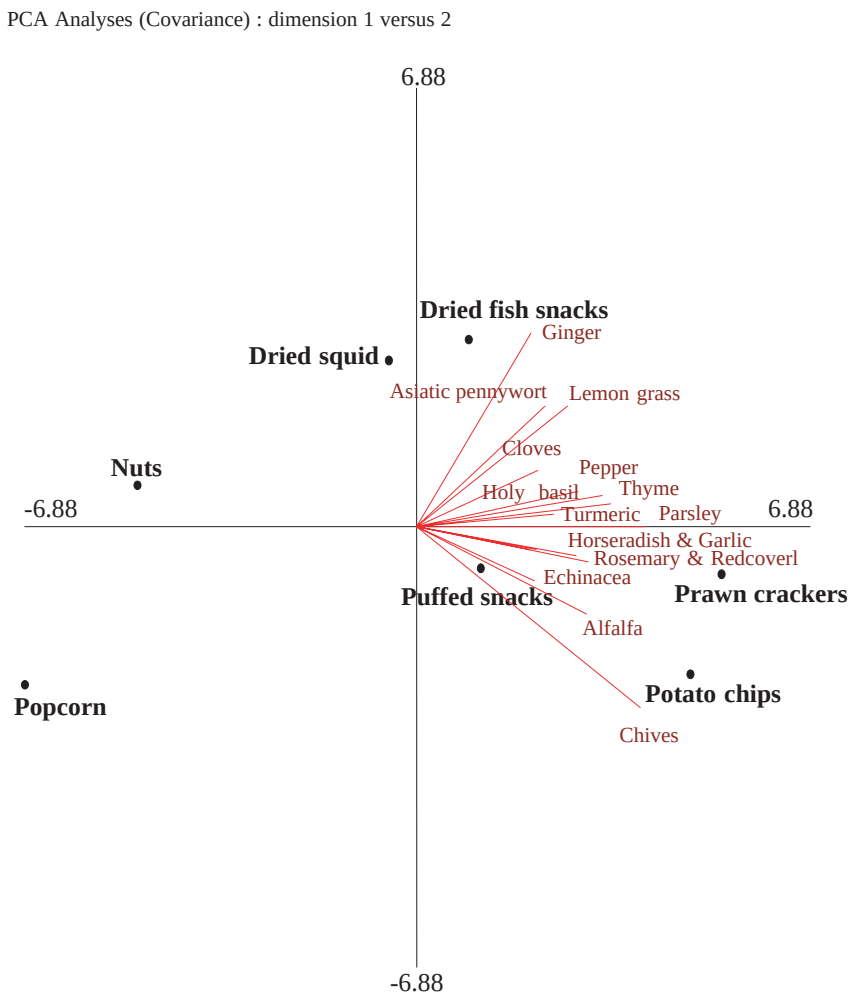


**Figure 4** Perceptual mapping of the suitability scores of using herbs as functional ingredients in Thai commercial snacks by New Zealand respondents (Explained variance = 92 per cent).

## LITURATURE CITED

Booth, R.G. 1990. Snack Food. Van Nostrand Reinhold. New York. 401 p.

Dole Food Company. 2001. Encyclopedia of Foods: a Guide to Healthy Nutrition. Academic Press. San Diego. 450 p.

Hehmhongsha, P. 1998. Herbs Used in Primary Health Care. Primary Health Care. Ministry of Public Health. Nonthaburi. 176 p.

Heasman, M. and J. Mellentin. 2001. The Functional Foods Revolution: Healthy People, Health Profits? Earthscan Publications. London. 313 p.

Lilien, G. L. and A. Rangaswamy. 1998. Marketing Engineering: Computer- Assisted Marketing Analysis and Planning. Addison Wesley Educational Publishers. Reading. 350 p.

Mazza, G. and B. D. Oomah. 2000. Herbs, Botanicals and Teas. Technomic Publishing Company. Lancaster. 416 p.

Neal, W.D. 1988. Overview of Perceptual Mapping. [cited 2001] Available from:URL:http:// www.sdrnet.com/ thoughtsonperceptualmapping.htm.

