ภาวะที่เหมาะสมสำหรับการผลิตไข่น้ำ *Wolffia globosa*

นางนิศาชล ฤาแก้วมา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ปีการศึกษา 2554

OPTIMAL CONDITIONS FOR PRODUCTION OF KHAI-NAM *Wolffia globosa*

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A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy Program in Biotechnology

Faculty of Science

Chulalongkorn University

Academic year 2011

Thesis Title	OPTIMAL CONDITIONS FOR PRODUCTION OF KHAI-NAM *Wolffia globosa*
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นิศาชล ฤาแก้วมา : ภาวะที่เหมาะสมสำหรับการผลิตไข่น้ำ *Wolffia globosa* (OPTIMAL CONDITIONS FOR PRODUCTION OF KHAI-NAM *Wolffia globosa*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.สมเกียรติ ปิยะธีรธิติวรกุล, อ. ที่ปรึกษาวิทยา นิพนธ์ร่วม: ดร. สรวิศ เผ่าทองศุข, 121 หน้า.

การศึกษาภาวะที่เหมาะสมสำหรับการผลิตไข่น้ำ จากการศึกษาด้านสัณฐานวิทยาของไข่น้ำจากบ่อธรรมชาติในอำเภอเมือง จังหวัดสกลนคร พบว่าเป็นไข่น้ำชนิด *Wolffia globosa* ไข่น้ำในสภาวะธรรมชาติมีการสืบพันธุ์แบบไม่อาศัยเพศโดยการแตกหน่อ ใช้ระยะเวลา 96 ชั่วโมง หรือ 4 วัน ตลอดการเก็บข้อมูลการเจริญเติบโตระยะเวลา 12 เดือน (มีนาคม 2551 – กุมภาพันธ์ 2552) พบว่าเดือนกรกฎาคม มีความหนาแน่นเซลล์สูงสุด 65.18 กรัม (น้ำหนักแห้ง) ต่อตารางเมตร และเดือนกุมภาพันธ์ มีความหนาแน่นเซลล์น้อยสุด 2.45 กรัม (น้ำหนักแห้ง) ต่อตารางเมตร ในส่วนของอัตราการผลิตพบว่าเดือนมิถุนายนมีอัตราการผลิตสูงสุด 1.05 กรัม (น้ำหนักแห้ง) ต่อตารางเมตรต่อวัน และเดือนสิงหาคมตรวจไม่พบการเพิ่มจำนวน ไข่น้ำจากบ่อธรรมชาติมีโปรตีน 33.3 เปอร์เซ็นต์ ไขมัน 5.0 เปอร์เซ็นต์ เยื่อใย 10.4 เปอร์เซ็นต์ มีกรดอะมิโนที่จำเป็นอยู่อย่างครบถ้วน และการปนเปื้อนจากเชื้อที่ก่อโรคพบน้อยมาก ซึ่งเหมาะสมจะเป็นอาหารของมนุษย์ ในห้องปฏิบัติการได้ศึกษาปัจจัยที่เหมาะสมต่อการเจริญเติบโตของไข่น้ำ พบว่า Hutner' medium ให้ผลผลิตของไข่น้ำสูงและช่วงชีวิตยาว (0.18±0.04 เซลล์ต่อตารางเมตรต่อวัน และ17.37±2.9 วัน) การเลี้ยงไข่น้ำด้วยแสงธรรมชาติพบว่าความเข้มแสงสูงในช่วงกลางวันไม่มีผลยับยั้งการสังเคราะห์แสงของไข่น้ำ แต่อุณหภูมิสูงมากกว่า 40 ^{o}C มีผลลบต่อการสังเคราะห์แสงของไข่น้ำ พีเอชที่เหมาะสมอยู่ในช่วง 5-7 และความหนาแน่นเริ่มต้นที่เหมาะสมคือ 5-20 % ของพื้นที่ผิวน้ำ เมื่อนำปัจจัยต่างๆไปทดลองร่วมกัน พบว่า ความเข้มแสงที่ 10,000 ลักซ์ พีเอช 6 และความหนาแน่นเริ่มต้นที่ 15 % ให้ผลผลิตสูงที่สุด การศึกษาระบบการเลี้ยงที่เหมาะสมการผลิตไข่น้ำ 5 ระบบคือ (1) ถังน้ำนิ่ง (2) ถังที่มีการพ่นอากาศ (3) ถังที่มีการกวนผสมที่ผิวหน้าน้ำ (4) ถังที่มีการพ่นละอองน้ำที่ผิวหน้าน้ำ และ (5) ถังที่มีการเลี้ยงบนชั้นที่อยู่เหนือน้ำโดยมีการพ่นละอองน้ำตลอดเวลา ในการทดลองเลี้ยง 28 วัน พบว่าถังที่มีการกวนผสมที่ผิวหน้าน้ำซึ่งเป็นระบบที่ให้น้ำไหลเวียนในแนวราบ ให้ผลผลิตสูงที่สุด 1.52±0.04 กรัม (น้ำหนักแห้ง) ต่อตารางเมตรต่อวัน เมื่อนำไข่น้ำมาวิเคราะห์ทางโภชนาการ พบว่าไข่น้ำมีโปรตีน 48.2 เปอร์เซ็นต์ ไขมัน 9.6 เปอร์เซ็นต์ เยื่อใย 14.5 เปอร์เซ็นต์ มีกรดอะมิโนที่จำเป็นอยู่อย่างครบถ้วน และการปนเปื้อนจากเชื้อที่ก่อโรคพบน้อยมาก

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5073835123 : MAJOR BIOTECHNOLOGY

KEYWORDS : *Wolffia globosa* / KHAI-NAM / WATERMEAL / DUCKWEED / OPTIMAL CONDITION / PRODUCTION /

NISACHOL RUEKAEWMA: OPTIMAL CONDITIONS FOR PRODUCTION OF KHAI-NAM *Wolffia globosa*. ADVISOR: ASSOC. PROF. SOMKIAT PIYATIRATITIVORAKUL, Ph.D., CO-ADVISOR: SORAWIT POWTONGSOOK, Ph.D., 121 pp.

Study on optimal conditions for the production of Khai-nam (water meal), *Wolffia globosa*, were carried out. Khai-nam was collected from the natural pond in Mueang district, Sakon Nakhon province, Thailand. According to morphological investigation, it was identified as *Wolffia globosa*. Under natural condition, asexual reproduction of Khai-nam took approximately 96 hours or 4 days for each generation. Highest density of *W. globosa* in natural pond was 65.18 g dry weight m^{-2} in July and the lowest density was 2.45 g dry weight m^{-2} in February. The highest productivity, 1.05 g dry weight $m^{-2}d^{-1}$, was found in June and no growth of Khai-nam was detected in August. Nutritional analysis of *W. globosa* from native pond was 33.3% protein with complete essential amino acids profile, 5.0% fat and 10.4% crude fiber. Bacterial analysis showed that Khai-nam from natural source had low bacterial contamination and therefore accepted for human consumption. Growth optimization of *W. globosa* was carried out under laboratory conditions. It was found that Hutner's medium provided high yield of 0.18±0.04 fronds $ml^{-1}d^{-1}$ with 17.37±2.9 days of life span. With outdoor cultivation, high light intensity during day time did not affect photosynthesis efficiency (quantum yield of PSII) measure by chlorophyll fluorescence technique but high temperature at 40 ^{o}C significantly reduced photosynthesis efficiency. The optimum pH for *W. globosa* was between 5 and 7 and the optimum initial density for *W. globosa* cultivation was 5 to 20% water surface area coverage. A factorial experiment on light intensity, initial pH and initial density on growth performance and quality of *W. globosa* indicated that 10,000 lux light intensity, initial pH of 6 and 15% initial density provided the highest yield. Finally, five outdoor culture systems for production of *W. globosa* were evaluated. The culture systems included (1) static tank, (2) vertical aeration tank, (3) horizontal surface agitation tank, (4) tank with top water spraying, and (5) layer culturing system with top water spraying. The cultivation period was 28 days. The results showed that the tank with horizontal circulation provided the highest yield of 1.52±0.04 g DW m^{-2} d^{-1}. The biomass produced had 48.2% protein with complete essential amino acids, 9.6% fat, and 14.5% crude fiber with low bacterial contamination.

Field of Study : Biotechnology Student's Signature .....

Academic Year : 2011 Advisor's Signature .....

Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor Assoc. Prof. Somkiat Piyatiratitivorakul and Dr. Sorawit Powtongsook for their advices, Assoc. Prof. Sirirat Rengpipat, Assoc. Prof. Thaithaworn Lirdwitayaprasit, Dr. Yupyn Chintapakorn and Assoc. Prof. Sukhoom Rowchi kindly serve as the thesis committee.

My acknowledgement is also expressed to Mr. Harnchoke Butweingpun, lecturer, from Sakon Nakhon Technical College for his useful suggestions and constructions about *Wolffia* culture systems; Prof. E. Landolt kindly supplied me the information of *Wolffia.*

I would like to thank you Miss Iing-on Tongcomdee, Miss Puncharas Gorcharoenwat, Mr. Dusit Srivilai and friends in Plankton Laboratory, Dept. Marine Science throughout Program in Biotechnology for their help in laboratory work and everything, moreover, Miss Supaporn Kodtha and Miss Srisuwan Huadkuntha from Sakon Nakhon Inland Fisheries Research and Development Center for the water analysis.

Throughout the long period of my doctoral program my parents and my parents in law have assisted me both financially and emotionally, moreover, I greatly appreciate to Miss Prapit Ruekaewma, sister in law, for take care our family and everything. My strongest and most important supporter and champion and will always be my husband Mr. Pramook Ruekaewma, who stuck by me through thick and thin and whose unfailing support made this dissertation possible. This dissertation is as much his achievement as mine. Last but not least I need to thank my daughter Chollathip Ruekaewma who does not complain about my absences and love me for who I am.

This research was supported by Rajamagala University of Technology Isan, Thai Government Scholarship in the Area of Science and Technology, NSTDA, Ministry of Science and Technology and The 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endownment Fund).

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ABBREVIATION

sp., spp.	species
mg	milligram
g	gram
kg	kilogram
ml	milliliter
l	liter
mm	millimeter
cm	centimeter
m	meter
ha	hectare
wt	weight
DW	dry weight
d	day
v/v	volume per volume
^{o}C	degree Celsius
rpm	round per minute
TN	Total Nitrogen
TKN	Total Potassium Nitrogen
PVC	polyvinyl chloride
CRD	completely randomized design
PSII	photosynthesis II

CHAPTER I

INTRODUCTION

Development of new foods is vital to the needs of rapid expanding in Asia because of rapid human population growth. *Wolffia* spp., watermeal or duckweed, is an aquatic plant generally found throughout Thailand and the neighbor countries. *Wolffia arrhiza* is used as food ingredient by Burmese, Laotian and Thai especially in the northeast and northern of Thailand. Watermeal or Khai-nam in Thai is an oval shape plant floating on pond water surface. Khai-nam is generally regarded as poor people's food and has attracted little attention as a potentially significant source of human food (Bhanthumnavin and McGary, 1971).

Furthermore, *W. arrhiza* exhibits high growth rate and consequently absorbs large amounts of nitrogen and phosphorus and its vegetative frond contains 40% protein on dry weight (Fujita, Mori and Kodera, 1999). Moreover, it may be feasible to use *W. arrhiza* and *W. globosa* to produce high protein animal feed (Naskar et al., 1986; Chantiratikul et al., 2010; Chantiratikul and Chumpawadee, 2011). *Wolffia* spp. also has a potential for a utilization and treatment of wastewater (Hillman and Culley, 1978; Edward et al., 1992).

In addition, researchers are using these plants to study basic plant development, plant biochemistry, photosynthesis, toxicity of hazardous substances, and much more.

Genetic engineers are cloning and modifying *Wolffia* spp. genes to inexpensively produce pharmaceutical products. Environmental scientists are using *Wolffia* spp. to remove unwanted substances from water (Cross, 2006: online). Although *Wolffia* spp. has been widely studied, the hygiene mass production of *Wolffia* spp. has received only little attention. In this research, we elucidate the effects of light intensity, temperature, pH, density and nutrients on growth and quality of *Wolffia* sp. and a suitable culture system for hygiene mass production of *Wolffia* sp. will be developed.

The objectives of this research are: 1) To study effect of light intensity, temperature, pH, density and nutrient on the performance of growth and quality of *Wolffia* sp. and 2) To develop a suitable culture system for the mass production of *Wolffia* sp. for human consumption.

CHAPTER II

LITERATURE REVIEW

INTRODUCTION

Wolffia is a genus of 11 species which is the smallest flowering plant on Earth. Some *Wolffia* species are shown in Figure 2-1., 2-2. and 2-3. Commonly called watermeal, these aquatic plants resemble specks of cornmeal floating on the water. *Wolffia* spp. are free-floating frond, green or yellow-green in color, and no roots. The flower is produced in a depression on the top surface of the plant body. It has one stamen and one pistil. Individuals often float together in pairs or form floating mats with related plants, such as *Lemna* spp. and *Spirodela* spp. (Figure 2-4.).

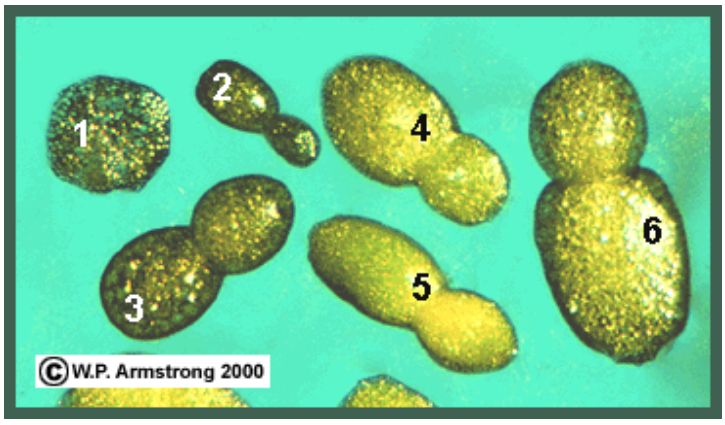


Figure 2-1. Dorsal view of six *Wolffia* species: 1. *W. microscopica* (India); 2. *W. globosa*; 3. *W. columbiana*; 4. *W. brasilliensis*; 5. *W. borealis*; 6. *W. arrhiza* (Germany) (Armstrong, 2000: online)

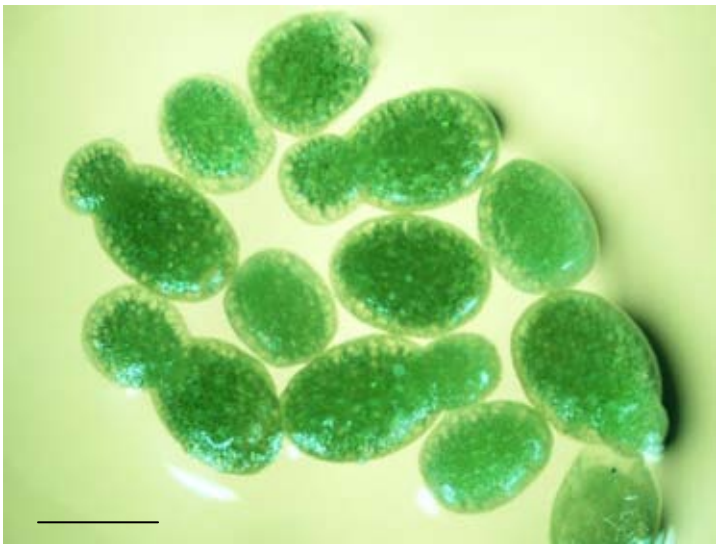


Figure 2-2. Dense population of *Wolffia* sp. at a natural pond in Mueang district, Sakon Nakhon province, Bar = 0.5 mm

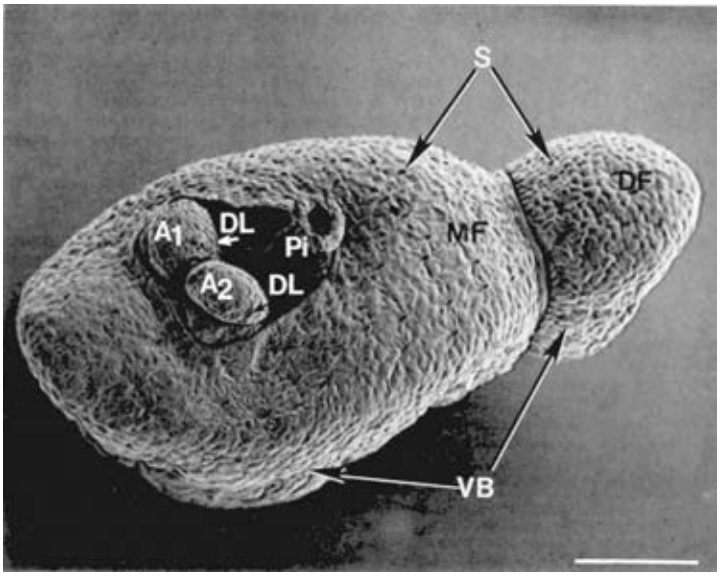


Figure 2-3. SEM of a two frond colony of *W. australiana* showing mother frond (MF), daughter frond (DF), stoma (S), and ventral bulge (VB). The mature flower consists of a pistil (Pi) and the two lobes of the anther labeled as A1 and A2. Each anther lobe has a dehiscence Line (DL). Bar=0.25 mm (Bernard, Bernard and Denny, 1990)

Most *Wolffia* species have a very wide distribution across several continents (Wikipedia, 2009: online). *Wolffia* is classified as:

Kingdom:	Plantae
(unranked):	Angiosperms
(unranked):	Monocots
Order:	Alismatales
Family:	Araceae
Subfamily:	Lemnoideae
Tribe:	Wolffieae
Genus:	***Wolffia***
Species:	*Wolffia angusta*
	Wolffia arrhiza
	Wolffia australiana
	Wolffia borealis
	Wolffia brasiliensis
	Wolffia columbiana
	Wolffia cylindracea
	Wolffia elongata
	Wolffia globosa
	Wolffia microscopica
	Wolffia neglecta

Figure 2-4. Floating *Wolffia* at surface of quiet a stream, often mixed with other Lemnaceae and aquatic plants such as *Lemna* spp. and *Spirodela* spp. (Armstrong, 2005: online)

Bhanthumnavin and McGarry (1971) observed and analyzed native methods of cultivating *W. arrhiza*. They reported protein, fat and crude fiber contents as 19.8%, 5.0% and 13.3% of dry weight, respectively. Moreover, in 1999, Jairakphan reported protein, fat and crude fiber contents of *W. arrhiza* collected from natural pond were 20.15%, 2.43% and 14.72%, respectively. Essential amino acid profile of the protein concentrate was; aspartic 1.21, threonine 0.64, serine 0.57, glutamic 1.67, proline 0.67, glycine 0.83, alanine 1.60, cystine 0.10, valine 0.94, methionine 0.20, isoleucine 0.69, leucine 1.30, tyrosine 0.37, phenylalanine 0.76, histidine 0.31, lysine 0.75, arginine 0.80, tryptophan 0.20 (g/100g of protein).

In another study reported protein, fat and crude fiber contents of *W. columblana* collected from anaerobic dairy waste lagoons on the LSU campus, the lagoons contained from 20 to 40 mg l^{-1} of TKN during the collection period. There were 44.7% protein, 6.6% fat and 11% crude fiber and essential amino acid profile of

the protein concentrate was listed; aspartic 5.63, threonine 2.55, serine 2.28, glutamic 5.76, proline 2.41, glycine 3.04, alanine 3.75, valine 3.49, methionine 0.87, isoleucine 3.06, leucine 5.83, tyrosine 2.17, phenylalanine 3.60, histidine 1.18, lysine 3.37, arginine 3.78 (g/100g of protein) (Rusoff, Blakeney and Culley, 1980).

ASEXUAL REPRODUCTION OF *Wolffia*

In laboratory condition, *W. arrhiza* has a vegetative reproduction by budding, with a generation time of approximately 4 days (Sakdisuwan, 1967; Vacharabhaya, 1969)

Bernard et al. (1990) reported that vegetative reproduction of *W. australiana* was by budding of new frond from one basal budding cavity. As many as two second generation (daughter) fronds, a third generation (granddaughter) frond and stipes of former second generation fronds present at one time were found in a budding cavity (Figure 2-5.). A heart shaped opening was found in the dorsal flower cavity and a pistil and a single stamen with a bilobed anther was found in the flower, having a red dehiscence line in each lobe. There was no significant difference in life span or fronds number produced by the three generations of parents studied. Life span were 17 days and 11 fronds were produced. Frond size at detachment decreased with increased age of the parent but all experimental plants continued to grow after detachment although small, late fronds did not grow as large as those produced early in the life.