Nordmark, B. 1999. Snack flavours' revolution. Food Technology in New Zealand 34 (5) : 26-27.

Painter, G. 1995. The Essential Herb Garden: Growing and Using Herbs in New Zealand. Penguin Books New Zealand. Auckland. 152 p.

Phuthiyanun, S. 1999. The Fundamental Thai Traditional Medicine: Herbs and Thai Traditional Medicine. 3rd ed. Institute of Thai Traditional Medicine. Department of Medicine. Ministry of Public Health. Nonthaburi. 448 p.

Promar International. 1997. Snack foods, part 1: the snack food market. Food Ingredients and Analysis International (Sep) : 19-22.

Pszczola, D.E. 1999. Ingredients: nutraceuticals that take the form of candy and snacks. Food Technology 53(10) : 74 -76, 78, 80.

Resurreccion A.V.A. 1998. Consumer Sensory Testing for Product Development. Aspen Publishers. Maryland. 254 p.

Thansettakij. 2001. Frito Lays re-launched "Mum Mun": using this good- view brand penetrate extruded snack segment. Thansettakij 21(1,607) : 8-11 July.

Thomas, S.C.Li. 2000. Medicinal Plants: Culture, Utilization and Phytopharmacology. Technomic Publishing Company. Lancaster. 517 p.

Tuley, L. 2000. Snacks go healthy. Food Manufacture (March) : 30-31.

Uhl, S.R. 2000. Handbook of Spices, Seasonings, and Flavourings. Technomic Publishing Company. Lancaster. 329 p.

Williams, D. 1999. Flavors for snack-food application. Perfumer and Flavorist 24 (Nov/ Dec) : 29, 31-32, 34.

Wuthithummawate, W. 1997. Thai Traditional Medicine: Summary of Herbs. 2nd Edition. Ordiun store. Bangkok. 480 p.

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# Recovery of Silver as Silver Nitrate from Waste Silver Chloride in Quantitative Analysis Laboratory

**Apisit Songsasen and Niti Poowanathai**


## ABSTRACT

Various methods for recovery of silver as silver nitrate from waste silver chloride in the quantitative analysis laboratory of the Department of Chemistry, Kasetsart University were investigated. Most of the methods based on the reduction of silver chloride to metallic silver and followed by the oxidation of metallic silver to silver nitrate by nitric acid. The electrolysis of silver (I) solution to give metallic silver also was investigated. The percent recovery and percent purity of the recovered-silver nitrate were in the range of 89.49-97.33 and 99.29-99.66, respectively. The reduction of silver chloride at 1,000 °C in electric furnace gave the highest percent recovery with high purity silver nitrate. The operation cost of each method was compared, and the reduction of silver chloride by formaldehyde, which gave 95.14% recovery and 99.29% purity, is recommended as a suitable method for recovery of silver as silver nitrate.

**Key words:** recovery of silver, silver chloride, silver nitrate

## INTRODUCTION

In general quantitative analysis laboratories, silver is commonly used as silver nitrate for qualitative and quantitative analysis of chloride, bromide and iodide ions, and generated silver halides as chemical waste. Because of the high cost of silver, a number of articles have appeared in the literature concerning reclamation of silver from its various compounds. Many of these methods use high temperature reactions (500-1,000°C), or hazardous materials such as cyanides, silver-ammonia solutions, concentrated base or aqua regia (Hayes and Steed, 1972; Foust, 1984; Hill and Bellows, 1986; Rawat and Kamoonpuri, 1986; Murphy *et al*., 1991).

As the price of silver nitrate increases and chemistry department budgets tighten, the recovery of silver from silver residues of analytical laboratory becomes necessary. Reverting wastes obtained in the experiment into useful materials can offset a portion of the cost. There are various procedures for recovering silver as silver nitrate from silver halide residue. Nevertheless, the search for the procedure using common and inexpensive laboratory reagents is also necessary.

This work focused on three general objectives: recovery of metallic silver from silver chloride waste from quantitative analysis laboratory of the Department of Chemistry (Kasetsart University) by various methods, conversion of the metallic silver back to silver nitrate, and comparison of percent purity and percent recovery of the products from each method. The cost for the recovery of silver from each procedure had also been investigated.

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## MATERIALS AND METHODS

### Recovery of metallic silver from silver chloride waste

The silver chloride residue obtained from quantitative analysis laboratory as waste was acidified with hydrochloric acid to ensure complete precipitation of silver as silver chloride. It was heated at 100°C until the residue appeared white. After cooling, the supernatant liquid was decanted, and silver chloride was filtered off on Whatman No. 42 paper on a Buchner funnel.

**Reduction of formaldehyde**

$2AgCl + 3NaOH + CH_2O \rightarrow 2Ag + HCO_2Na + 2NaCl + 2H_2O$

A 4.10 g of NaOH (Merck, AR. grade) was dissolved in 100 ml of water in a beaker and 5 g of finely ground silver chloride was added with rapid stirring, followed by 3 ml of 37% formaldehyde (BDH, Lab grade). The beaker was covered with a watch glass and stirring continued. After 10 minutes the mixture was heated to 60-70°C, and stirring was continued for a total time of about 1 hour. By the end of this time the metallic silver will have agglomerated into small shiny pellets. The pellets were collected on a sintered glass filter, washed several times with water, then acetone and air dried.

**Reduction with glucose and fructose**

$2AgCl + 3NaOH + C_6H_{12}O_6 \rightarrow 2Ag + C_6H_{11}O_7Na + 2NaCl + 2H_2O$

A 2.7 g of NaOH (Merck, AR. grade) was dissolved in 100 ml of water, and 5 g of silver chloride was suspended in the resulting solution. A 3.675 g portion of glucose (Ajax, Lab grade) or fructose (Fluka, Lab. grade) was added. The reaction mixture immediately turned black. It was covered with a watch glass and heated to 70-80°C with stirring for about 1 hour. The resulting silver pellets were collected on a sintered glass filter, washed several times with water, then acetone and air dried.

**Reduction with sodium borohydride**

$8Ag^+ + BH_4^- + 8OH^- \rightarrow 8Ag + B(OH)_4^- + 4H_2O$

A 0.75 g of $NaBH_4$ (Fluka, AR. grade) was dissolved in 50 ml of ammonia solution (5 g AgCl / 50 ml concentrated ammonia solution) in a beaker. A vigorous reaction took place, a powdery precipitate of silver was observed in about 1 minute. The beaker was covered with a watch glass and heated to 70-80°C with stirring for about 1 hour. The silver pellets were collected on a sintered filter, washed several times with water, then acetone and air dried.

**Reduction with zinc, copper and magnesium powder**

$2Ag^+ + Zn \rightarrow 2Ag + Zn^{2+}$

$2Ag^+ + Cu \rightarrow 2Ag + Cu^{2+}$

$2Ag^+ + Mg \rightarrow 2Ag + Mg^{2+}$

For 5 g of silver chloride which dissolved in 50 ml of concentrated ammonia solution, 1.5 g of zinc (Mallinckrodt, Lab grade) or 1.15 g of copper (Merck, Lab grade) or 0.43 g of magnesium (Riedel-de Haen, Lab. grade) powder was used. A portion of metal powder was added in the silver-ammonia solution. The mixture was stirred at 40°C for 1 hour, and the resulting precipitated was collected on a sintered glass filter and washed with water several times. The resulting precipitated was washed with 6M $H_2SO_4$ to wash out the excess metal powder.

**Silver electrodeposition method**

A 2.5 g of AgCl was dissolved in 100 ml of 5% KCN (Carlo Erba, Lab grade) solution. A 2.4 g of potassium carbonate (Unilab, Lab grade) was added into the silver chloride solution. A carbon rod electrode was used as the cathode and a stainless steel rod electrode as the anode. The electric current was adjusted to 1-3 A, and the solution was stirred gently and electrolyzed for 15-45 minutes. The silver will have deposited as a coarse adherent coating on the carbon rod electrode. The concentration of cyanide ion left in the solution after the electrolysis was determined by Volhard's method (Bassett *et al*., 1978).