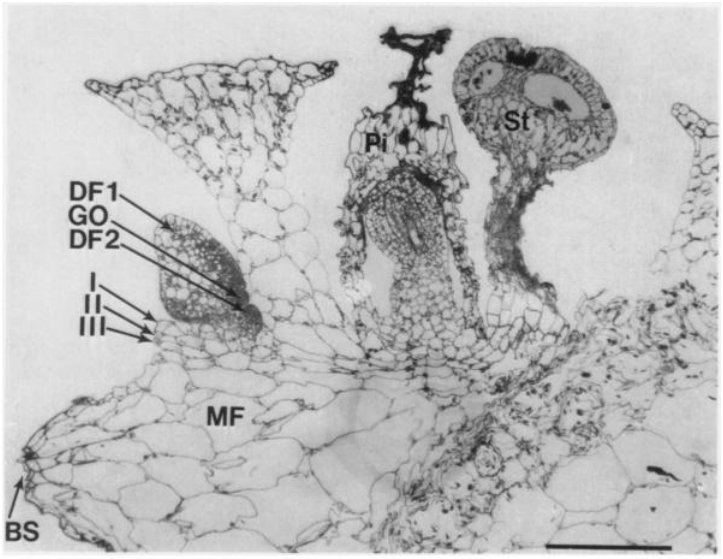


Figure 2-5. Light micrograph of a longitudinal section of *W. australiana* showing pistil (Pi) and stamen (St) in flower cavity. Daughter fronds (DF1 and DF2) and granddaughter (GO) as well as stipes (I, II and III) of three earlier fronds can be seen in the budding cavity. Note bud detachment (BS) area where mother frond (MF) detached from its parent, Bar = 0.1 mm (Bernard et al., 1990)

Lemon, Posluszny and Husband (2001) reported rate of vegetative reproduction development in duckweeds (Lemnaceae) *W. borealis*, by measuring the number of daughter fronds produced over the life span of mother fronds. Life span of *W. borealis* was 15.8 days and produced 9.8 daughter fronds (number), thus *W. borealis* exhibited the reproductive rate of 0.62 fronds per day. Vegetative reproduction production in the Lemnaceae forms a continuum from *Wolffia*, which develops relatively small (0.5–1.5 mm) and numerous propagules are released before maturity.

FACTORS EFFECTING ON *Wolffia*

Duckweed is cultured in axenic (sterile) conditions using chemically defined media under artificial lights and growth rates were recorded that far exceeded growth rates measured under natural conditions. Duckweed populations are limited mostly by light, temperature and nutrients (Hillman, 1961). Temperature is the most important factors determining growth rates of free floating macrophytes in the field (Heide et al., 2006). Moreover, crowding is also an important factor in limitation of duckweed growth (Driever, Van Nes and Roijackers, 2005; Frederic et al., 2006).

Cultivating *W. arrhiza* was observed in native methods and analyzed. Small scale cultivation by rain fed was carried out by villagers living near provincial urban centers in northern Thailand. The pH of water was between 6.5 to 7.0 and shaded by bamboo groves. *W. arrhiza* remains in its edible vegetative from November to July and inedible sexually reproducing from August to October. On 9 months of productivity the calculated annual yield was 265 tons wet wt ha^{-1}or 10.5 tons dry wt ha^{-1}(Bhanthumnavin and McGarry, 1971).

Naskar et al. (1986) reported that sewage effluent is rich in nutrients and therefore can serve as a culture medium for the duckweed *W. arrhiza*. Growth rate and total biomass production of *W. arrhiza* were investigated. With 100% sewage effluent the total extrapolated production of duckweed was 100.5 tons $ha^{-1}year^{-1}$.

W. arrhiza (Landolt, 1986) is a small circular floating weed with a size of 1 mm in length and lives in tropical and subtropical lakes and marshes. In summer, the vegetative frond of *W. arrhiza* grows quickly and absorbs large amounts of nutrients. In autumn and winter, however, the frond changes to a resting form, called “turion”.

The turion contains a large amount of starch and sinks to the bottom due to the change in its density. The vegetative frond contains a large amount of protein but a small amount of starch. Turions were induced effectively under a high plant density, an ample quantity of light and a long illumination period. Turions can be easily induced from vegetative frond artificially under a high plant density and high light strength using a culture solution with a low nutrient concentration, as in water treated by cultivation of the vegetative frond (Fujita et al., 1999).

Suppadit et al. (2008) and Suppadit (2011) reported that a biomass of 12 g of *W. arrhiza* $l^{-1}$of shrimp farm and quail farm effluent with treatment period of 30 days provided the best conditions for the growth of *W. arrhiza*.

N-P-K fertilizer (16 -16- 16) at 100 mg l^{-1} was added with tap water for *W. arrhiza* cultivation and adjusted pH at 5-6 under opened building at light intensity more than 5,000 lux throughout 30 days- culture. 2 kg wet wt m^{-2} were found in this study. Beta-carotene was 600 mg wet wt m^{-2} in 24 days- culture (Panwanidumrong, 2009).

The biomass production of duckweed on the tank was conducted with two treatments. With a TN concentration of about 2 mg l^{-1} in both treatment, the first treatment was the duckweed grew on the water surface of three round tanks with a radius of 0.9 m and a height of 0.9 m. the water surface area was 2.54 m^{-2} in each tank. The second treatment was that duckweed grew in three 30x30 cm square frame made of 10 cm diameter PVC pipe and those PVC square frames were floating in another tank with same size as those used in the first treatment. The daily growth rates of duckweed in three big tanks and three small square PVC frames were 0.099 kg wet wt

m^{-2} (361 tons wet wt ha^{-1} annually) and 0.127 kg wet wt m^{-2} (464 tons wet wt ha^{-1} annually), respectively (Fedler and Duan, 2011).

Most bioregenerative life support systems, BLSS, are based on gravitropic higher plants which exhibit growth and seed generation disturbances in microgravity. When used for a lunar or martial base the reduced gravity may induce a decreased productivity in comparison to Earth. Therefore, the implementation of aquatic biomass production modules in higher plant and/or hybrid BLSS may compensate for this and offer, in addition, the possibility to produce animal protein for human nutrition. These are plant production bioreactors for the species mentioned above and another suitable candidate, the lemnacean (duckweed) species, *W. arrhiza.* Moreover, combined intensive aquaculture systems with a closed food loop between herbivorous fishes and aquatic and land plants are being developed which may be suitable for integration into a BLSS of higher complexity (Bluem and Paris, 2001).

PRACTICAL APPLICATIONS OF *Wolffia*

As a new source of inexpensive protein

It is known that *W. arrhiza* Wimm. was used as a vegetable by Burmese, Laotians and people in the northeast and northern of Thailand for many generations. The name in Thai, Khai-nam, suggests the oval shape of the plant (length 1.5 mm, width 1.0 mm). Khai-nam is generally regarded as poor people's food and has attracted little attention as a potentially significant source of human food (Bhanthumnavin and McGarry,1971).

The value of duckweed as a source of feed for fish and poultry has been promoted by the World Bank, especially in developing countries (Skillicorn, Spira and Journey, 1993). Researcher in Thailand demonstrated the value of using *W. globosa* as a dietary protein replacement on performance and carcass characteristics in broilers (Chantiratikul et al., 2010). *W. arrhiza* also has potential as a feed ingredient of fish farming (Naskar et al., 1986). Its amino acid composition is similar to those of animal protein than plant protein, having high lysine and methionine content, two amino acids normally deficient in plant products (Dewanji, 1993). Finally, dried duckweed can provide vitamins, minerals and pigments such as beta carotene in livestock diets, reducing the need to add these compounds to rations and thus the feed producer money.

Perhaps the most promising use of duckweed is as a feed for pond fish such as carp and tilapia, *W. arrhiza* alone supported the growth of two species of Indian carp and four species of Chinese carps as well as one species of barb (Naskar et al., 1986). Mature poultry can utilize dried duckweed as a partial substitute for vegetable protein such as soybean meal in cereal grain based diets.

As an alternative means of wastewater treatment

Considerable work was done in the 1970's and 1980's on the use of duckweed genera, especially *lemna*, as a means of treating wastewater of both agricultural and domestic origin. A part of a facultative treatment system, duckweed can cover treatment ponds and reduce the growth of algae in these ponds as well as reduce nitrogen in the effluent from these ponds through ammonia uptake and denitrification (Alaerts, Mahbubar and Kelderman, 1996). Duckweed can also be part of constructed

wetland systems, either as a component of a wetland receiving wastewater of as plants that polish nutrients from wetland treated effluents.

Researcher used *Wolffia* for treatment on effluent from shrimp farms and quail farms (Suppadit et al., 2008; Suppadit, 2011) and guidelines for the use of duckweed to remove ammonia and phosphorus from effluent from an algae culture system were given by Koles, Petrell and Bagnall (1987).

As an inexpensive and accurate way of toxicity testing

Due to its small size and ease of growth, duckweed species make ideal organisms for toxicity testing (Lakatos et al., 1993). Duckweed species have been used to test the toxicity of oils (King and Coley, 1985) and *Wolffia* was used as bioindicator of zinc and cooper contamination in natural water resources (Pla-on, 2005).

CHAPTER III

MATERIALS AND METHODS

The optimized conditions for production of Khai-nam, *Wolffia* sp., were carried out into 3 parts, i.e. (1) biology investigation of Khai-nam, (2) effects of culture media, light intensity, temperature, initial pH and initial density on Khai-nam production in laboratory and (3) outdoor mass culture systems for Khai-nam. The laboratory experiments were conducted at Marine Plankton Culture Laboratory, Department of Marine Science, Chulalongkorn University, Bangkok. The outdoor mass culture systems were conducted at Town Tan Tor agricultural farm, Mueang district, Sakon Nakhon province.

BIOLOGICAL INVESTIGATION OF KHAI-NAM

The biological investigation of Khai-nam, *Wolffia* sp., in nature was carried out in 4 steps, i.e. (1) species identification of Khai-nam isolated from natural pond, (2) investigation of asexual reproduction in Khai-nam, (3) estimating production rate in natural pond and (4) proximate analysis and microbial determination in Khai-nam. The biological investigation of Khai-nam was conducted in a natural pond at Mueang district, Sakon Nakhon province.

Identification Khai-nam *Wolffia* sp. from the natural pond

Khai-nam, watermeal, *Wolffia* sp. was collected from a small pond in Mueang district, Sakon Nakhon province and identified following Landolt (1994) (Figure 3-1., 3-2., 3-3.).

Key to the species (Landolt, 1994)

- Surface of the fronds 11/3-21/2 times as long as wide, 11/2-3 times as deep as wide, with the greatest width at the surface of the water (nearly no translucent edge visible from above); stigma with pigment cells

  - Fronds mostly > 0.9 mm long, with 50-120 stomata…..……....*W. australiana*

  - Fronds mostly < 0.9 mm long, with 8-20 stomata

    - Fronds whitish green at the surface with intensely green margins,

      2 - 3 times as deep as wide……...……...*W. angusta*

    - Fronds intensely green at the surface without green colored margins,

      11/2-2 times as deep as wide……...……..… *W. neglecta*

- Surface of the fronds 1-12/3 times as long as wide, 3/4-11/2 as deep as wide, with the greatest width below the surface of the water (at least laterally a translucent edge visible from above); stigma without pigment cells

  - Fronds intensely green and mostly shiny at the surface,

    with mostly >30 stomata.....…… *W. arrhiza*

  - Fronds not shiny, pale green to rather intensely green, with < 30 stomata

    - Fronds mostly < 0.6 mm wide, 11/4-12/3 as long as wide

      - Fronds with no translucent edge at the tip,

        with 15-30 stomata……..…… *W. cylindracea*

      - Fronds with distinct translucent edge at the tip, mostly

        < 20 stomata……..…… *W. globosa*

    - Fronds mostly > 0.6 mm wide, 1-11/3 as long as wide. *W. columbiana*

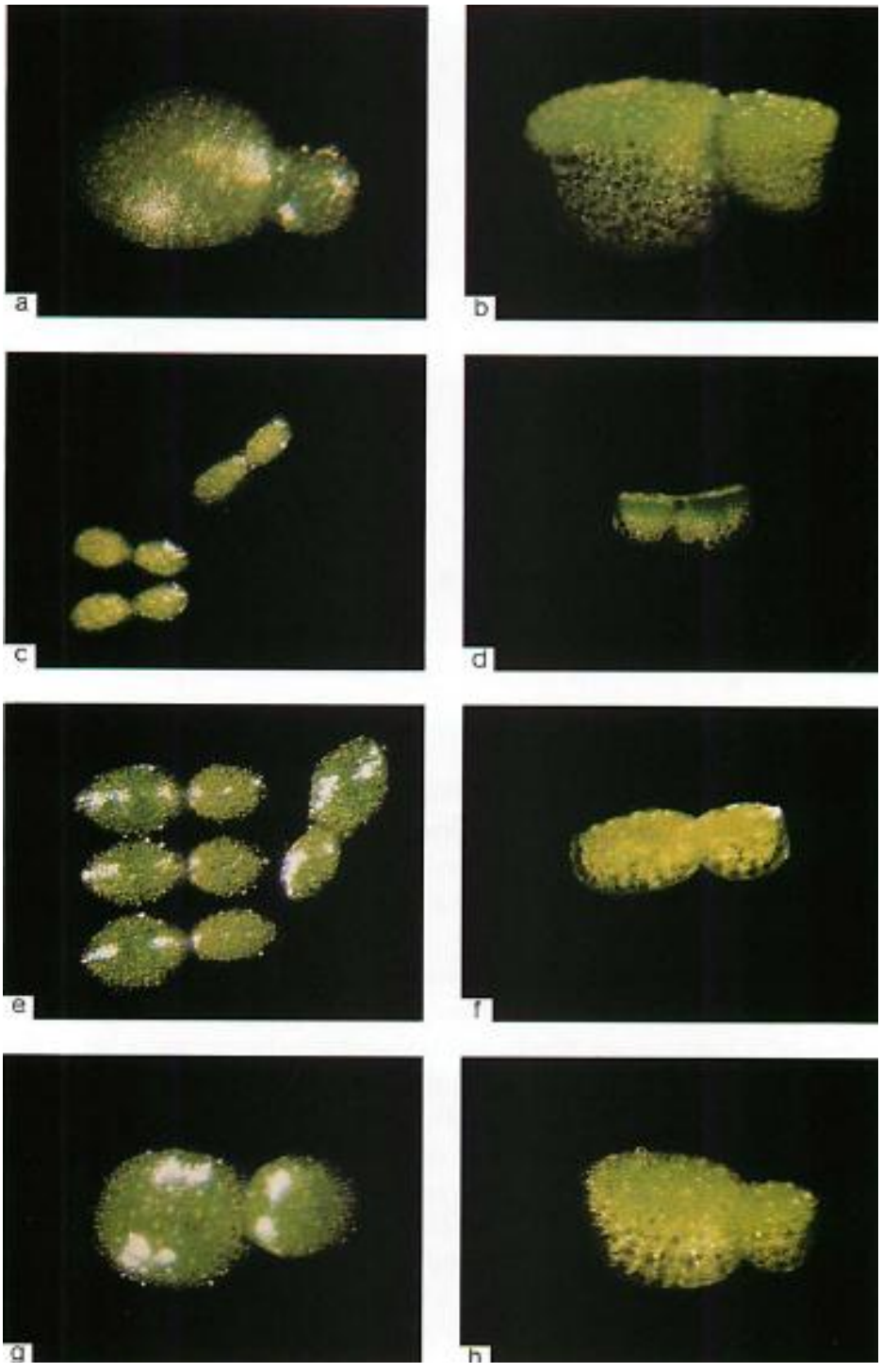


Figure 3-1. The *Wolffia* species of the section *Wolffia* from above (left) and from the side (right) (x 8) (Landolt, 1994)

a., b.: *Wolffia australiana* c, d.: *Wolffia angusta*

e., f.: *Wolffia neglecta* g., h.: *Wolffia arrhiza*

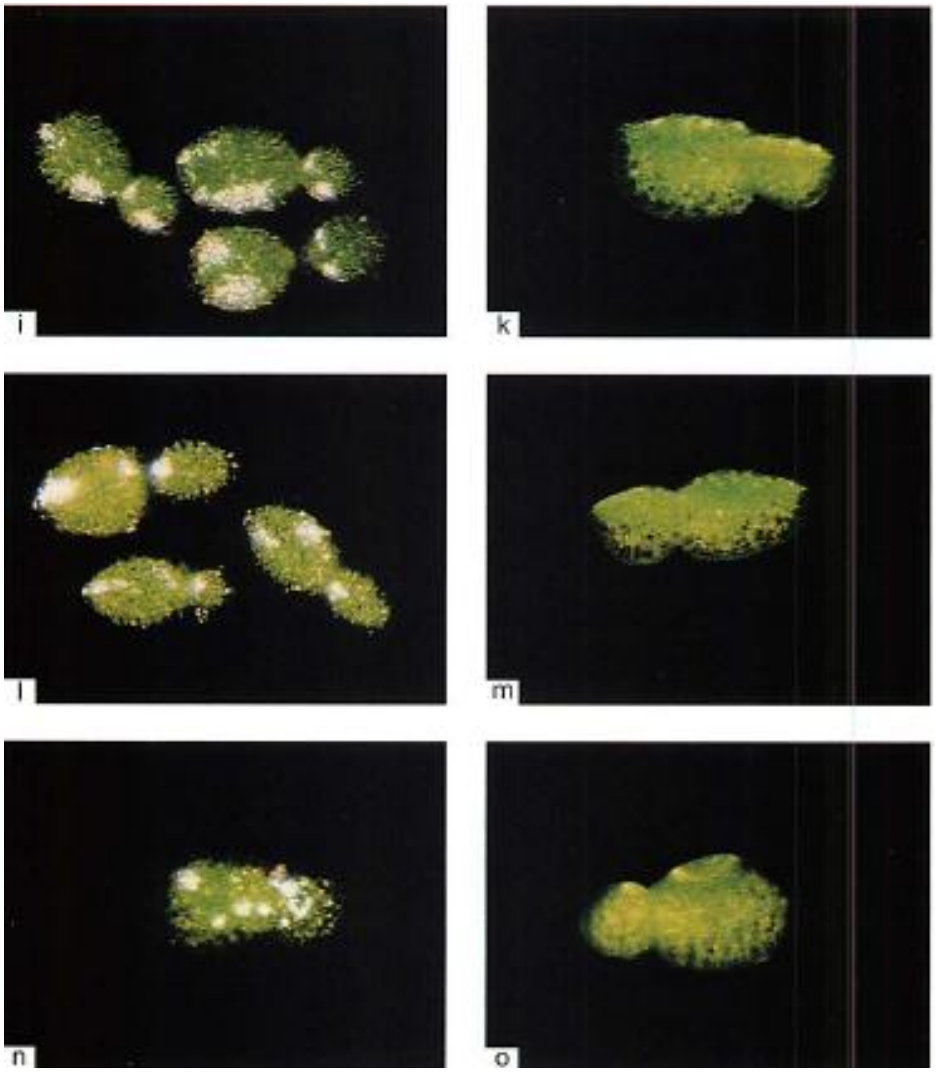


Figure 3-2. The *Wolffia* species of the section *Wolffia* from above (left) and from the side (right) (x 8) (Landolt, 1994)

i., k.: *Wolffia cylindracea* 1., m.: *Wolffia globosa*

n., o.: *Wolffia columbiana*

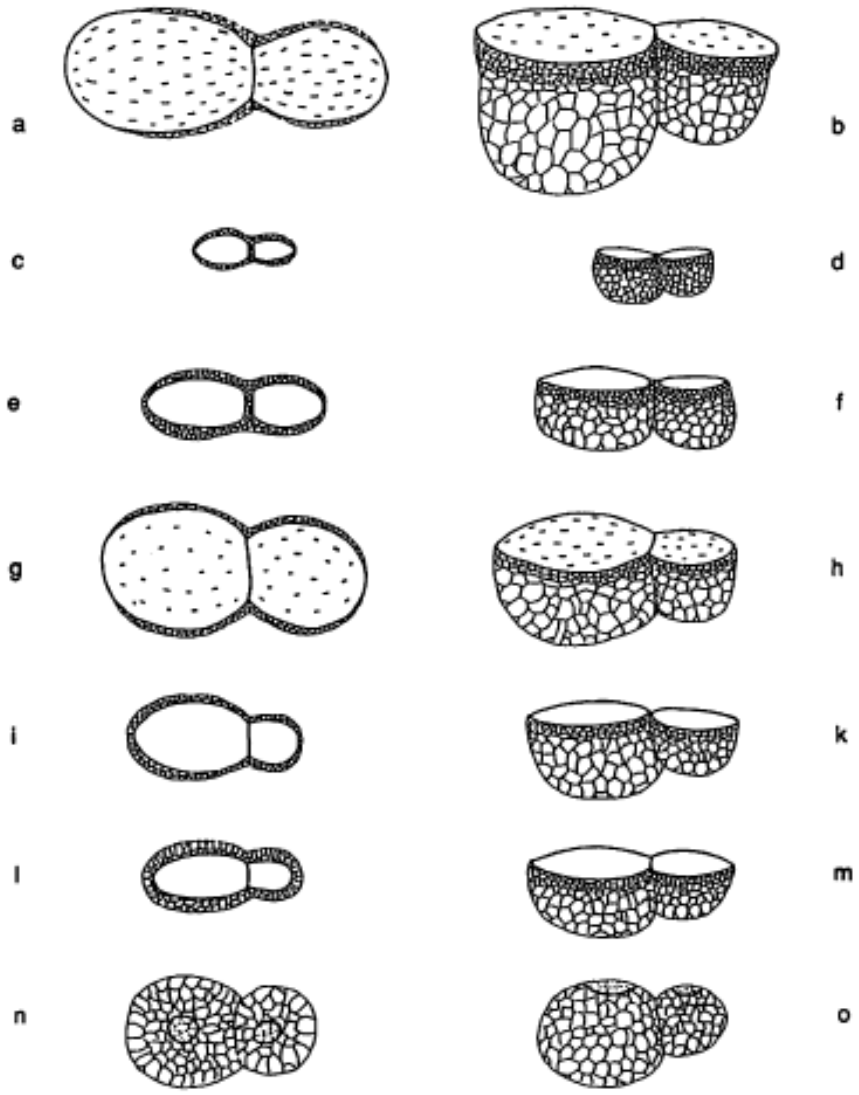


Figure 3-3. Drawings of the *Wolffia* species of the section *Wolffia* from above (left) and from the side (right) (x 8) (Landolt, 1994)

a., b.: *Wolffia australiana* c, d.: *Wolffia angusta*

e., f.: *Wolffia neglecta* g., h.: *Wolffia arrhiza*

i., k.: *Wolffia cylindracea* 1., m.: *Wolffia globosa*

n., o.: *Wolffia columbiana*

Asexual reproduction of Khai-nam *W. globosa*

The collected frond of *W. globosa* at natural pond in Meuang district, Sakon Nakhon province was examined under a light microscopy and photographs were taken every 6 hours to find a releasing stage of the daughter frond from single mother frond.

Production rate of Khai-nam *W. globosa* in natural pond

W. globosa at natural pond (30 x 60 x 1.5 m) in Meuang district, Sakon Nakhon province (N 17^{o} 08. 021′, E 104^{o} 06.780′) (Figure 3-4.) was random collected around the pond.

A strainer, which was made from iron structure (diameter 32 cm) and cover with filter cloth, was used for *W. globosa* collection. When expanded *W. globosa* full the pond, sample was random collected 10 points around the pond. The strainer was dipped under water surface area after 10-15 minute lifting the strainer for the sample collection at water surface area. If the blow sample go to total up at pond corner by the wind, *W. globosa* was collected of all in the pond. The dry weight was determined. Dry weight was determined by drying *W. globosa* for 24 hours in an oven at 70 ^{o}C (Driever et al., 2005).

The experiment was run every month in a year period. The environment conditions of light intensity (measured by luxmeter), light period, temperature (measured by thermometer), pH (measured by pH meter), dissolved oxygen, ammonia, nitrite, alkaline and hardness were recorded. For dissolved oxygen, ammonia, nitrite, alkaline and hardness were analyzed following APHA (1995)

Figure 3-4. The nature pond filled with *W. globosa*

Proximate analysis and microbial determination

W. globosa was collected locally at a small pond in Mueang district, Sakon Nakhon province, during maximum density in 2009, July, and transferred to the laboratory for proximate analysis (AOAC, 2005, 2008), amino acid profile (Petritis, Elfakir and Dreux, 2002) and microbial determination (USFDA/CFSAN/BAM, 2009: online, Chapter 3, 4, 12).

FACTORS EFFECTING ON KHAI-NAM *W. globosa* PRODUCTION

The factors effecting on the growth of *W. globosa*, in the laboratory were carried out in 6 steps, i.e. (1) culture media experiment for *W. globosa*, (2) effect of light intensity on photosynthesis effect of *W. globosa*, (3) effect of temperature on

photosynthesis effect of *W. globosa*, (4) effect of initial pH on the growth of *W. globosa*, (5) effect of initial density of *W. globosa* and (6) a factorial experiment on light intensity, initial pH and initial density on the growth and quality of *W. globosa*. The laboratory experiments were conducted at Marine Plankton Culture laboratory, Department of Marine Science, Chulalongkorn University, Bangkok.

Culture media experiment

Four culture media were selected for the study. There were natural pond-water medium from pond 1 and 2 where *W. globosa* has been found at Mueang district, Sakon Nakhon province; modified Hoagland's medium (Sakdisuwan, 1967) (Appendix 1.); modified Hutner's media (Hutner, 1953) (Appendix 2.) and distilled water as a control.

W. globosa collected locally at a small pond in Mueang district, Sakon Nakhon province was cleaned by placing in a 20% bleach (sodium hypochlorite) solution for several seconds to a minute, then rinsed with sterile water (Rains, 1993) and transferred into the above 5 media. Individual frond of *W. globosa* was grown in 24 well plates with 2 ml of each media (Figure 3-5.) at controlled temperature 25 °C with 12 hours photoperiod of 4000 lux light intensity.

When the mother fronds (G0) had produced a daughter frond (G1), the daughter frond was removed from the mother frond after recording the frond size and the period of frond generation. The first daughter frond was transferred to new culture medium which was the same as mother frond medium. The daughter frond had produced a granddaughter frond (G2), the first granddaughter frond was removed from the daughter frond then recording the frond size and the period of frond generation.

The first granddaughter frond was transferred to new culture medium which was the same as mother frond medium. The granddaughter frond had produced a great-granddaughter frond (G3), the first great-granddaughter frond was removed from the granddaughter frond then recording the frond size and the period of frond generation. The daughter frond number and the life span of each mother frond were recorded.

Production rate of each culture medium was calculated by total number of daughter frond divided by the life span of the mother frond (Lemon et al., 2001) and divided by the culture area.

All data were analyzed using descriptive statistics and analysis of variance (ANOVA) followed by Duncan's multiple range comparison tests at $p \geq 0.05$.

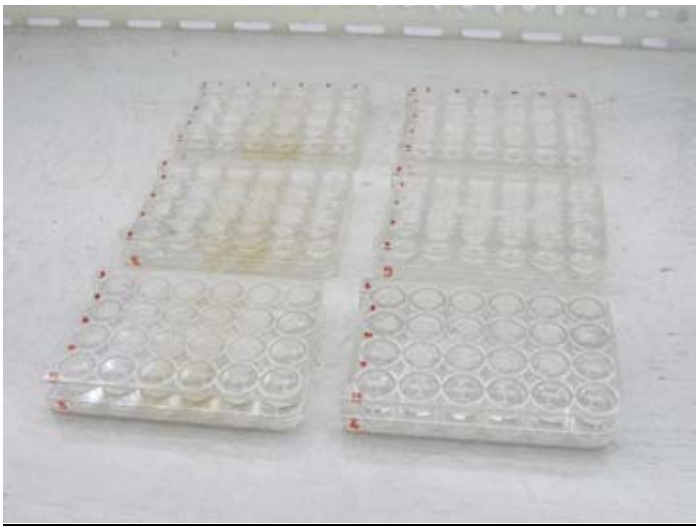


Figure 3-5. *W. globosa* cultivation in 24 wells tissue cultured plates, only individual frond was cultured in each well.

Effect of light intensity on photosynthesis of Khai-nam *W. globosa*

Effect of light intensity on photosynthesis of *W. globosa* was done by cultivating in Hutner's medium. Approximately $5x10^7$ fronds of *W. globosa* were placed in a quadrilateral plastic box (0.38x0.58x0.1 m) contained 22 l of Hutner's medium and exposed with various light intensity of natural light. Photosynthesis efficiency was measured using chlorophyll fluorescence technique (Kitajima and Butler, 1975) by Fv/Fm, where Fv meant variable fluorescence and Fm meant maximum fluorescence. The *W. globosa* was measured at 7.00 am, 9.00 am, 11.00 am, 13.00 pm, 15.00 pm and 17.00 pm.

Fv/Fm meant optimal quantum yield were calculated by following equation (Kitajima and Butler, 1975).

$$Fv/Fm = (Fm - Fo) / Fm$$

Where Fo is the dark adapted initial minimum fluorescence, Fm is maximal fluorescence measured during the first saturation pulse after dark adaptation.

Effect of temperature on photosynthesis of Khai-nam *W. globosa*

W. globosa was cultured in Hutner's medium as the technique in light intensity experiment and adjusted to the tested temperature of 10, 15, 20, 25, 30, 35 and 40^oC. Approximately $1x10^5$ fronds of *W. globosa* were placed in tube containing 5 ml of Hutner's medium, the tubes then were placed in plastic boxes (25x35x20 cm) and about 15 cm of water was added for temperature control by an electrical heater for the tested temperature of 20, 25, 30, 35 and 40^oC (Figure 3-6.). Temperature of the water

was checked by a thermometer. The other temperature of 10 and 15 ^{o}C were carried out in the incubator. The experiment was run under a light intensity of 5000 lux after 3 hours of acclimation photosynthesis was determined. Photosynthesis efficiency was measured using chlorophyll fluorescence technique by Fv/Fm (Kitajima and Butler, 1975).

Figure 3-6. Temperature control unit for *W. globosa* grown in various temperatures for photosynthesis study

Effect of initial pH on growth of Khai-nam *W. globosa*

Effect of seven initial pH values; 4, 5, 6, 7, 8, 9 and 10 were investigated on growth and size of *W. globosa*. Hutner's medium was prepared and adjusted to the tested pH value. *W. globosa* stock maintained in Marine Plankton Culture laboratory

from the previous experiment was transferred into each pH medium which was prepared as seven pH values for about 2 weeks. Twenty two fronds cm^{-2} of *W. globosa*, with an average frond size of 0.36 mm^2, were transferred into a tube (r=0.85 cm, h=15 cm). The tube was filled with 10 ml media of each pH medium. Triplicates were applied in each pH media. All experiments were run at temperature of 25 ^{o}C with 12 hours photoperiod and light intensity of 4000 lux.

Frond numbers and frond size of *W. globosa* in each pH media were collected at five days interval for growth determination. Production rate (yield) and relative growth rate (RGR) were collected and calculated by following equation (Guy, Granoth and Gale, 1990).

$$\text{Production rate (Yield, Y)} = (N_2 - N_1) / t$$

$$\text{Relative growth rate (RGR)} = (\ln N_2 - \ln N_1) / t$$

Where N_2 is the final growth (frond number, wet weight or dry weight), N_1 is the initial growth and t is time (hour or day).

All data were analyzed using descriptive statistics and analysis of variance (ANOVA) followed by Duncan's multiple comparison tests. F-values with a probability level < 0.05 were considered as statistical significance.

Effect of initial density on Khai-nam *W. globosa* culture

Initial density of *W. globosa* 0.10, 0.50, 1, 5, 10, 20, 30 and 40% surface area was investigated on performing growth and size effects. Hutner's medium was prepared as same manner in the previous experiment.

W. globosa maintained in Marine Plankton Culture laboratory from the previous experiment was used for the experiment. *W. globosa* frond size about 0.36 mm^2at density of 0.44, 1.32, 2.64, 14.1, 27.75, 55.51, 83.26 and 111.01 fronds cm^{-2} (equal to 0.10, 0.50, 1, 5, 10, 20, 30 and 40% of cultured surface area, respectively) were prepared in tubes (r=0.85 cm, h=15 cm). The tube was filled 10 ml cultured media in temperature of 25 ^{o}C and 12 hours photoperiod at light intensity of 4000 lux. The experiment was run in triplicates.

Fronds numbers and frond size of each treatment were determined every five days. Production rate (yield) and relative growth rate were calculated by following Guy, Granoth and Gale (1990). All data were analyzed using descriptive statistics and analysis of variance (ANOVA) followed by Duncan's multiple comparison tests. F-values with a probability level < 0.05 were considered as statistical significance.

A factorial experiment on light intensity, initial density and pH growth and quality of Khai-nam *W. globosa*

A CRD involved factorial experiment of light intensity, initial pH and initial density to determine an optimal condition for growth and quality of *W. globosa* was investigated. Light intensity of 2,000, 6,000 and 10,000 lux, pH of 5, 6 and 7 and initial density of 5, 10, 15 and 20% surface area (14.10, 27.75, 41.63 and 55.51 fronds m^{-2}, respectively) were selected for the experiment (see detail in Table 3-1.).

Fronds of *W. globosa* maintained in Marine Plankton Culture laboratory with Hutner's medium were evaluated in 3 x 3 x 4 factorial experiment. *W. globosa*, frond size about 0.36 mm^2, were cultured in tubes (r=0.85 cm, h=15 cm). The tube was

filled 10 ml media and all treatments were run in triplicates. All tubes were grown in controlled temperature at 25 ^{o}C with 12 hours photoperiod.

Frond numbers and size of each treatment were counted and measured every seven days for growth determination. Production rate (yield) and relative growth rate were calculated by following Guy, Granoth and Gale (1990). All data was analyzed using descriptive statistics and analysis of variance (ANOVA) followed by Duncan's multiple comparison tests. F-values with a probability level < 0.05 were considered as statistical significance.

Table 3-1. Treatment combination of 3x3x4 CRD involved factorials experiments

Treatment	Factors		
	Light intensity (lux)	Initial pH	Initial density (% surface area)
1	2,000	5	5 (14.10 fronds m^{-2})
2	2,000	5	10 (27.75 fronds m^{-2})
3	2,000	5	15 (41.63 fronds m^{-2})
4	2,000	5	20 (55.51 fronds m^{-2})
5	2,000	6	5 (14.10 fronds m^{-2})
6	2,000	6	10 (27.75 fronds m^{-2})
7	2,000	6	15 (41.63 fronds m^{-2})
8	2,000	6	20 (55.51 fronds m^{-2})
9	2,000	7	5 (14.10 fronds m^{-2})
10	2,000	7	10 (27.75 fronds m^{-2})
11	2,000	7	15 (41.63 fronds m^{-2})
12	2,000	7	20 (55.51 fronds m^{-2})

Table 3-1. Treatment combination of 3x3x4 CRD involved factorials experiments (continue)

Treatment	Factors		
	Light intensity	Initial pH	Initial density(% of surface area)
1 3	6,000	5	5 (14.10 fronds m^{-2})
14	6,000	5	10 (27.75 fronds m^{-2})
15	6,000	5	15 (41.63 fronds m^{-2})
16	6,000	5	20 (55.51 fronds m^{-2})
17	6,000	6	5 (14.10 fronds m^{-2})
18	6,000	6	10 (27.75 fronds m^{-2})
19	6,000	6	15 (41.63 fronds m^{-2})
20	6,000	6	20 (55.51 fronds m^{-2})
21	6,000	7	5 (14.10 fronds m^{-2})
22	6,000	7	10 (27.75 fronds m^{-2})
23	6,000	7	15 (41.63 fronds m^{-2})
24	6,000	7	20 (55.51 fronds m^{-2})
25	10,000	5	5 (14.10 fronds m^{-2})
26	10,000	5	10 (27.75 fronds m^{-2})
27	10,000	5	15 (41.63 fronds m^{-2})
28	10,000	5	20 (55.51 fronds m^{-2})
29	10,000	6	5 (14.10 fronds m^{-2})
30	10,000	6	10 (27.75 fronds m^{-2})
31	10,000	6	15 (41.63 fronds m^{-2})
32	10,000	6	20 (55.51 fronds m^{-2})
33	10,000	7	5 (14.10 fronds m^{-2})
34	10,000	7	10 (27.75 fronds m^{-2})
35	10,000	7	15 (41.63 fronds m^{-2})
36	10,000	7	20 (55.51 fronds m^{-2})

OUTDOOR CULTURE SYSTEM OF KHAI-NAM *W. globosa*

Outdoor mass culture of *W. globosa* for growth rate and quality determination was designed in 2 experiments. Firstly, 5 different culture systems to determine production rate of *W. globosa.* Secondly, the culture system yielding the higher production was selected for *W. globosa* quality study.

Mass culture systems were conducted at an agricultural farm, in Mueang District, Sakon Nakhon Province, Thailand.

Five different culture systems; 1) a static culture, 2) a vertical aeration culture, 3) a horizontal movement culture, 4) a system with top water spraying, and 5) a above water layer culturing system with water spraying on the top (see Figure 3-7. for details) were used for mass culture of *W. globosa*.

A static culture had no any circulation during culture period. A vertical aeration culture system, an air stone (400 l hours^{-1} of pressure) was used to circulate the water vertically. A horizontal movement culture, a blade paddle wheel driving by a mini-motor (3,500 rpm) was used to circulate the water horizontally. For top spraying and a layer culturing system with top spraying, water in these 2 systems was moved from the bottom of the culture to the top and sprayed (900 l hours^{-1}) over the surface area. For a layer culture one, a plate of plankton net was placed few centimeters over water surface and water spraying above the net provided. All culture systems were run in black cylinder plastic tanks with an area of 0.152 m^2 with 40 cm high. All cultures were setup in a warehouse which was covered by transparent plastic sheet for protecting rain water.

At culture, 50 liters of Modified Hutner's medium with pH 6 was prepared. Depth of the culture was maintained to 30 cm by adding the freshwater to recover the evaporation. The plastic tank was inoculated with frond density of *W. globosa* as 15% surface area (24 g $tank^{-1}$). The period of this experiment was done in October 2010 to February 2011 under ambient temperature and light conditions.

Environmental parameters such as light intensity, temperature, pH, dissolved oxygen, NO^{-}_{3}-N and PO_4-P (APHA, 1995) of all the outdoor culture systems were monitored every 7 days. Wet weight and frond size of *W. globosa* were investigated after a culture of 28 days. Besides, dry weight was also determined by oven dried at 70 ^{o}C for 24 hours (Driever, Van Nes and Roijackers, 2005).

The five culture systems were run in triplicates. Data were analyzed using descriptive statistics and one way analysis of variance (ANOVA) followed by Duncan's multiple comparison tests. F-values with a probability level < 0.05 were considered as statistical significance

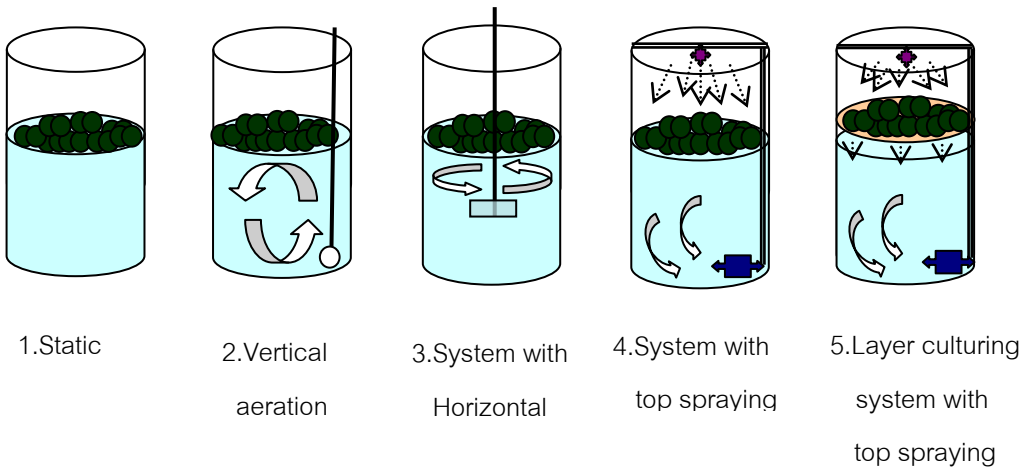


Figure 3-7. Five different culture systems for out door mass production of *W. globosa*

Culture of Khai-nam *W. globosa* for proximate analysis and microbial determination

A system with horizontal flow (Figure 3-7) was used for mass culture of *W. globosa* for proximate analysis and microbial determination. The culture was 28 days in 5 replicates then harvested and dried at 70 ^{o}C for 24 hours. The samples of *W. globosa* were analysis for proximate analysis (AOAC, 2005, 2008), amino acid profile (Petritis, Elfakir and Dreux, 2002) and microbial determination (USFDA/CFSAN/BAM, 2009: online, Chapter 3, 4, 12).

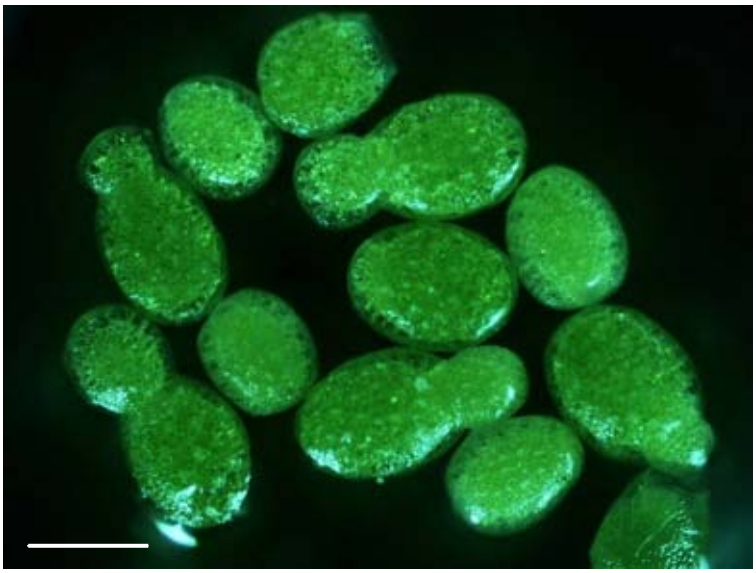
CHAPTER IV

RESULTS

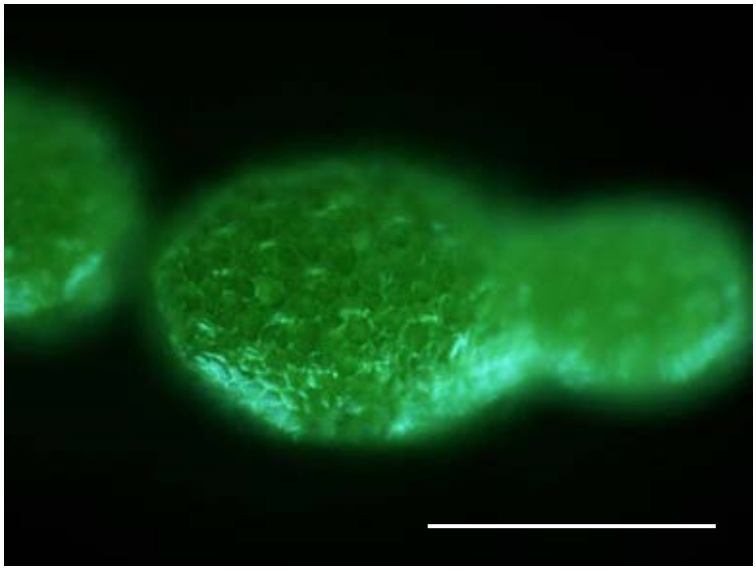
BIOLOGY INVESTIGATION OF KHAI-NAM IN NATURE

Identification of Khai-nam *Wolffia* sp. from the natural pond

Khai-nam collected from a natural pond in Mueang district, Sakon Nakhon province was identified using key of Landolt (1994). The characteristics of Khai-nam were described as stomata number about 16 -18 and the morphology (Figure 4-1) of the fronds was; ellipsoid, with the greatest width distinctly below the surface of the water (all around a translucent edge visible from above), 0.4 – 0.9 mm length, 0.3 – 0.6 mm width, 11/3-12/3 times as long as wide, 3/4 -11/3 as deep as wide, pale green on the surface; cells below the epidermis only slightly smaller than the cells at the bottom of the frond; the lower submerged part of the frond pointing straight down. These characteristics can be described the Khai-nam as *Wolffia globosa*.



a



b

Figure 4-1. a, b *Wolffia* sp. collected at a natural pond in Mueang district, Sakon Nakhon province, Bar = 0.5 mm

Asexual reproduction of Khai-nam *W. globosa*

W. globosa frond was oval shaped with 0.6 mm width, 0.9 mm length. From a single mother frond, a daughter developed in 66 hrs as 4 steps; ¼ of mother frond size, ½ of mother frond size, ¾ of mother frond size and balance with mother frond size. The mother prepared to release the daughter in 6 hours and the mother frond released the daughter frond within 24 hours. Frond of *W. globosa* which collected from natural pond in the northeast of Thailand used 96 hours for a doubling time (Figure 4-2). Environment conditions in the natural pond were 12: 12 hours of light: dark period, 25 – 28 ^{o}C of air temperature, 22 – 26 ^{o}C of water temperature, pH at 6.8 – 7.0, Dissolved Oxygen of 1 – 5 mg l^{-1}, NO_3^-- N of 20 – 25 mg l^{-1} and 10 – 15 mg l^{-1} of PO_4-P.