**Silver recovery with high temperature method**

A dried residue of silver chloride was

thoroughly mixed with an equivalent of potassium carbonate. The mixture was placed in a graphite crucible and baked in a furnace at 1,000°C. At this temperature the reduced silver melts, forming a puddle at the bottom of the crucible. The crucible was filled with the mixture no more than three-fourth full as foaming occurs. After 1 hour, the crucible was removed and silver ingot was obtained.

**Preparation of silver nitrate from recovered silver**

Approximately 15 ml of water was added into 3.7 g of recovered silver and heated to 50°C, then concentrated $HNO_3$ (Carlo Erba, Lab grade) was added dropwise with occasional swirling until all the silver has dissolved. The solution was filtered through the Whatman No. 42 paper to remove undissolved material. The filtrate was concentrated on a hot plate until precipitation occurred. The excess $HNO_3$ was neutralized in a desicator filled with NaOH. The recovered silver nitrate was characterized by X-ray powder diffractometer. The purity of the recovered silver nitrate was determined by atomic adsorption spectrophotometer using PERKIN ELMER, AAnalyst 800.

## RESULTS AND DISCUSSION

The X-ray powder diffraction (XPD) spectrum of the recovered silver nitrate is shown in Figure 1. The spectrum shows a strong peak at 35.80 degrees with two weak peaks at 54.96 and 75.37 degrees, indicated that only silver nitrate in the product. The percent purity and recovery of silver nitrate from waste silver chloride from each method is shown in Figure 2 and 3, respectively.

Silver in the form of $[Ag(NH_3)_2]^+$ ion or $AgOH_{(aq)}$ was easily reduced by aqueous borohydride, formaldehyde, reducing sugar (glucose and fructose), as well as active metals such as zinc, magnesium and copper. The electrolysis of $[Ag(CN)_2]^-$ ion gave quite a promising yield of recovered-silver nitrate. High temperature method using potassium carbonate ($K_2CO_3$) as reducing agent also gave a good yield of recovered-silver nitrate when the experiment was performed on a high temperature furnace.

Among all the reducing agents used in the experiments, sodium borohydride and formaldehyde seem to be more powerful than other reducing agents. As seen in Figure 3, the aqueous sodium borohydride and the aqueous formaldehyde procedures gave 95.59% and 95.14% recovery of