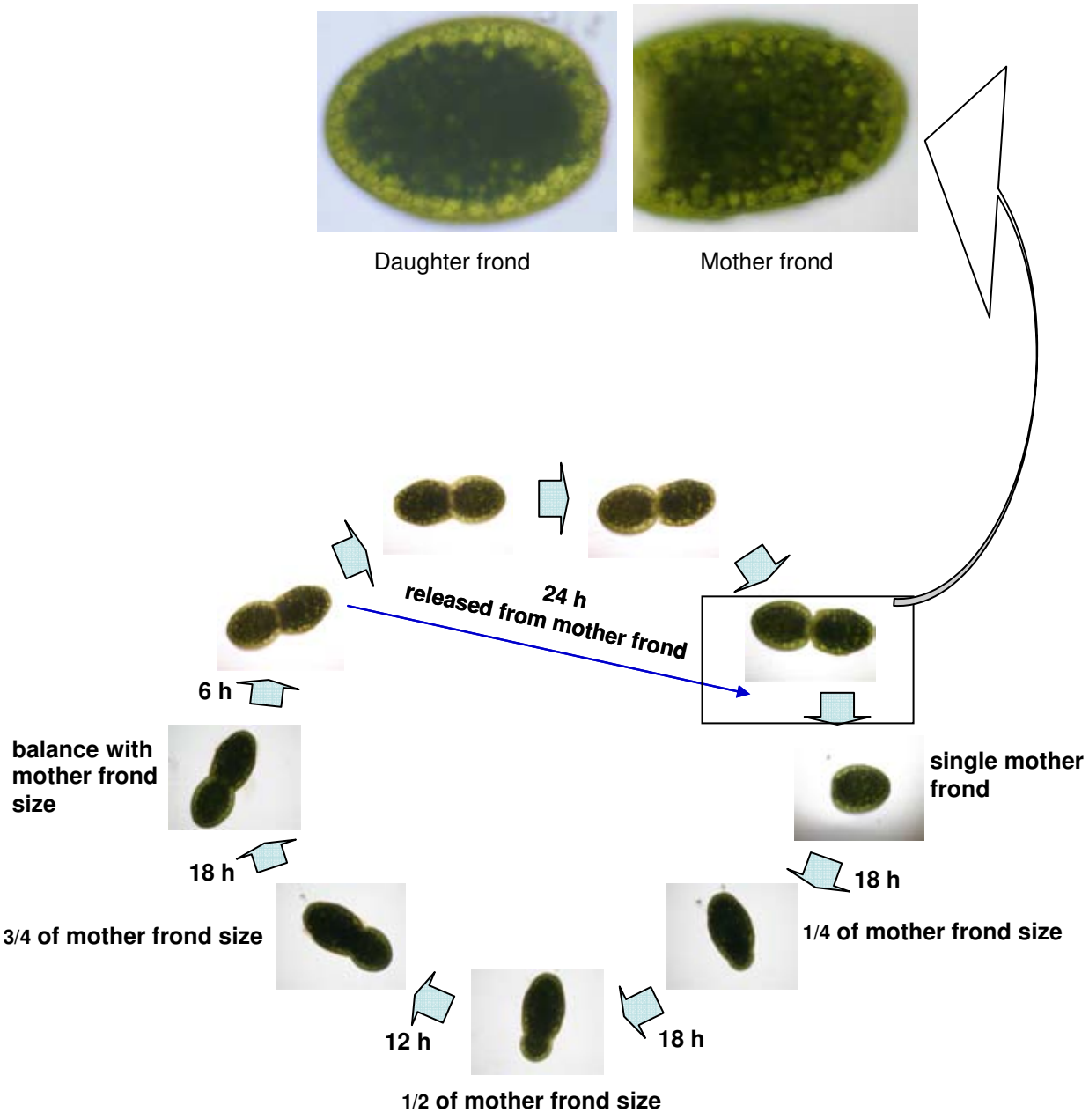


Figure 4-2. Asexual reproduction of *W. globosa*

Production rate of Khai-nam *W. globosa* on natural pond

Production of *W. globosa* was collected at natural pond every month for a year (March, 2008 – February, 2009) period. The result is shown in Figure 4-3. It indicated that production of *W. globosa* was high in June, July and August. The maximal production peak 65.18 g dry weight m^{-2} was found in July. It appeared that *W. globosa* reached their maximal production during the rainy season. During drought season with low temperature in December to February *W. globosa* production declined.

The environment condition in the natural pond during data collecting (March, 2008 – February, 2009) is showed in Table 4-1.

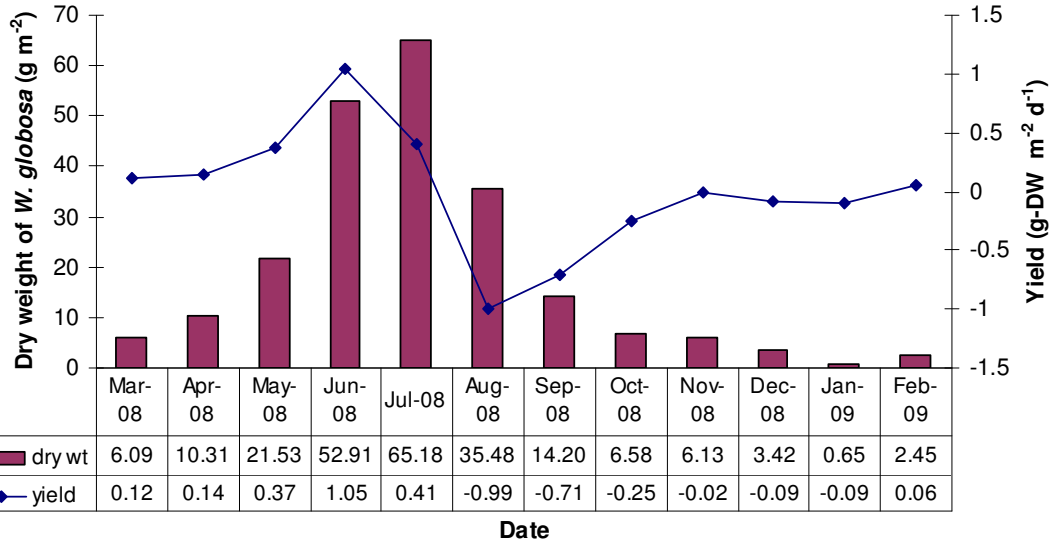


Figure 4-3. Production of *W. globosa* at the natural pond during the year of 2008

Table 4-1. Environment condition (average) in the natural pond during March 2008 – February 2009

Parameters	Months											
	Mar-08	Apr-08	May-08	Jun-08	Jul-08	Aug-08	Sep-08	Oct-08	Nov-08	Dem-08	Jan-09	Feb-09
Light intensity (Max) ($x10^4$ lux)	23	25	24	23	22	21	20	21	20	190	20	20
Light : Dark period (hrs)	12: 12	12: 12	12: 12	12: 12	12: 12	12: 12	12: 12	12: 12	11: 13	10: 14	11: 13	12: 12
Temperature (air) (^oC)	22.2	34.8	33.6	32.7	34.2	31	33.7	33.6	30.9	28	30	30
Temperature (water) (^oC)	24.6	31.9	31.3	31.4	32.4	29.7	32.2	31	32.2	28.9	30.2	37
pH	7.5	8.2	7.3	7.3	8.5	6.7	6.7	6.8	7	9	8	8.7
Dissolved Oxygen (mg l^{-1})	3	3.4	1.5	4.5	5.5	4	6	1	2	1	2	3
Ammonia (mg l^{-1})	0.38	0	0.2	1	0.5	4	2.5	3	3	2	0.5	1
Nitrite (mg l^{-1})	0	0	0.1	0	0	0.1	0.1	0.1	0.1	0.1	0.1	0
Alkalinity (mg l^{-1})	162	90	90	70	60	50	90	100	90	94	136	180
Hardness (mg l^{-1})	87	100	50	100	50	100	50	50	50	50	50	60

Proximate analysis and microbial determination (Table 4-2.)

Proximate analysis of *W. globosa*, collected locally at a small pond in Mueang district, Sakon Nakhon province, was 33.3% protein, 5.0% fat, 10.4% crude fiber. Amino acid profiles indicated that *W. globosa* has fully essential amino acid with high level of cystine. Furthermore, microbial determination showed that *W. globosa* had little contamination of pathogenic bacteria.

Table 4-2. Proximate analysis of *W. globosa* (dry matter) and microbial determination form the natural pond in Mueang district, Sakon Nakhon province

Components	values
Protein (%)	33.3
Fat (%)	5.0
Crude fiber (%)	10.7
Amino acid (mg/100g of Protein)	
Aspatic acid	3539
Threonine *	662
Serine	982
Glutamic acid	2557
Proline	1279
Glycine	1507
Alanine	3128
Cystine	5457
Valine *	1849
Metionine *	571
Isoleucine *	685
Leucine *	2032
Tyrosine	890
Phenylalanine *	502
Histidine *	228
Lysine *	1530
Arginine *	1393
Tryptophan *	46
Microbial analysis	
Total plate count, cfu/g	7.6×10^5
MPN *E. coli* /g	< 3
Staphylococcus aureus, cfu/g	< 10 (ND)
Salmonella spp. / 25 g	ND

***** denoted the essential amino acid for human

FACTORS EFFECTING ON KHAI-NAM *W. globosa* PRODUCTION

Culture media experiment

Statistically significant differences ($p<0.05$) were found among the 5 culture media (Figure 4-4.). Frond of *W. globosa* in natural water 2 had longer life span as 19.07±2.65 days than other media. The life span in other culture media were 17.37±2.9, 15.87±3.81, 14.57±3.0 and 13.95±4.07 days in Hutner's medium, natural water 1, Hoagland's medium and distilled water (control), respectively. There was no significant difference between the life span of the natural water 2 and Hutner's medium.

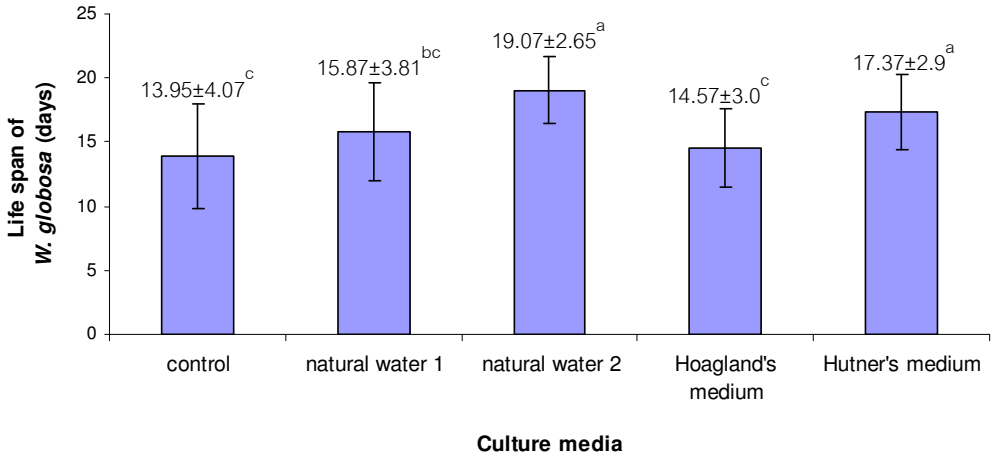


Figure 4-4. Life span of *W. globosa* in 5 different culture media
a, b and c denoted significant difference in mean ($p<0.05$)

The mean number of daughter fronds produced was significantly different among culture media. *W. globosa* in Hutner's medium and control produced the highest number of daughter fronds (mean=6.17±0.96 fronds) and the lowest (mean=2.25±1.25 fronds), respectively (Figure 4-5.). There was no significant difference between the daughter fronds number of Hutner's medium and Hoagland's medium.

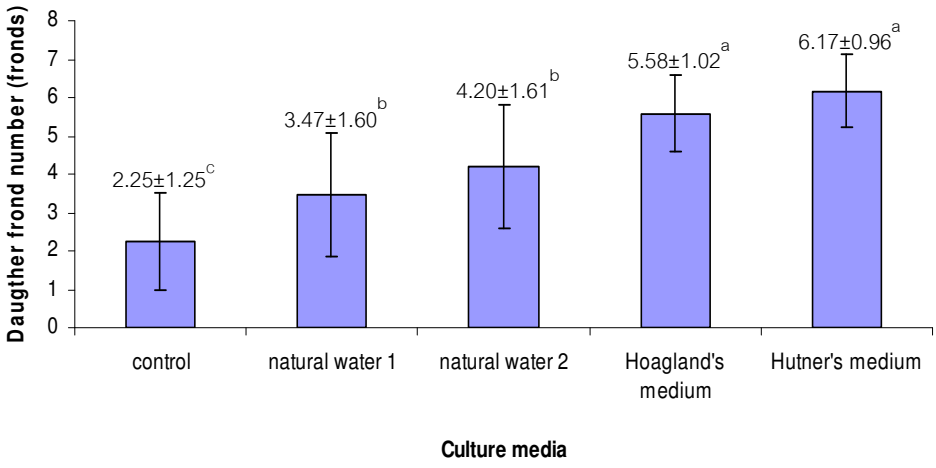


Figure 4-5. Daughter frond (G1) number of *W. globosa* in culture media
a, b and c denoted significant difference in mean ($p<0.05$)

Production rate of *W. globosa* in Hoagland's medium which provided 0.20±0.03 fronds $ml^{-1}d^{-1}$ was significantly higher than other culture media (0.11±0.05, 0.11±0.05 and 0.09±0.05 fronds $ml^{-1}d^{-1}$ in natural water 1, 2 and control, respectively) (Figure 4-6.). However, production rate of *W. globosa* cultured in Hoagland's medium and Hutner's medium (0.18±0.04 fronds $ml^{-1}d^{-1}$) was not significant difference.

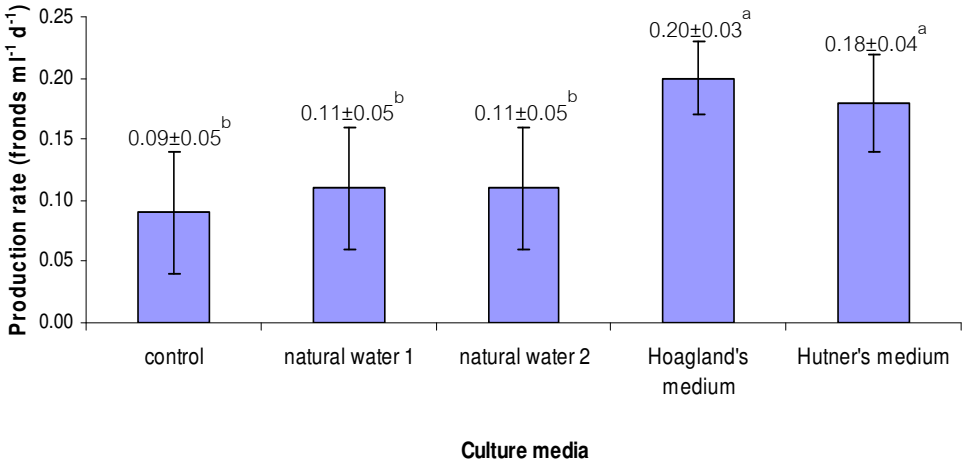
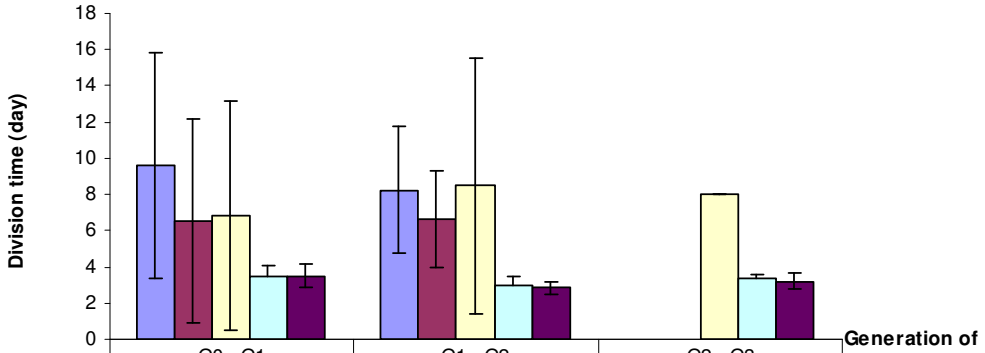


Figure 4-6. Production rate of *W. globosa* in 5 different culture media
a and b denoted significant difference in mean ($p<0.05$)

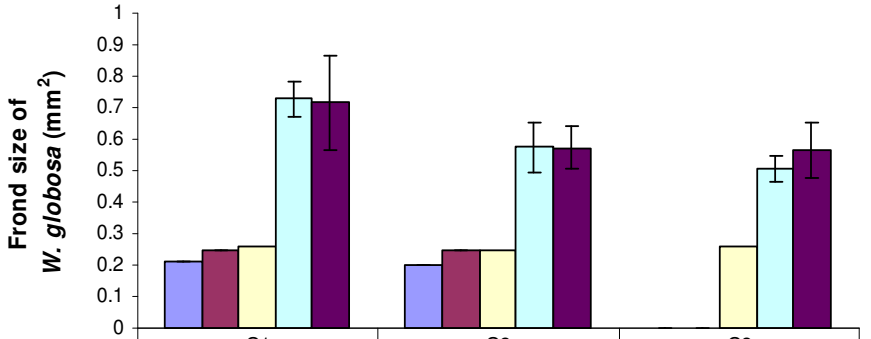
Division time or doubling time of *W. globosa* in 5 different culture media in Figure 4-7. indicated that the mean division time of *W. globosa* was significantly different ($p<0.05$) among culture media. For first division (G0-G1), fronds of *W. globosa* cultured in Hoagland's medium (3.46±0.55 days) and Hutner's media (3.50±0.61 days), division time was much significantly shorter than in others (6.53±5.60, 6.83±6.30 and 9.60±6.24 days of the natural water 1, natural water 2 and control, respectively). For later division, the results were still as the same sequence of the first division. However, in the third division (G2-G3) *W. globosa* cultured in control and natural water 1 could not occur.



	G0 - G1	G1 - G2	G2 - G3
control	9.60 ± 6.24^{c}	8.25 ± 3.5^{b}	0
natlural w ater 1	6.53 ± 5.60^{b}	6.64 ± 2.66^{b}	0
natural w ater 2	6.83 ± 6.30^{bc}	8.5 ± 7.07^{b}	8 ^{b}
Hoagland's medium	3.46 ± 5^{a}	2.94 ± 0.51^{a}	3.38 ± 0.23^{a}
Hutner's medium	3.50 ± 0.61^{a}	2.84 ± 0.35^{a}	3.19 ± 0.46^{a}

Figure 4-7. Division time in the generation of *W. globosa* on culture media
a, b and c denoted significant difference in mean of column ($p<0.05$)

Frond sizes of *W. globosa* in 5 different culture media were significantly difference ($p<0.05$) (Figure 4-8.). The daughter frond (G1) in Hoagland's medium produced the biggest frond of 0.73±0.06 mm^2 with significant difference from others, except the culture of Hutner's medium. The smallest fronds were found in the control one (non nutrients). In granddaughter frond (G2), the fronds in Hoagland's medium and Hutner's medium had the biggest size 0.57±0.08 mm^2. In G3, the similar result was observed.



	G1	G2	G3
control	0.21 [b]	0.2 [b]	0
natlural w ater 1	0.25 [b]	0.25 [b]	0
natural w ater 2	0.26 [b]	0.25 [b]	0.26 [b]
Hoagland's medium	0.73 ± 0.06[a]	0.57 ± 0.08[a]	0.51 ± 0.04[a]
Hutner's medium	0.72 ± 0.15[a]	0.57 ± 0.07[a]	0.57 ± 0.06[a]

Figure 4-8. Frond size in generation of *W. globosa* in 5 different culture media a and b denoted significant difference in mean of column ($p<0.05$)

The results of culture media effects on life span, daughter frond number, production rate, division time and frond size of *W. globosa,* indicated that Hutner's medium provided a better performance than the other culture media. Therefore, Hutner's medium was selected for the next experiments.

Light intensity effect on photosynthesis of Khai-nam *W. globosa*

Effect of light intensity (natural condition) on photosynthesis of *W. globosa* was investigated under the natural light. The various light intensity and temperature ranged 500 to 100,000 lux and 22 to 35 ^{o}C, respectively (Figure 4-9.) were used to determine effects on chlorophyll fluorescence values; Fv (variable fluorescence) and Fm (maximum fluorescence). The result showed the value of Fv/Fm more than 0.8 (Figure 4-10.). It indicated no effect of day light intensity on photosynthesis of *W. globo*sa in natural condition.

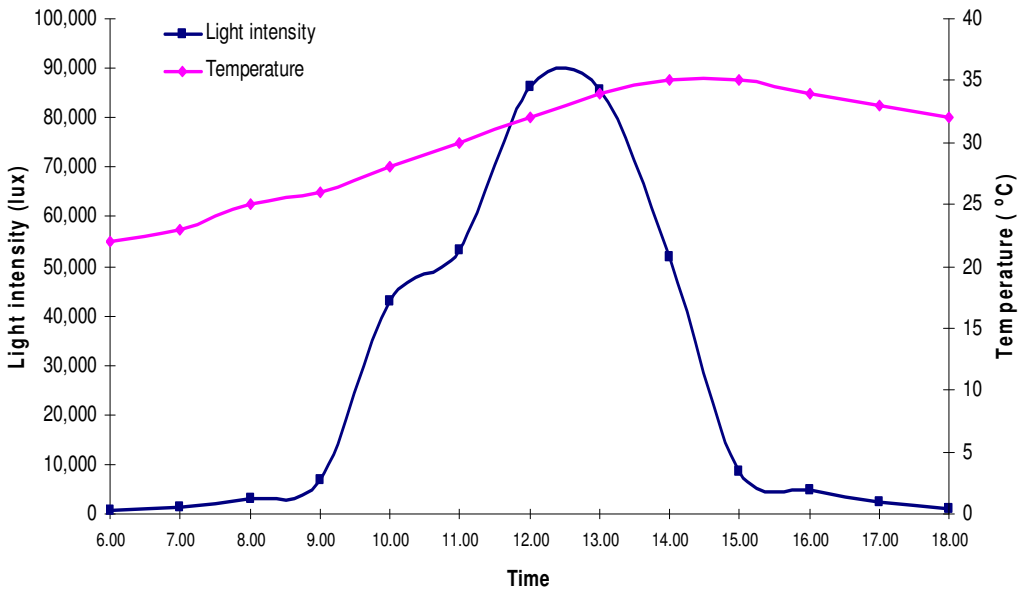


Figure 4-9. Various light intensity and temperature during a day on December 2009

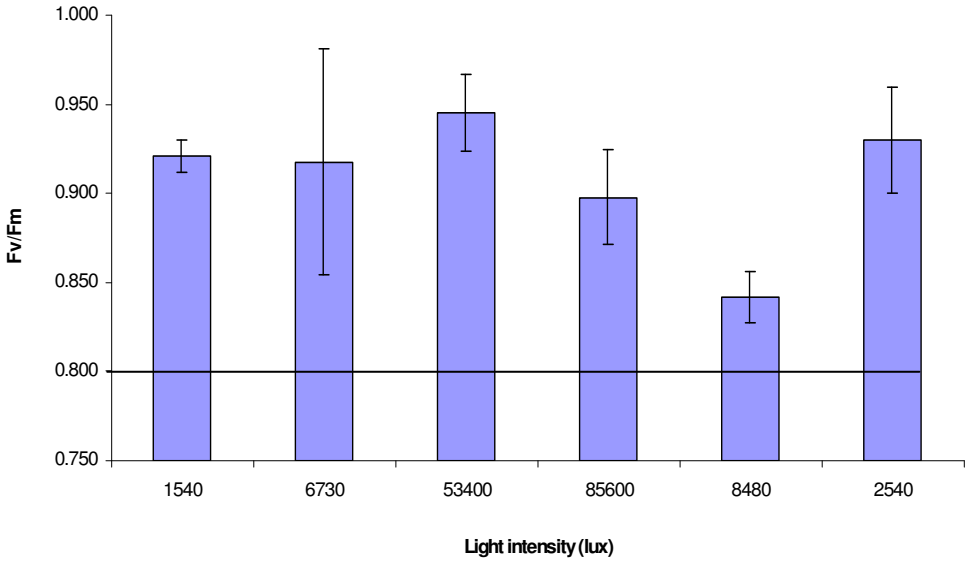


Figure 4-10. Effect of light intensity on photosynthesis efficiency of *W. globosa*

Effect of temperature on photosynthesis on Khai-nam *W. globosa*

Various temperatures ranged 10-40 °C with light intensity 5000 lux was used to determine effects on chlorophyll fluorescence values; Fv (variable fluorescence) and Fm (maximum fluorescence) ratio. The result showed the value of Fv/Fm more than 0.8 under temperatures during 10-35 °C (Figure 4-11.), indicated that temperatures between 10 and 35 °C were no effect on photosynthesis of *W. globosa*. It optimal quantum yield was determined under these temperature. On the contrary, fronds of *W. globosa* in the temperature at 40 °C showed Fv/Fm ratio less than 0.8 (Figure 4-11), indicating that the temperature at 40 °C reduced photosynthesis of *W. globosa*.

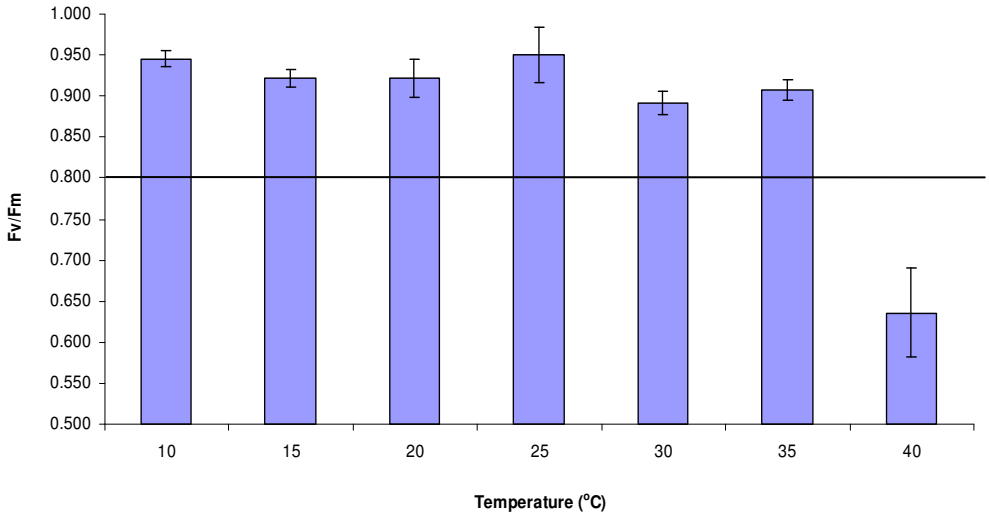


Figure 4-11. Effect of temperature on photosynthesis efficiency of *W. globosa*

Effect of initial pH on performance of growth in Khai-nam *W. globosa*

Frond numbers of *W. globosa* were evaluated in seven initial pH; 4, 5, 6, 7, 8, 9 and 10 with every 5 days observation (Figure 4-12). The results showed that the initial pH at 5, 6 and 7 provided positive frond number throughout the entire period of cultivation and produced higher frond number of 367.0±10.0, 390.7±12.0 and 331.5±10.4 fronds m^{-2}, respectively in 25 days of cultivation. At pH 4 and 10 frond production increased rapidly and then decreased after day 5 until the end of the experiment. The initial pH at 8 and 9 provided little positive frond numbers of 50.4±9.6 and 38.7±6.2 fronds m^{-2}, respectively, at the end of cultivation.

Production rate of *W. globosa* calculated from frond numbers indicated that the initial pH 6 provided the highest yield of 10.3±0.4 fronds $m^{-2}d^{-1}$ significantly difference with others ($p<0.05$), except pH 5 (9.9±0.4 fronds $m^{-2}d^{-1}$) The production for pH 7, 8, 9, 4 and 10 were 9.23±0.4, 0.93±0.3, 0.6±0.2, - 0.7±0.1 and – 0.7 fronds $m^{-2}d^{-1}$, respectively (Figure 4-13).

Frond size of *W. globosa* cultured in initial pH 4 to 10 showed in Figure 4-14, the results showed that frond size at day 10 was bigger than the early or later days. Initial pH 6 provided bigger frond size (0.48±0.12 mm^2) than others. Initial pH 7, 5, 8, 9, 4 and 10 gave frond size of 0.47±0.11, 0.45±0.12, 0.44±0.12, 0.39±0.09, 0.37±0.12 and 0.36±0.10 mm^2, respectively. After 10 days the fronds size of all pH decreased until the end of experiment. The fronds size after 30 days is showed in Figure 4-15. *W. globosa* grew in all pH, frond size decreased dramatically with time of culture.

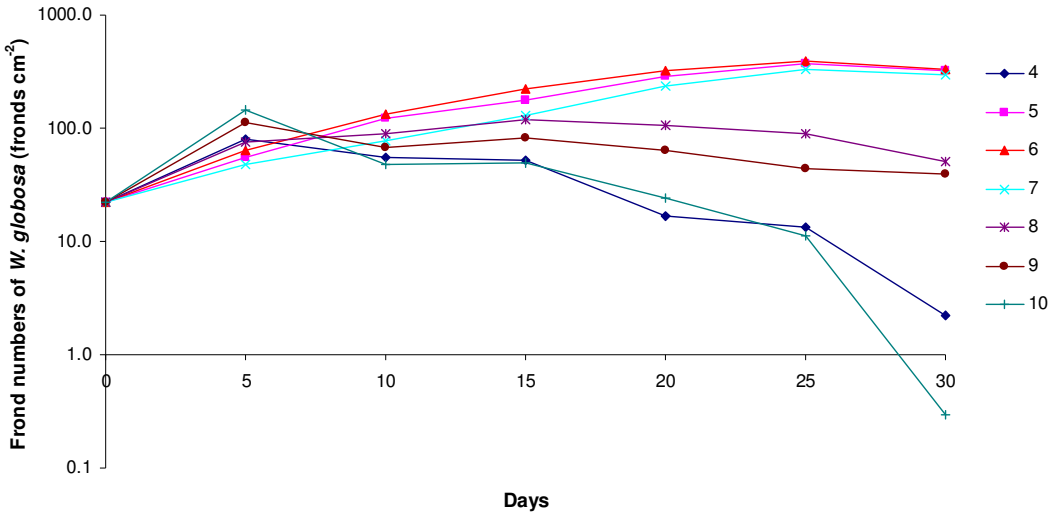


Figure 4-12. Effect of initial pH on growth of *W. globosa*

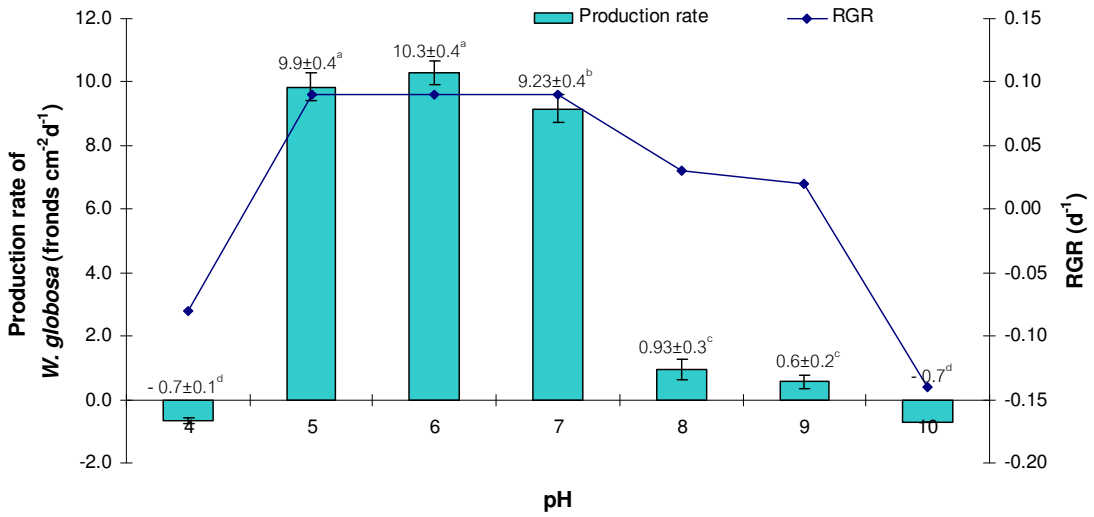


Figure 4-13. Effect of initial pH on the production rate and relative growth rate (RGR) after 30 days - culture

a, b, c and d denoted significant difference in mean ($p<0.05$)

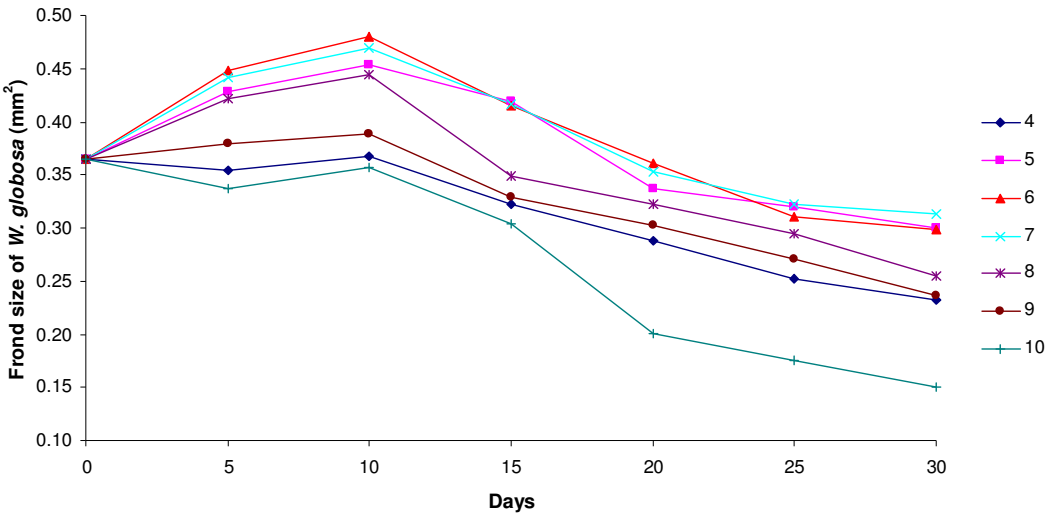


Figure 4-14. Frond size of *W. globosa* cultured in various pH 4 to 10

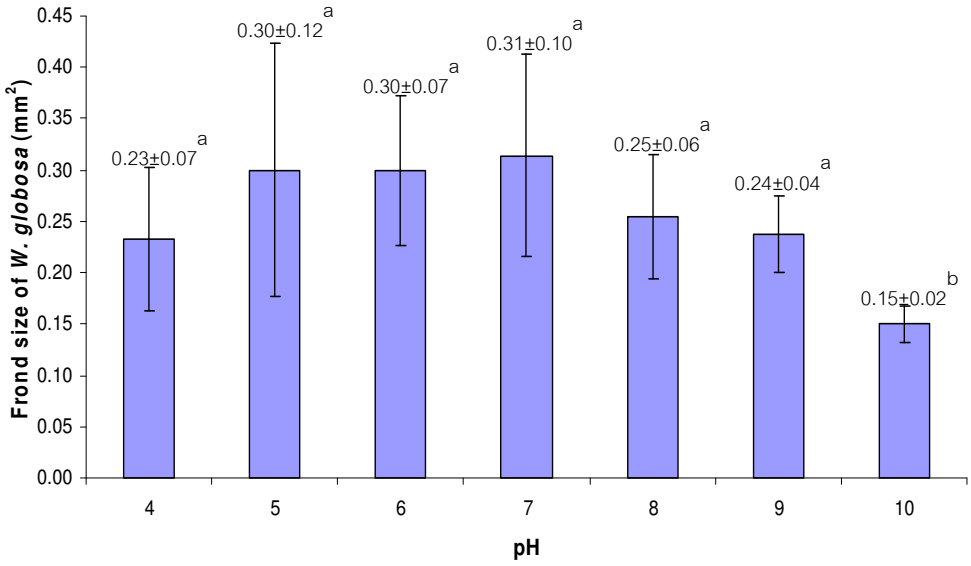


Figure 4-15. Frond size of *W. globosa* after 30 days- cultured in pH 4 to 10

a and b denoted significant difference in mean ($p<0.05$)

Effect of initial density on Khai-nam *W. globosa* culture

Frond numbers of *W. globosa* were evaluated in eight initial densities 0.1, 0.5, 1, 5, 10, 20, 30 and 40% of surface area. Growth of *W. globosa* was determined every 5 days and result is showed in Figure 4-16. Production rate of *W. globosa* calculated from frond number indicated that 10% of surface area provided the highest yield of 8.47±0.20 fronds $cm^{-2}d^{-1}$ and significantly difference to other density ($p<0.05$). The productions in 20, 5, 30, 1, 40, 0.5 and 0.1% of surface area were 8.22±0.21, 7.91±0.18, 6.01±0.23, 5.60±0.19, 4.23±0.32, 3.56±0.17 and 1.77±0.18 fronds $m^{-2}d^{-1}$, respectively (Figure 4-17). Relative growth rate (RGR) of *W. globosa* in 0.1% surface area was the highest (0.16 d^{-1}). RGR's 0.15, 0.14, 0.12, 0.12, 0.09, 0.07 and 0.06 d^{-1} were found in 0.5, 1, 5, 10, 20, 30 and 40% of surface area, respectively.

Frond size of *W. globosa* cultured in various initial densities is showed in Figure 4-18. The results showed that frond size in 0.1% surface area was the biggest 0.41±0.03 mm^2 in 25 days of culture. Fronds size of *W. globosa* decreased in 20, 30 and 40% surface area throughout the entire cultivation. The fronds size after 30 days is showed in Figure 4-19. There was significant difference among initial densities, 0,1% of surface area provided the biggest frond size 0.40±0.03 mm^2 and 40% of surface gave the lowest frond size of 0.27±0.02 mm^2.