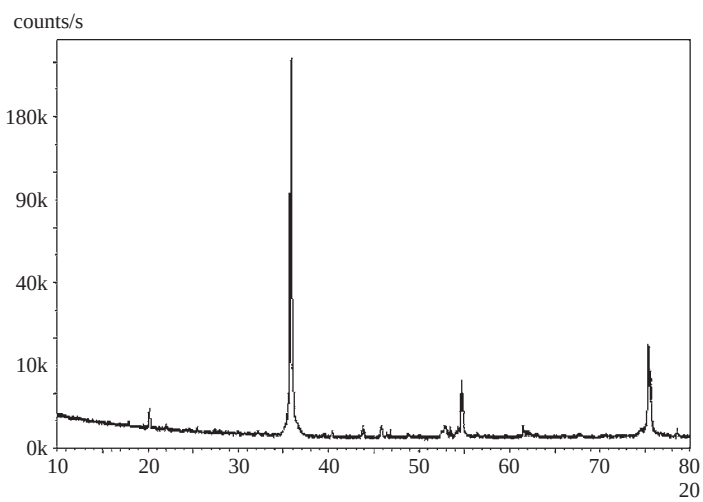


**Figure 1** X-ray powder diffraction (XPD) spectrum of the recovered silver nitrate.

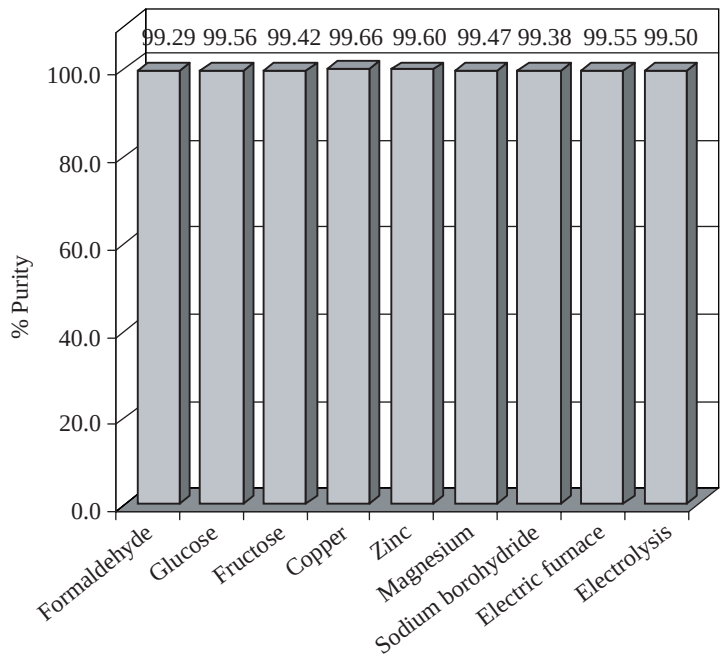


**Figure 2** Percent purity of recovered-silver nitrate prepared by various methods.

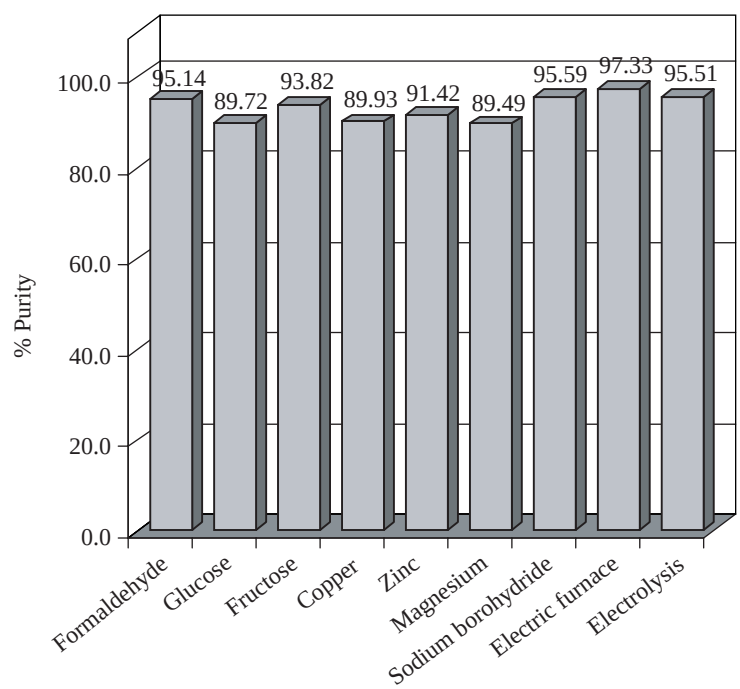


**Figure 3** Percent recovery of silver nitrate prepared by various methods.

silver nitrate, respectively, which were higher than when other reducing agents were used. This may be because both of them were used in aqueous form while the others in solid form, and the reduction potential of sodium borohydride and formaldehyde were very low compared to other reducing agents used (Table 1). Another reason is the feature of recovered-metallic silver (Table 2). When both formaldehyde and sodium borohydride were used as the reducing agent, the size of recovered-metallic silver were small and shiny which was easier to react and change to silver nitrate. Although the reduction of silver ion by reducing metals also gave small size silver pellet, the color of the pellet was not shiny which may indicate the presence of $Ag_2O$ at the surface of silver metal which made them difficult to react with nitric acid in the final step.

The electrolysis of $[Ag(CN)_2]^-$ when stainless steel were used as both anode and cathode gave quite a promising result (95.51% recovery). However, this method is not recommended due to a quantitative amount of cyanide ion left in the post-electrolysis solution. Using this method, the initial concentration of cyanide ion was 0.75 molar, and it was found that the concentration of cyanide ion after the electrolysis was 0.18 molar which was still too high and could cause some problems to the environment.

The reduction of silver chloride by formaldehyde and fructose seems to be a suitable method (Table 3), both in the quality of recovered-silver nitrate and the operation cost. Although the operating cost of the reduction of silver chloride at 1,000°C in electric furnace was double to the reduction by formaldehyde, this method may be recommended if the high purity of recovered-silver nitrate is required.