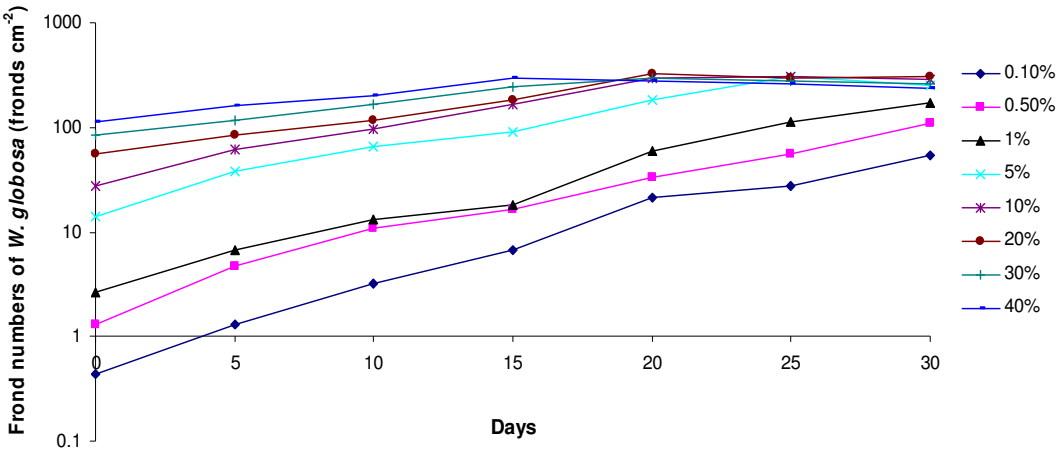


Figure 4-16. Effect of initial density on growth of *W. globosa*

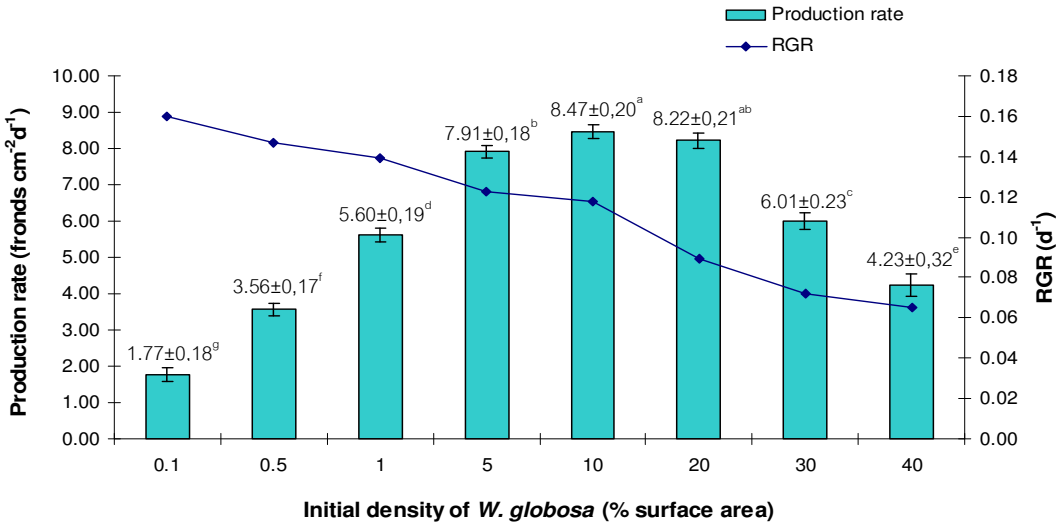


Figure 4-17. Effect of initial density on the production rate and relative growth rate (RGR) after 30 days - culture

a, b, c, d, e, f and g denoted significant difference in mean ($p<0.05$)

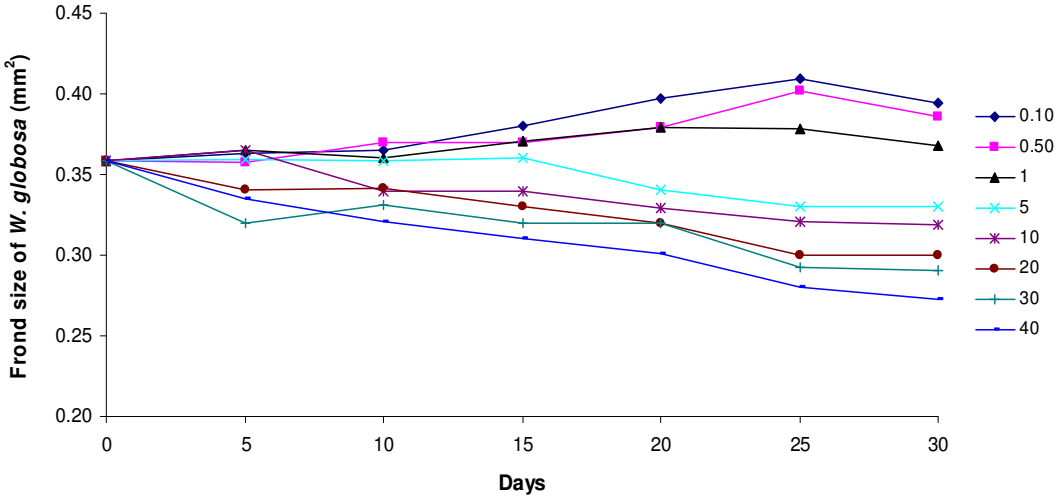


Figure 4-18. Frond size of *W. globosa* in various initial densities

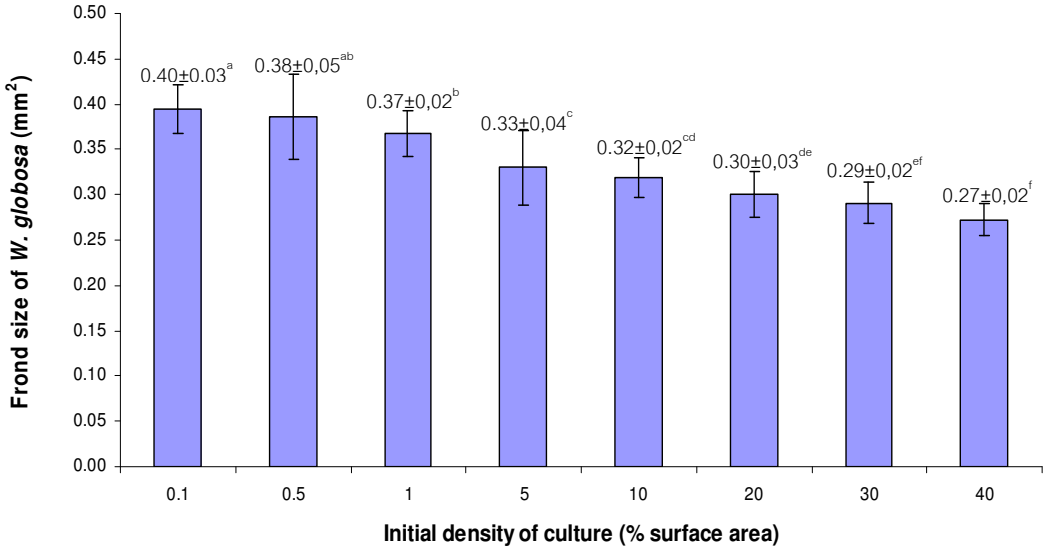


Figure 4-19. Frond size of *W. globosa* after 30 days- cultured in various initial densities

a, b, c, d, e and f denoted significant difference in mean ($p<0.05$)

Factorial experiment on light intensity, initial density and initial pH on the performance of growth and quality of Khai-nam *W. globosa*

3x3x4 completely randomized design involved factorials consisted of light intensity (2,000, 6,000 and 10,000 lux), initial pH (5, 6 and 7) and initial density (5, 10, 15 and 20% surface area) was performed on growth and quality of *W. globosa*. Frond number and size were evaluated. The results showed that frond number of all treatment were increased throughout the entire cultivation 28 days, however, after 21 days-culture the fronds number in most treatment, decreased at the end of experiment, the factor of light intensity 10,000 lux, pH at 6 and 15 % surface area (41.63 fronds cm^{-2}) provided the highest fronds number 442±8 fronds cm^{-2}. The factor of light intensity 2,000 lux, pH at 5 and 5% of surface area (14.10 fronds cm^{-2}) provided the lowest fronds number 59±7 fronds cm^{-2}(Figure 4-20.).

Production rate of *W. globosa* calculated from frond number indicated that the factor of light intensity 10,000 lux, pH at 6 and 15% surface area (41.63 fronds cm^{-2}) provided the highest yield 14.29±0.28 fronds $cm^{-2}d^{-1}$ and relative growth rate of 0.08 d^{-1} was significantly difference to other treatments ($p<0.05$). The factor of light intensity 2,000 lux, pH at 5 and 5% of surface area (14.10 fronds cm^{-2}) provided the lowest yield 1.59±0.24 fronds $cm^{-2}d^{-1}$ with relative growth rate of 0.05 d^{-1}. The factor of light intensity 10,000 lux, pH at 6 and 5% surface area (14.10 fronds cm^{-2}) provided the highest relative growth rate 0.10 d^{-1}. The factor of light intensity 2,000 lux, pH at 5 and 20% of surface area (55.51 fronds cm^{-2}) provided the lowest relative growth rate of 0.03 d^{-1} (Figure 4-21.).

Frond size of *W. globosa* cultured in 3x3x4 factorial experiment is showed in Figure 4-22. The results showed that frond size in the factor of light intensity 2,000 lux, pH at 5 and 5% surface area (14.10 fronds cm^{-2}) provided the biggest 0.38 mm^2 in 14 days – culture. The factor of light intensity 10,000 lux, pH at 6 and 20% of surface area (55.51 fronds cm^{-2}) provided very small frond size of 0.27 mm^2. The fronds size after 28 days-culture is showed in Figure 4-23. The factor of light intensity 2,000 lux, pH at 6 and 5% surface area (14.10 fronds cm^{-2}) provided the biggest 0.34 mm^2 and the factor of light intensity 10,000 lux, pH at 6 and 20% of surface area(55.51 fronds cm^{-2}) provided very small frond size of 0.27 mm^2, nevertheless, there was no significant difference among treatments.

From this experiment, treatment at 31 consists of initial pH at 6 and initial density at 15% surface area was used for the outdoor culture systems.

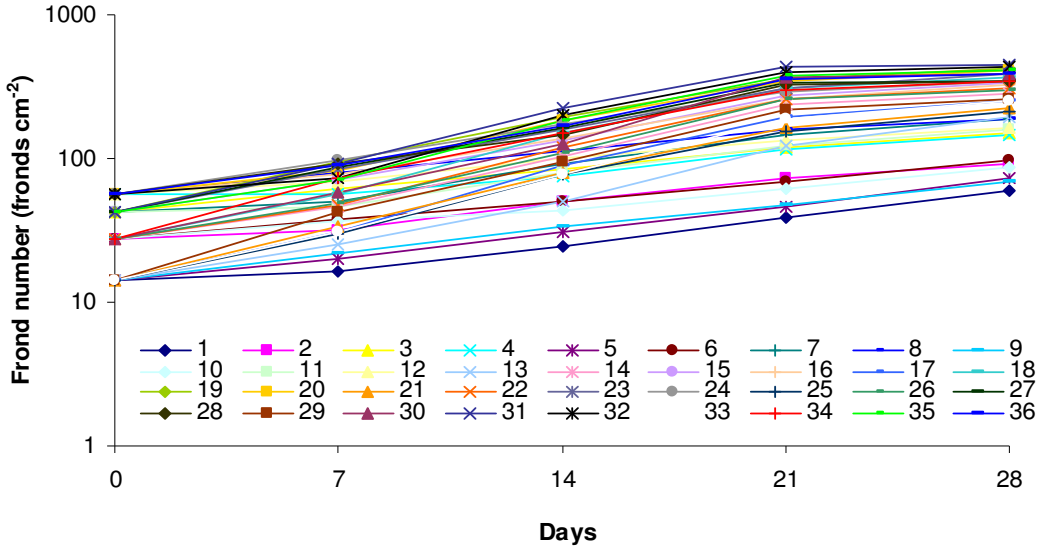


Figure 4-20. Effect of 3x3x4 factorial on growth of *W. globosa*

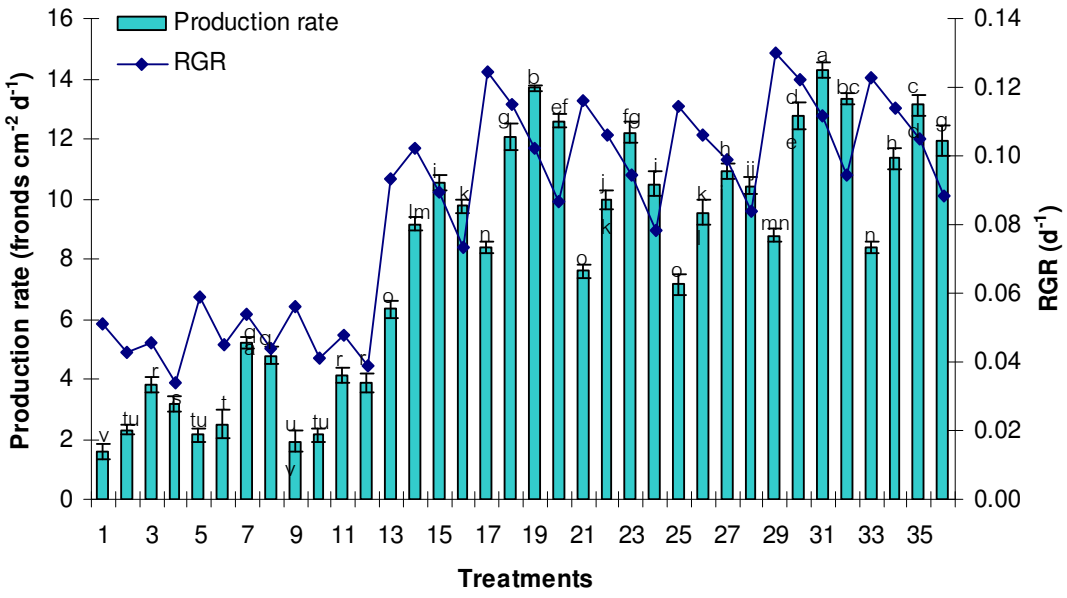


Figure 4-21. Production rate and relation growth rate of *W. globosa* under 3x3x4 factorial experiment after 28 days-culture

a to v denoted significant difference in mean ($p<0.05$)

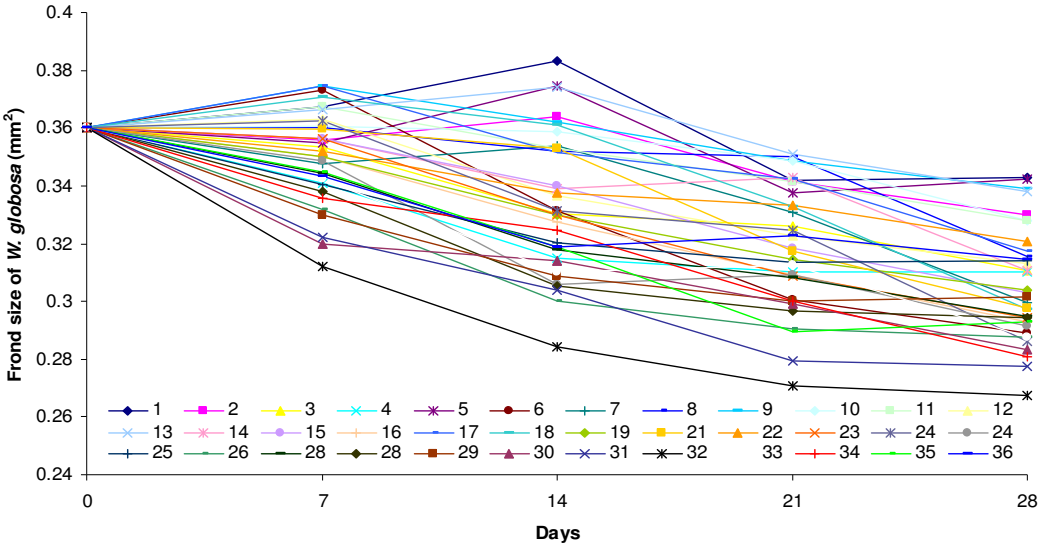


Figure 4-22. Frond size of *W. globosa* under 3x3x 4 factorial experiments

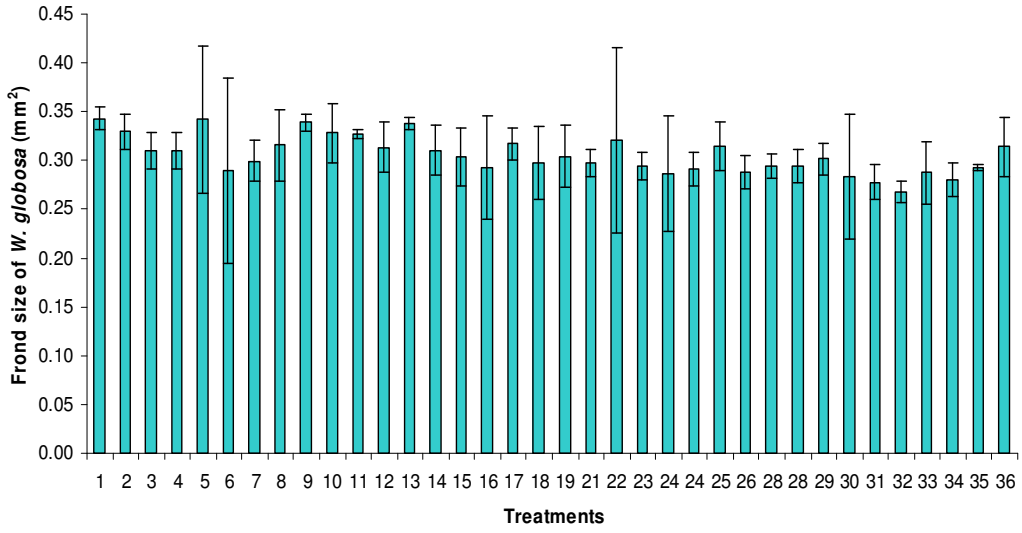


Figure 4-23. Fronds size of *W. globosa* under 3x3x4 factorial experiment after 28 days-culture

OUTDOOR CULTURE SYSTEM OF KHAI-NAM *W. globosa*

Culture systems test

Mass culture of *W. globosa* was evaluated in five different culture systems, a static, a vertical aeration, a system with horizontal, a system with top spraying and a layer culturing system with top spraying (Figure 4-24.), throughout the entire period of outdoor cultivation (28 days).

W. globosa was cultivated under the laboratory condition and then transferred to the outdoor in five different culture systems. Growth by wet weight of *W. globosa* determined every 7 days (Figure 4-25.) indicated that a system with horizontal flow provided the highest yield of 1,073.46±54.32 g m^{-2} and significantly difference to others in 28 days ($P<0.05$). The productions in other systems were 877.89±86.67, 783.03±123.36, 726.62±190.32 and 641.14±40.64 g m^{-2} in the system with top spraying, static, vertical aeration and layer culturing system with top spraying, respectively.

Dry weight of *W. globosa* was also evaluated and the result showed that a system with horizontal flow provided the highest yield of 42.94±2.17 g m^{-2} and significantly difference with others in 28 days ($P<0.05$). The productions in other system were 35.123.47±, 31.32±4.93, 29.06±7.61 and 25.65±1.63 g m^{-2} in the system with top spraying, static, vertical aeration and layer culturing system with top spraying, respectively (Figure 4-26.).

A 21 days-culture of *W. globosa* in a system with horizontal flow produced 1.52±0.04 g dry weight $m^{-2}d^{-1}$ which was significantly higher than those in other systems (1.18±0.17, 0.96±0.27, 0.94±0.04 and 0.86±0.09 g dry weight $m^{-2}d^{-1}$ for system with top spraying, vertical aeration, static and layer culturing system with top spraying, respectively) (Figure 4-27.).

Frond size of *W. globosa* in 7 days was 0.59±0.09, 0.53±0.03, 0.49±0.03, 0.48±0.08 and 0.47±0.06 mm^2 in vertical aeration, system with horizontal flow, system with top spraying, layer culturing system with top spraying and static, respectively, however, each culture system was not significantly different (Figure 4-28.).

The environment condition during the outdoor cultivation was showed in Table 4-3. All culture systems had similar values of environment factors.

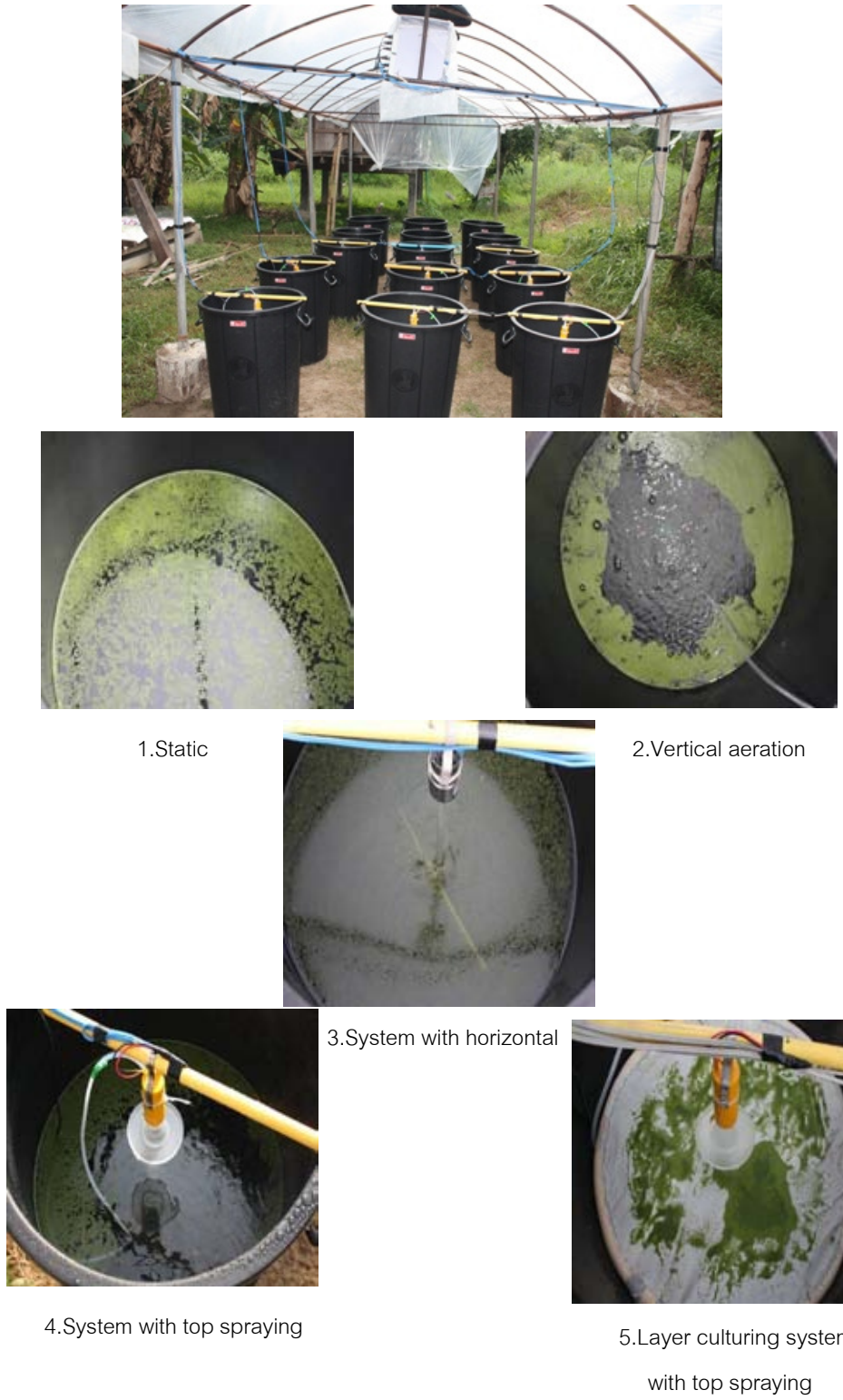
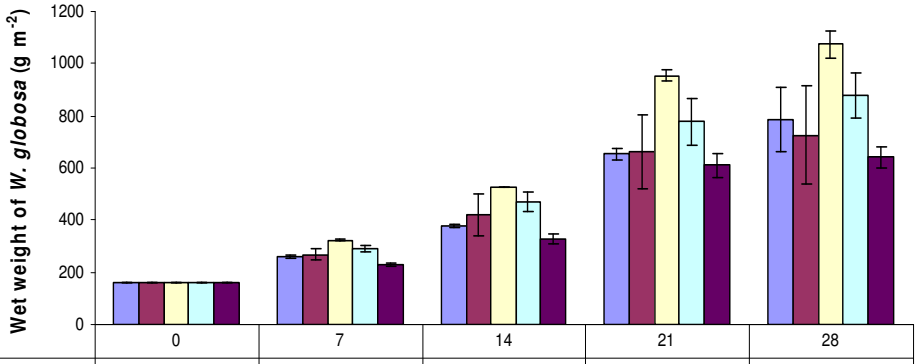


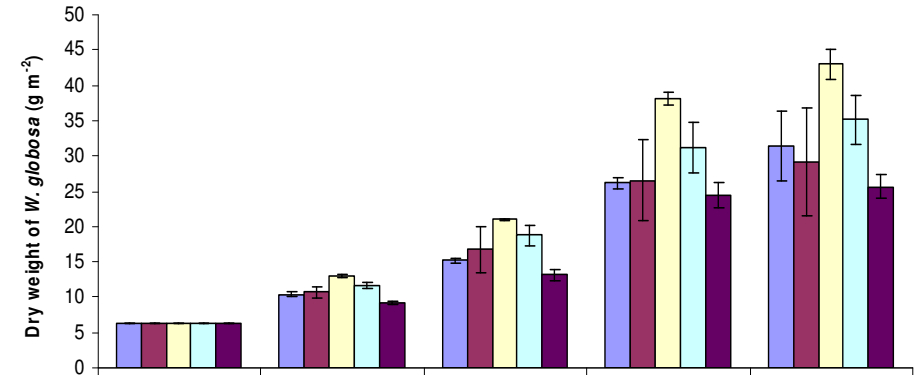
Figure 4-24. *W. globosa* in 5 different culture systems



	0	7	14	21	28
static	157.89	259.45 ±7.75^{c}	378.49 ±6.04^{cd}	653.53 ±21.65^{bc}	783.03 ±123.36^{bc}
vertical aeration	157.89	267.26 ±21.33^{c}	419.23 ±80.46^{bc}	662.96 ±142.8^{bc}	726.62 ±190.32^{bc}
horizontal flow	157.89	324.23 ±5.43^{a}	524.14 ±0.35^{a}	954.34 ±22.31^{a}	1073.46 ±54.32^{a}
top spraying	157.89	290.81 ±12.87^{b}	468.42 ±37.14^{ab}	777.70 ±90.86^{b}	877.89 ±86.67^{ab}
Layer culturing system	157.89	229.17 ±5.04^{d}	329.01 ±18.89^{d}	611.89 ±46.49^{c}	641.14 ±40.64^{c}

Figure 4-25. Wet weight of *W. globosa* cultivated in 5 different culture systems.

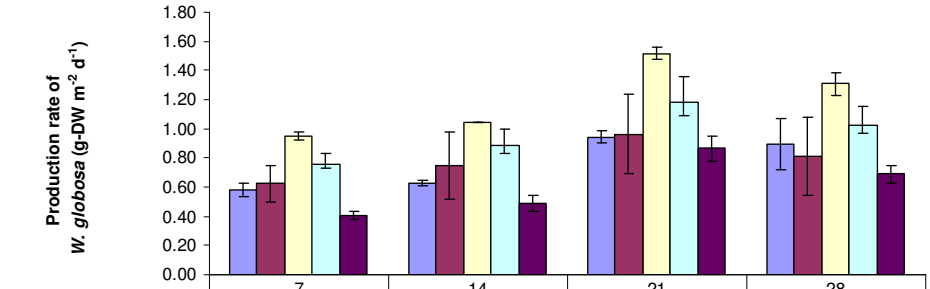
a, b, c and d denoted significant difference in mean of column ($p<0.05$)



	0	7	14	21	28
static	6.32	10.38 ±0.31^{c}	15.14 ±0.24^{cd}	26.14 ±0.87^{bc}	31.32 ±4.93^{bc}
vertical aeration	6.32	10.69 ±0.85^{c}	16.77 ±3.22^{bc}	26.52 ±5.71^{bc}	29.06 ±7.61^{bc}
horizontal flow	6.32	12.97 ±0.22^{a}	20.97 ±0.01^{a}	38.17 ±0.89^{a}	42.94 ±2.17^{a}
top spraying	6.32	11.63 ±0.51^{b}	18.74 ±1.49^{ab}	31.11 ±3.63^{b}	35.12 ±3.47^{ab}
Layer culturing system	6.32	9.17 ±0.20^{d}	13.16 ±0.76^{d}	24.48 ±1.86^{c}	25.65 ±1.63^{c}

Figure 4-26. Dry weight of *W. globosa* cultivated in 5 different culture systems

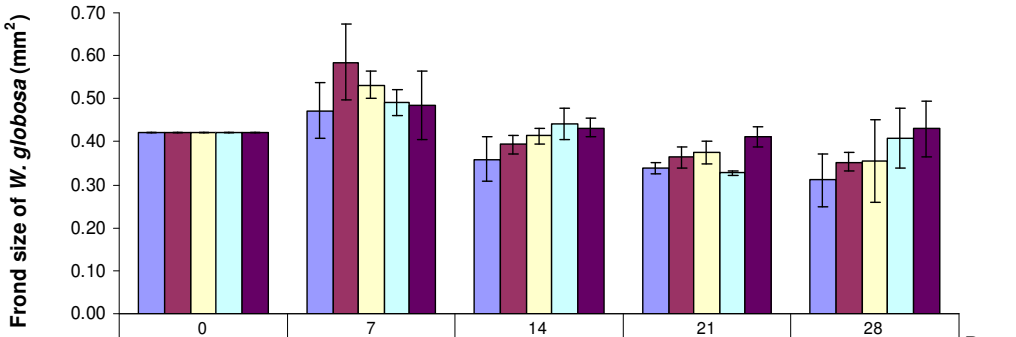
a, b, c and d denoted significant difference in mean of column ($p<0.05$)



	7	14	21	28
static	0.58±0.04^{c}	0.63±0.02^{Cd}	0.94±0.04^{bc}	0.89±0.18^{bc}
vertical aeration	0.62±0.12^{c}	0.75±0.23^{bc}	0.96±0.27^{bc}	0.81±0.27^{bc}
horizontal flow	0.95±0.03^{a}	1.05±0.00^{a}	1.52±0.04^{a}	1.31±0.08^{a}
top spraying	0.76±0.07^{b}	0.89±0.11^{ab}	1.18±0.17^{b}	1.03±0.12^{ab}
Layer culturing system	0.41±0.03^{d}	0.49±0.05^{d}	0.86±0.09^{C}	0.69±0.06^{C}

Figure 4-27. Production rate of *W. globosa* cultivated in 5 different culture systems

a, b, c and d denoted significant difference in mean of column ($p<0.05$)



	0	7	14	21	28
static	0.42	0.47±0.06	0.36±0.05^{b}	0.34±0.01^{c}	0.31±0.06
vertical aeration	0.42	0.59±0.09	0.39±0.02^{ab}	0.36±0.03^{bc}	0.35±0.02
horizontal flow	0.42	0.53±0.03	0.41±0.02^{ab}	0.38±0.03^{ab}	0.35±0.09
top spraying	0.42	0.49±0.03	0.44±0.04^{a}	0.33±0.01^{c}	0.41±0.07
Layer culturing system	0.42	0.48±0.08	0.43±0.02^{a}	0.41±0.02^{a}	0.43±0.06

Figure 4-28. Frond size of *W. globosa* cultivated in 5 different culture systems

a, b and c denoted significant difference in mean of column ($p<0.05$)

Table 4-3. Environment condition during the outdoor cultivation (October - November 2010) in five culture systems

Parameters	value
Light intensity	Max 98,000 lux
Light : Dark period	About 12: 12 hours
Temperature (air)	20 -36.5 ^{o}C
Temperature (medium)	17 -31 ^{o}C
pH	5.8 – 7.4
Dissolved Oxygen	5.5 – 15.5 mg l^{-1}
NO_3^--N	40-50 mg l^{-1}
PO_4-P	30-40 mg l^{-1}

Culture of Khai-nam *W. globosa* for proximate analysis and microbial determination

W. globosa was cultivated in modified Hutner's medium (1953) at pH 6 with 30 cm depth of tank. The culture was run an outdoor and harvested after 28 days. The proximate analysis and microbial determination form the system with horizontal flow is shown in Table 4-4. It indicated that *W. globosa* contained 48.2% protein, 9.6% fat, 14.5% crude fiber and fully amino acids profile. Furthermore, microbial determination showed a limit number or non pathogen microbial.

Table 4-4. Proximate analysis of *W. globosa* (dry matter) and microbial determination form the system with horizontal flow

Component	value
Protein (%)	48.2
Fat (%)	9.6
Crude fiber (%)	14.5
Amino acid (mg/100g of Protein)	
Aspatic acid	4137
Threonine *	1124
Serine	2048
Glutamic acid	4378
Proline	2450
Glycine	2530
Alanine	3213
Cystine	1928
Valine *	2410
Metionine *	843
Isoleucine *	1205
Leucine *	3896
Tyrosine	1365
Phenylalanine *	924
Histidine *	402
Lysine *	3333
Arginine *	2369
Tryptophan *	120
Microbial analysis	
Total plate count, cfu/g	1.7×10^6
MPN *E. coli* /g	< 3
Staphylococcus aureus, cfu/g	< 10 (ND)
Salmonella spp. / 25 g	ND

***** denoted the essential amino acid for human

CHAPTER V

DISCUSSIONS

INVESTIGATION OF KHAI-NAM BIOLOGY IN NATURAL POND

Khai-nam collected from the natural pond in Mueang district, Sakon Nakhon province, is identified as *Wolffia globosa* because of its morphology and the nucleotide sequence (Sangdee et al., 2010). *Wolffia*, duckweed, is the most reduced plant, its general form and shoot architecture has been difficult to study and interpret (Landolt, 1986, 1998). Systematic studies agree that evolution in the family has proceeded from a more complex to more reduced forms, the physically smallest and simplest genus, *Wolffia,* represents the more derived condition (Daubs, 1965; Den Hartog, 1975; Landolt, 1986; Les, Landolt and Crawford, 1997).

Sangdee et al. (2010) reported that 18 samples of *Wolffia* spp. collected from the north-east of Thailand provided the nucleotide sequence of maturase K and 5' *trnK* intron consist of 431 and 443 bps, respectively. The nucleotide sequences showed highly homology with muturase K and 5' *trnK* of *Wolffia globosa* in GenBank databases, indicated that these 18 *Wolffia* samples are *W. globosa.*

Asexual reproduction of Khai-nam *W. globosa*

The result of frond division time of *W. globosa* in this study is comparable with those found in other studies. One known estimate of the production rate for *W. borealis* was 0.62 fronds per day (Lemon et al., 2001), frond division time of 38.64 hours frond^{-1}, which is much lower than 40.36 hours frond^{-1} reported in this study. The differences between this study and the other may due to environmental conditions and illustrate the phenotypic plasticity of these plants (Landolt, 1986). Other study estimated for the frond division time of *W. arrhiza* was 4 days per frond (Sakdisuwan, 1967), which is similarly reported in this study.

Production rate of Khai-nam *W. globosa* on natural pond

Bhanthumnavin and McGarry (1971) reported that a 9 month period (November - July), productivity of *W. arrhiza* in northern Thailand (the small scale opened pond cultivation used rain water in Chiangmai) was 3.89 g dry weight $m^{-2}d^{-1}$. In addition, duckweeds reproduce by vegetative reproduction and are characterized by rapid clone growth. Yields equivalent in outdoor tanks maximum yields approached 5 g dry weight $m^{-2}d^{-1}$ (Said et al., 1979). Comparing this experiment with 2 reports, low yield (1.05 g dry weight m^{-2} d^{-1}) in the natural pond at Mueang district, Sakon Nakhon province is found. The various production rates as reported in varies considerably due to species, age of the plant, nutrients and other environment conditions.

Proximate analysis and microbial determination

The comparison protein, fat, crude fiber content and essential amino acid profile of the protein concentrate between *W. globosa* in this experiment and *W. arrhiza* (Jairakphan, 1999) (Table 5-1.). The result showed that *W. globosa* collected from the natural pond provided higher protein content and fat content of 33.3% and 5.0%, respectively, than *W. arrhiza* which was collected from natural pond, however, crude fiber content in *W. arrhiza* was produced higher than another one.

Trytophan, the essential amino acid profile of the protein concentrate in *W. arrhiza* was provided higher than *W. globosa.*

The result of this comparison was similar the previous reports. The crude protein content of *Wolffia* obtained from natural waters (ponds, lakes, ditches, streams and paddy fields) has been reported to range from 7 to 20% (Tan, 1970; Bhanthumnavin and McGarry, 1971; Jairakphan, 1999). Grown in enriched waters containing mineral media or effluents from agricultural and municipal waste lagoons, the protein content (30 – 45 %) was greatly increased over that from natural waters with low nutrients (Rusoff et al., 1980; Fujita et al., 1999; Chantiratikul et al., 2010). However, *W. globosa* collected the natural pond provided high protein, fat, crude fiber content and essential amino acid profile of the protein concentrate.

Table 5-1. Proximate analysis of *W. arrhiza* and *W. globosa* (dry matter)

Components	*Wolffia arrhiza* (Jairakphan, 1999)	*Wolffia globosa* (Natural pond in this study)
Protein (%)	20.15	33.3
Fat (%)	2.43	5.0
Crude fiber (%)	14.72	10.7
Amino acid(mg/100g of Protein)		
Aspatic acid	1209	3539
Threonine	641	662
Serine	565	982
Glutamic acid	1669	2557
Proline	674	1279
Glycine	831	1507
Alanine	1595	3128
Cystine	104	5457
Valine	944	1849
Metionine	201	571
Isoleucine	685	685
Leucine	1300	2032
Tyrosine	374	890
Phenylalanine	758	502
Histidine	309	228
Lysine	751	1530
Arginine	804	1393
Tryptophan	201	46

FACTORS EFFECTING ON KHAI-NAM *W. globosa* PRODUCTION

Culture media experiment

Five different culture media were compared on life span, daughter frond number, production rate, division time and frond size of *W. globosa*. The results found that Hoagland's medium provided shorter life span of *W. globosa* than that of the others, but yield of *W. globosa* cultured in Hoagland's and Hutner's media was higher (0.40 and 0.36 fronds d^{-1}, respectively) comparing to others. On the contrary, the natural water pond 2 had longer life span of 19.07 days than that of others, but the yield was the lowest as the control one. Moreover, daughter frond number in Hutner's medium gave the highest of 6.17 fronds throughout life span of mother frond. Lemon et al. (2001) reported that *W. borealis* grown in 33% v/v strength Hutner's medium, adjusted to pH 6.5, provided life span 15.8 days, daughter frond 9.8 fronds and production rate 0.62 fronds d^{-1}, which are similar life span of *W. globosa* in this study. Life span of *W. globosa* in this study (17.37 days) is longer than the 1.57 days of *W. borealis*, but daughter frond number of *W. borealis* is more than the 3.63 fronds of *W. globosa*. Moreover, production rate of *W. borealis* is more than the 0.26 fronds d^{-1} of *W. globosa*. The differences may due to environmental conditions and illustrate the phenotypic plasticity of the plants (Landolt, 1986).

Bernard et al. (1990) studied flower structure, anatomy and life history of *W. australiana* reported that life span was 17 days and 11 fronds were produced. Frond size at detachment decreased with increased age of plant, which is similarity the present experiment as size decrease with increasing generation of *W. globosa.*

Hillman (1961) reported that most duckweed grew better in 1/3 strength Hutner's medium, but in dilution of 1/100 reduced growth in many species. In general, *Wolffia* L. *trisulca* or those with thin fronds grew better than others on dilute media (Landolt, 1957)

Effect of light intensity on photosynthesis effecting of Khai-nam *W. globosa*

The Fv/Fm testing is used the primary method for checking and choosing factors which is relative the photosynthesis such as light intensity and temperature. Fv/Fm is a dark adapted test used to determine maximum quantum yield. This ratio is an estimate of the maximum portion of absorbed quanta used in PSII reaction centers. It is import to property dark adapt samples for this test. Fo will be raise and Fm will be lowered if dark adaptation is inadequate. Since dark adaptation requirement can vary with species, varieties, mutants and sun vs. shade leaves testing should be done to ensure proper dark adaptation (Kitajima and Butler, 1975). The present experiment used the same basic information for examining. In 2009, December is winter season in Thailand thus this data will be representation in winter season. Temperature of water surface was between 22 and 35 ^{o}C and light intensity of was between 500 and 100,000 lux (about 9.25 to 1,850 μE $m^{-2}s^{-1}$) are no effect on photosynthesis of *W. globosa.*

Effect of temperature on photosynthesis effecting Khai-nam *W. globosa*

The Fv/Fm testing is used as primary method for checking and choosing factors which is relative the photosynthesis such as light intensity and temperature**.** Fv/Fm is a dark adapted test used to determine maximum quantum yield and Fv/Fm take only a second to make a measurement. In this experiment, temperature at 40 ^{o}C had effect on photosynthesis of *W. globosa*. Wedge and Burris (1982) studied effects of light and

temperature on duckweed photosynthesis and reported that duckweeds had a temperature optimum between 30 and 35 ^{o}C. Plants show decreasing photosynthesis only when the temperature exceeds 40 ^{o}C (Hew, Krotkov and Canvin, 1969).

Effect of initial pH on performance of growth in Khai-nam *W. globosa*

Effect of pH on growth of *W. globosa* is clearly indicated that initial pH at 4, 8, 9 and 10 had no suitability for culturing *Wolffia.* Initial pH at 5, 6 and 7 are suitable for culturing *W. globosa*. *Wolffia* has an optimum growth at pH 5 and growth declined with increasing pH (McLay, 1976) In natural ponds, the pH at 6.5 to 7.0 of the water was an optimized pH (Bhanthumnavin and McGarry, 1971). In controlled laboratory condition, pH at 5 to 6 was suitable pH for the optimal growth of *Wolffia* (Rowchi and Somboon, 2007). Hillman (1961) reported that *Lemna* and *Wolffia* grew well in pH at 4.5 to 7.5 and outer limits at 3.5 and 8.5, moreover, there may be small differences in the pH tolerance of various species.

Effect of initial density on Khai-nam *W. globosa* culture

This experiment indicated that relative growth rate (RGR) and frond size of *W. globosa* decreased with increase of initial density. It is similar the other researchers found. Driever et al. (2005) suggested that crowding was an important factor in limitation of duckweed growth. Growth rates of duckweed is produced decreasing with increasing density, moreover, it could well be described as growth limitation by biomass. Suppadit et al. (2008) reported that the initial biomass level of 4 g l^{-1} yielded the highest increase in biomass, whereas the initial biomass level of 16 g l^{-1} provided the greatest decrease in biomass, this might be because of the low survival of *W. arrhiza*.

A factorial experiment on light intensity, initial density and initial pH on the performance of growth and quality of Khai-nam *W. globosa*

Effect of light intensity, initial pH and initial density using 3x3x4 factorial design consist of light intensity at 2,000, 6,000 and 10,000 lux, initial pH at 5, 6 and 7 and initial density at 5, 10, 15 and 20% surface area, on growth of *W. globosa* was evaluated. This result indicated high light intensity (6,000 and 10,000 lux) is suitability for *Wolffia* cultivation. It is similar the other study, Hillman (1961) reported that the effect of light duration and intensity appear to be relatively uncomplicated. Light intensities below about 7,000 lux, the multiplication rate of studied duckweed increases with increasing daily duration of expire, reaching a maximum under continuous light (Clark, 1925; Landolt, 1957).

Landolt (1957) reported that fluorescent was used as light supplemented to supply various photoperiods and intensities on a large number of lemnaceae. Multiplication rate increased with intensity until a maximal value was reached.

Light intensity provided very high effect on the growth of *W. globosa*, moreover, it has the effect with other factor such as pH and density (Figure 4-21.). The effect of initial pH and initial density depend on light intensity. When high light intensity (6,000 and 10,000 lux), the various of initial pH and initial density are showed the difference distinct on the production rate and relative growth rate (RGR). However, in low light intensity (2,000 lux) is not found the difference between the initial pH and density.

OUTDOOR CULTURE SYSTEM OF KHAI-NAM *W. globosa*

Culture systems test

The present study, the system with horizontal flow produced higher wet weight and dry weight than other systems in 28 days (1073.46 and 42.94 g m^{-2}, respectively). Moreover, the system with horizontal flow produced higher production rate than other one in 21 days- culture (1.52 g-DW $m^{-2}d^{-1}$). This might be because of too dense and some of *W. globosa* died after the 21 days- culture. This is similar to trend that was reported by Suppadit et al. (2008), Suppadit (2011) and Cheng and Stomp (2009).