## CONCLUSION

The methods for recovery of silver as silver nitrate from silver chloride waste have been compared. The reduction of silver chloride by formaldehyde and fructose has proved to be a suitable method both in percent yield and operation cost. The reduction of silver chloride at 1,000°C is suitable if the high purity of recovered-silver nitrate is required. These methods can be applied for the recovery of silver from the general quantitative analysis laboratories.

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**Table 1** Standard reduction potential of some metals and compounds.

| Reaction | $E^o$/V |
|---|---|
| $Ag^+ + e^- \rightarrow Ag$ | 0.800 |
| $Cu^{2+} + 2e^- \rightarrow Cu$ | 0.342 |
| $Mg^{2+} + 2e^- \rightarrow Mg$ | -2.356 |
| $Zn^{2+} + 2e^- \rightarrow Zn$ | -0.762 |
| $H_2BO_3^- + 5H_2O + 8e^- \rightarrow BH_4^- + 8OH^-$ | -1.240 |
| $HCO_2^- + 2H_2O + 2e^- \rightarrow CH_2O + 3OH^-$ | -1.160 |

**Table 2** The appearance of recovered-metallic silver prepared by various methods.

| Procedure | Appearance of recovered-metallic silver |
|---|---|
| 1. Using sodium borohydride as reducing agent | Small size shiny pellets (~3 mm) |
| 2. Using formaldehyde as reducing agent | Small size shiny pellets (~3 mm) |
| 3. Using glucose as reducing agent | Medium size pellets (~5 mm) |
| 4. Using fructose as reducing agent | Medium size pellets (~5 mm) |
| 5. Using copper as reducing agent | Small size pellets (~3 mm) |
| 6. Using zinc as reducing agent | Small size pellets (~3 mm) |
| 7. Using magnesium as reducing agent | Fine brown-gray powder, |
| 8. Electrolysis | Shiny film deposited on the electrode |
| 9. High temperature (using electric furnace) | Large size shiny pellet (~8 mm) |

**Table 3** Cost of recovered-silver nitrate by various methods and commercial price.

| Method | Cost of silver nitrate (Baht/gram) |
|---|---|
| 1. Using formaldehyde as reducing agent | 2.66 |
| 2. Using glucose as reducing agent | 3.25 |
| 3. Using fructose as reducing agent | 3.00 |
| 4. Using copper as reducing agent | 5.42 |
| 5. Using zinc as reducing agent | 4.71 |
| 6. Using magnesium as reducing agent | 5.18 |
| 7. Using sodium borohydride as reducing agent | 10.34 |
| 8. High temperature (using electric furnace) | 7.28 |
| 9. Electrolysis | 6.43 |
| 10. Commercial price | 21.94* |

* Average price for 100 g/package from 3 companies.

## LITERATURE CITED

Bassett, J., R.C. Denney, G.H. Jeffery, and J. Mendham. 1978. Vogel's Textbook of Quantitative Inorganic Analysis. 4th ed., Longman, New York. 925 p.

Foust, D.F. 1984. Recovery of silver and cobalt from laboratory wastes. J. Chem. Educ. 61 (10) : 924.

Hayes, J.M. and S.P Steed. 1972. Procedure for recovering elemental silver from silver residues. J. Chem. Educ. 49 : 156.

Hill, J.W. and L. Bellows. 1986. Production or recovery of silver for laboratory use. J. Chem. Educ. 63 (4) : 357.

Murphy, J.A., A.H. Ackerman, and J.K. Heeren. 1991. Recovery of silver from and some use for waste silver chloride. J. Chem. Educ. 68 (7) : 602-604.

Rawat, J.P. and S.M. Kamoonpuri. 1986. Recovery of silver from laboratory wastes. J. Chem. Educ. 63 (6) : 537-538.

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สมาชิกเลขที่.....

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ลงชื่อ.....เหรัญญิก

(.....)

วันที่.....

# ใบรับรอง

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ตำแหน่ง.....สถานที่ทำงาน.....

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ศึกษา.....จริง

ลงชื่ออาจารย์ที่ปรึกษา.....

(.....)

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