Production rate in the vertical aeration system was not significantly different throughout 28 days of culture. This might be because of vertical movement interfering normal living of *W. globosa*, since this plant always floats only on the water surface. When it is follow water circulation as verticality. It provide low yield. In 28 days-culture, *W. globosa* in the vertical aeration system produce production rate lower than the static water culture.

For the layer culturing system with top spraying, this culture system provide the lowest yield (wet weight, dry weight and production rate) throughout cultivation, indicated that a layer culturing system with top spraying is unsuitable for mass culture of *W. globosa.* This might be because of frond of *W. globosa* pile up very much on the layer (Figure 4-24.), it is not spread by the water. Therefore, frond surface of *W. globosa* is low the photosynthesis effecting low yield.

Fedler and Duan (2011) studied biomass production for bioenergy using recycled wastewater in a natural waste treatment system and reported the biomass

production of duckweed (containing both *Lemna* and *Wolffia*) in the tank with a TN concentration of about 2 mg l^{-1}. The water surface area was 2.54 m^2 (a radius of 0.9 mm and a height of 0.9 m). The average daily growth rates of duckweed was 99 – 127 g wet weight $m^{-2}d^{-1}$. The mean long –term extrapolated yield of *Lemna* and mixed *Lemna – Wolffia* was 0.003 g dry weight m^{-2} d^{-1} (Edwards et al., 1992). Maximum yield of duckweed was 15 g dry weight m^{-2} d^{-1} using domestic sewage (Oron et al., 1984, 1988; Gaigher and Short, 1986). Nasker et al. (1986) reported a dry weight yield of *W. arrhiza* grown in different concentrations of sewage, ranging from 0.002 to 0.003 g $m^{-2}d^{-1}$. For yield of *Wolffia* in this experiment all 5 different culture system provide 0.51 to 1.90 g dry weight m^{-2} d^{-1}.

Culture of Khai-nam *W. globosa* for proximate analysis and microbial determination

The comparison protein, fat, crude fiber content and essential amino acid profile of *W. globosa* were compared to *W. arrhiza* (Jairakphan, 1999) and *W. columbiana* (Rusoff et al., 1980) as showed in Table 5-2. The result revealed that *W. globosa* grown in a system with Hutner's medium provided higher protein and fat content of 48.2% and 9.6%, respectively, than those of *W. Columbiana* collected from anaerobic dairy waste lagoons on the LSU campus, the lagoons contained from 20 to 40 mg l^{-1} of TKN during the collection period. *W. arrhiza* and *W. globosa* which was collected from natural pond, however, crude fiber content in *W. arrhiza* was produced higher than other one.

The essential amino acid profile of the protein concentrate in *W. columbiana* was provided higher than *W. arrhiza* and *W. globosa.* On the contrary, cystine and tryptophan were found in only *W. arrhiza* and *W. globosa*.

W. globosa grown in culture system was produced protein, fat, crude fiber content and essential amino acid profile of the protein concentrate higher than *W. globosa* grown in the natural pond (except Cystine) and *W. arrhiza* collected from natural pond (except Tryptophan).

The result of this comparison was similar the previous reports. The crude protein content of *Wolffia* obtained from natural waters (ponds, lakes, ditches, streams and paddy fields) has been reported to range from 7 to 20% (Tan, 1970; Bhanthumnavin and McGarry, 1971; Jairakphan, 1999). Grown in enriched waters containing mineral media or effluents from agricultural and municipal waste lagoons, the protein content (30 – 45 %) was greatly increased over that from natural waters with low nutrients (Rusoff et al., 1980; Fujita et al., 1999; Chantiratikul et al., 2010).

Table 5-2. Proximate analysis of *Wolffia* (dry matter)

Component	*Wolffia columbiana* (Rusoff et al., 1980)	*Wolffia arrhiza* (Jairakphan, 1999)	*Wolffia globosa* (in this study)	
			Culture system	Natural pond
Protein (%)	44.7	20.15	48.2	33.3
Fat (%)	6.6	2.43	9.6	5.0
Crude fiber (%)	11.0	14.72	14.5	10.7
Amino acid (mg/100g of Protein)				
Aspatic acid	5630	1209	4137	3539
Threonine	2550	641	1124	662
Serine	2280	565	2048	982
Glutamic acid	5760	1669	4378	2557
Proline	2410	674	2450	1279
Glycine	3040	831	2530	1507
Alanine	3750	1595	3213	3128
Cystine		104	1928	5457
Valine	3490	944	2410	1849
Metionine	870	201	843	571
Isoleucine	3060	685	1205	685
Leucine	5830	1300	3896	2032
Tyrosine	2170	374	1365	890
Phenylalanine	3600	758	924	502
Histidine	1180	309	402	228
Lysine	3370	751	3333	1530
Arginine	3780	804	2369	1393
Tryptophan		201	120	46

CHAPTER VI

CONCLUSION AND RECOMMENDATION

1. Khai-nam, watermeal, was collected from the local natural pond in Mueang district, Sakon Nakhon province is identified as *Wolffia globosa*.
2. Asexual reproduction of *W. globosa* in the natural pond water needs a complete cycle for 96 hours or 4 days.
3. Production rate of *W. globosa* on natural pond from Sakon Nakhon province provided high growth of 65.18 g dry weight m^{-2} on July and the lowest was 2.45 g dry weight m^{-2}on February. However, June and August provided the highest yield 1.05 g dry weight $m^{-2}d^{-1}$and the lowest yield -0.99 g dry weight $m^{-2}d^{-1}$, respectively.
4. *W. globosa* collected locally at a small pond in Mueang district, Sakon Nakhon province produce 33.3% protein, 5.0% fat, 10.4% crude fiber and fully amino acid and it does not find or a little find the pathogen microbial.
5. Hutner's medium is suitable for *W. globosa* culture.
6. Light intensity under the natural light is not effect on photosynthesis of *W. globosa.*
7. Temperature at 40 ^{o}C has an negative effect on photosynthesis of *W. globosa.*
8. The pH at 5 to 7 of medium can produce high yield of *W. globosa.*
9. Initial density for *W. globosa* cultivation is 5 to 20 % surface area.

10. A factorial experiment on light intensity, initial pH and initial density on the performance of growth and quality of *W. globosa* indicate that the light intensity at 10,000 lux, initial pH at 6 and 15% surface area of initial density produce high yield.
11. The system with horizontal movement in outdoor, *W. globosa* show high yield and it can produce 48.2% protein, 9.6% fat, 14.5% crude fiber and fully amino acids.

RECOMMENDATION

Previous study conduct mass product from different sewage, nutrient resource and record base data about effecting factors, a culture system for *Wolffia* or duckweed has received little attention. I think that the culture system of *Wolffia* should develop for the highest yield.

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APPENDIX

Appendix 1. modified Hoagland's medium consist of (mg/l) (Sakdisuwan, 1967)

$CaCl_2$	554
$NaNO_3$	849
KNO_3	505
$MgSO_4$	240
$KH2PO_4$	136
Fe-EDTA	5
Nitsch's minor element solution	1

Nitsch's minor element stock solution consist of (mg/l)

$ZnSO_4$	100
$MnCl_2$	2000
$H3BO_3$	1000
$CuSo_4$	11
NaCl	13
$CoCl_2$	20
$NaMoO_4$	20

Appendix 2. modified Hutner's medium (Fe) consist of (mg/l) (Hutner, 1953)

$(NH_4)_2SO_4$	33
$NaNO_3$	42
K_2HPO_4	80
$CaCl_2$	27
$NaNO_3$	41
$MgSO_4$	100
$FeSO_4$	5
$MnSO_4$	3
$ZnSO_4$	13
H_3BO_3	3
Na_2MoO_4	5
$CuSO_4$	0.8
$CoSO_4$	0.2
Fe-EDTA	100

Appendix 3. Statistical analysis

Dependent Variable: Lifespan

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	319.460272	79.865068	7.26	<.000
Error	93	1023.346667	11.003728		
Corrected Total	97	1342.806939			

R-Square	Coeff Var	Root MSE	Lifespan Mean
0.237905	20.70864	3.317187	16.01837

Source	DF	Type I SS	Mean Square	F Value	Pr > F
media	4	319.4602721	79.8650680	7.26	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
media	4	319.4602721	79.8650680	7.26	<.0001

Duncan's Multiple Range Test for Lifespan

Alpha	0.05
Error Degrees of Freedom	93
Error Mean Square	11.00373
Harmonic Mean of Cell Sizes	18.75

Number of Means	2	3	4	5
Critical Range	2.151	2.264	2.339	2.393

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	media
A	19.067	15	nw2
B A	17.375	24	Hut
B C	15.867	15	nw1
C	14.575	24	Ho
C	13.950	20	con

Appendix 3. Statistical analysis (continue)

Dependent Variable: G1number

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	213.4397959	53.3599490	33.75	<.0001
Error	93	147.0500000	1.5811828		
Corrected Total	97	360.4897959			

R-Square	Coeff Var	Root MSE	G1number Mean
0.592083	27.88013	1.257451	4.510204

Source	DF	Type I SS	Mean Square	F Value	Pr > F
media	4	213.4397959	53.3599490	33.75	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
media	4	213.4397959	53.3599490	33.75	<.0001

Duncan's Multiple Range Test for G1number

Alpha	0.05
Error Degrees of Freedom	93
Error Mean Square	1.581183
Harmonic Mean of Cell Sizes	18.75

Number of Means	2	3	4	5
Critical Range	.8155	.8582	.8865	.9072

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	media
A	6.1667	24	Hut
A	5.5833	24	Ho
B	4.2000	15	nw2
B	3.4667	15	nw1
C	2.2500	20	con

Appendix 3. Statistical analysis (continue)

Dependent Variable: productionrate

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.19408867	0.04852217	26.68	<.0001
Error	93	0.16914500	0.00181876		
Corrected Total	97	0.36323367			

R-Square	Coeff Var	Root MSE	productionrate Mean
0.534336	29.49472	0.042647	0.144592

Source	DF	Type I SS	Mean Square	F Value	Pr > F
media	4	0.19408867	0.04852217	26.68	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
media	4	0.19408867	0.04852217	26.68	<.0001

Duncan's Multiple Range Test for productionrate

Alpha	0.05
Error Degrees of Freedom	93
Error Mean Square	0.001819
Harmonic Mean of Cell Sizes	18.75

Number of Means	2	3	4	5
Critical Range	.02766	.02911	.03007	.03077

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	media
A	0.19583	24	Ho
A	0.18167	24	Hut
B	0.11333	15	nw2
B	0.11200	15	nw1
B	0.08650	20	con

Appendix 3. Statistical analysis (continue)

Dependent Variable: divisiontimeG1

Source	DF	Sum ofSquares	Mean Square	F Value	Pr > F
Model	4	570.636735	142.659184	7.58	<.0001
Error	93	1750.325000	18.820699		
Corrected Total	97	2320.961735			

R-Square	Coeff Var	Root MSE	divisiontimeG1 Mean
0.245862	75.98780	4.338283	5.709184

Source	DF	Type I SS	Mean Square	F Value	Pr > F
media	4	570.6367347	142.6591837	7.58	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
media	4	570.6367347	142.6591837	7.58	<.0001

Duncan's Multiple Range Test for divisiontimeG1

Alpha	0.05
Error Degrees of Freedom	93
Error Mean Square	18.8207
Harmonic Mean of Cell Sizes	18.75

Number of Means	2	3	4	5
Critical Range	2.814	2.961	3.059	3.130

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	media
A	9.600	20	con
B A	6.833	15	nw2
B	6.533	15	nw1
C	3.500	24	Hut
C	3.458	24	Ho

Appendix 3. Statistical analysis (continue)

Dependent Variable: divisiontimeG2

Source	DF	Sum ofSquares	Mean Square	F Value	Pr > F
Model	4	199.2959821	49.8239955	14.77	<.0001
Error	40	134.9040179	3.3726004		
Corrected Total	44	334.2000000			

R-Square	Coeff Var	Root MSE	divisiontimeG2 Mean
0.596337	43.72534	1.836464	4.200000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
media	4	199.2959821	49.8239955	14.77	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
media	4	199.2959821	49.8239955	14.77	<.0001

Duncan's Multiple Range Test for divisiontimeG2

Alpha	0.05
Error Degrees of Freedom	40
Error Mean Square	3.3726
Harmonic Mean of Cell Sizes	4.912281

Number of Means	2	3	4	5
Critical Range	2.368	2.490	2.570	2.627

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	media
A	8.500	2	nw2
A	8.250	4	con
A	6.643	7	nw1
B	2.938	16	Ho
B	2.844	16	Hut

Appendix 3. Statistical analysis (continue)

Dependent Variable: divisiontimeG3

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	21.09742647	10.54871324	80.10	<.0001
Error	14	1.84375000	0.13169643		
Corrected Total	16	22.94117647			

R-Square	Coeff Var	Root MSE	divisiontimeG3 Mean
0.919631	10.19719	0.362900	3.558824

Source	DF	Type I SS	Mean Square	F Value	Pr > F
media	2	21.09742647	10.54871324	80.10	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
media	2	21.09742647	10.54871324	80.10	<.0001

Duncan's Multiple Range Test for divisiontimeG3

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	0.131696
Harmonic Mean of Cell Sizes	2.4
Number of Means	2 3
Critical Range	.7105 .7445

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	media
A	8.0000	1	nw2
B	3.3750	8	Ho
B	3.1875	8	Hut

Appendix 3. Statistical analysis (continue)

Dependent Variable: frondsizeG1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.59182303	0.14795576	11.57	0.0002
Error	14	0.17898750	0.01278482		
Corrected Total	18	0.77081053			

R-Square	Coeff Var	Root MSE	frondsizeG1 Mean
0.767793	17.48031	0.113070	0.646842

Source	DF	Type I SS	Mean Square	F Value	Pr > F
media	4	0.59182303	0.14795576	11.57	0.0002

Source	DF	Type III SS	Mean Square	F Value	Pr > F
media	4	0.59182303	0.14795576	11.57	0.0002

Duncan's Multiple Range Test for frondsizeG1

Alpha 0.05

Error Degrees of Freedom 14

Error Mean Square 0.012785

Harmonic Mean of Cell Sizes 1.538462

Number of Means	2	3	4	5
Critical Range	.2765	.2897	.2979	.3034

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	media
A	0.7300	8	Ho
A	0.7163	8	Hut
B	0.2600	1	nw2
B	0.2500	1	nw1
B	0.2100	1	con

Appendix 3. Statistical analysis (continue)

Dependent Variable: frondsizeG2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.29237895	0.07309474	13.64	<.0001
Error	14	0.07500000	0.00535714		
Corrected Total	18	0.36737895			

R-Square	Coeff Var	Root MSE	frondsizeG2 Mean
0.795851	14.10403	0.073193	0.518947

Source	DF	Type I SS	Mean Square	F Value	Pr > F
media	4	0.29237895	0.07309474	13.64	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
media	4	0.29237895	0.07309474	13.64	<.0001

Duncan's Multiple Range Test for frondsizeG2

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	0.005357
Harmonic Mean of Cell Sizes	1.538462

Number of Means	2	3	4	5
Critical Range	.1790	.1876	.1928	.1964

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	media
A	0.57500	8	Ho
A	0.57000	8	Hut
B	0.25000	1	nw2
B	0.25000	1	nw1
B	0.20000	1	con

Appendix 3. Statistical analysis (continue)

Dependent Variable: divisiontimeG3

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	21.09742647	10.54871324	80.10	<.0001
Error	14	1.84375000	0.13169643		
Corrected Total	16	22.94117647			

R-Square	Coeff Var	Root MSE	divisiontimeG3 Mean
0.919631	10.19719	0.362900	3.558824

Source	DF	Type I SS	Mean Square	F Value	Pr > F
media	2	21.09742647	10.54871324	80.10	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
media	2	21.09742647	10.54871324	80.10	<.0001

Duncan's Multiple Range Test for divisiontimeG3

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	0.131696
Harmonic Mean of Cell Sizes	2.4
Number of Means	2 3
Critical Range	.7105 .7445

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	media
A	8.0000	1	nw2
B	3.3750	8	Ho
B	3.1875	8	Hut

Appendix 3. Statistical analysis (continue)

Dependent Variable: productionratepH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	495.1523810	82.5253968	979.11	<.0001
Error	14	1.1800000	0.0842857		
Corrected Total	20	496.3323810			

R-Square	Coeff Var	Root MSE	productionratepH Mean
0.997623	6.881174	0.290320	4.219048

Source	DF	Type I SS	Mean Square	F Value	Pr > F
pH	6	495.1523810	82.5253968	979.11	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
pH	6	495.1523810	82.5253968	979.11	<.0001

Duncan's Multiple Range Test for productionratepH

Alpha	0.05					
Error Degrees of Freedom	14					
Error Mean Square	0.084286					
Number of Means	2	3	4	5	6	7
Critical Range	.5084	.5327	.5477	.5579	.5651	.5703

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	pH
A	10.3000	3	6
A	9.8333	3	5
B	9.2000	3	7
C	0.9667	3	8
C	0.6000	3	9
D	-0.6667	3	4
D	-0.7000	3	10

Appendix 3. Statistical analysis (continue)

Dependent Variable: frondsizepH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	0.14480311	0.02413385	3.59	0.0034
Error	79	0.53055619	0.00671590		
Corrected Total	85	0.67535930			

R-Square	Coeff Var	Root MSE	frondsizepH Mean
0.214409	30.20897	0.081951	0.271279

Source	DF	Type I SS	Mean Square	F Value	Pr > F
pH	6	0.14480311	0.02413385	3.59	0.0034

Source	DF	Type III SS	Mean Square	F Value	Pr > F
pH	6	0.14480311	0.02413385	3.59	0.0034

Duncan's Multiple Range Test for frondsizepH

Alpha	0.05
Error Degrees of Freedom	79
Error Mean Square	0.006716
Harmonic Mean of Cell Sizes	9.639344

Number of Means	2	3	4	5	6	7
Critical Range	.07430	.07818	.08075	.08263	.08408	.08525

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	pH
A	0.31533	15	7
A	0.30067	15	5
A	0.29933	15	6
A	0.25400	15	8
A	0.23733	15	9
A	0.23286	7	4
B	0.15000	4	10

Appendix 3. Statistical analysis (continue)

Dependent Variable: productionratedensity

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	123.4207958	17.6315423	381.22	<.0001
Error	16	0.7400000	0.0462500		
Corrected Total	23	124.1607958			

R-Square	Coeff Var	Root MSE	productionratedensity Mean
0.994040	3.758389	0.215058	5.722083

Source	DF	Type I SS	Mean Square	F Value	Pr > F
density	7	123.4207958	17.6315423	381.22	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
density	7	123.4207958	17.6315423	381.22	<.0001

Duncan's Multiple Range Test for productionratedensity

Alpha 0.05

Error Mean Square 0.04625

Number of Means	2	3	4	5	6	7	8
Critical Range	.3722	.3903	.4017	.4094	.4150	.4192	.4224

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	density
A	8.4667	3	10%
B A	8.2233	3	20%
B	7.9067	3	5%
C	6.0133	3	30%
D	5.6000	3	1%
E	4.2300	3	40%
F	3.5633	3	0.50%
G	1.7733	3	0.10%

Appendix 3. Statistical analysis (continue)

Dependent Variable: frondsizedensity

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.14507500	0.02072500	23.42	<.0001
Error	72	0.06372000	0.00088500		
Corrected Total	79	0.20879500			

R-Square	Coeff Var	Root MSE	frondsizedensity Mean
0.694820	8.953785	0.029749	0.332250

Source	DF	Type I SS	Mean Square	F Value	Pr > F
density	7	0.14507500	0.02072500	23.42	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
density	7	0.14507500	0.02072500	23.42	<.0001

Duncan's Multiple Range Test for frondsizedensity

Alpha 0.05

Error Degrees of Freedom 72

Error Mean Square 0.000885

Number of Means	2	3	4	5	6	7	8
Critical Range	02652	02790	.02882	.02949	.03000	.03042	.03076

Means with the same letter are not significantly different.

Duncan Grouping		Mean	N	density
	A	0.39600	10	0.10%
B	A	0.38400	10	0.50%
	B	0.36700	10	1%
	C	0.32900	10	5%
D	C	0.31900	10	10%
D	E	0.30000	10	20%
F	E	0.29100	10	30%
	F	0.27200	10	40%

Appendix 3. Statistical analysis (continue)

Dependent Variable: ProductionRate

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	35	1719.196933	49.119912	554.01	<.0001
Error	72	6.383733	0.088663		
Corrected Total	107	1725.580667			

R-Square	Coeff Var	Root MSE	ProductionRate Mean
0.996301	3.666534	0.297763	8.121111

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	35	1719.196933	49.119912	554.01	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	35	1719.196933	49.119912	554.01	<.0001

Duncan's Multiple Range Test for ProductionRate

Alpha	0.05
Error Degrees of Freedom	72
Error Mean Square	0.088663

Number of Means	2	3	4	5	6	7	8	9	10	11	12
Critical Range	.4847	.5099	.5266	.5388	.5483	.5558	.5621	.5673	.5718		

Number of Means	13	14	15	16	17	18	19
Critical Range	.5757	.5791	.5821	.5848	.5872	.5893	.5913

Number of Means	20	21	22	23	24	25	26	27	28
Critical Range	.5930	.5946	.5961	.5974	.5986	.5997	.6007	.6016	.6025

Number of Means	29	30	31	32	33	34	35	36
Critical Range	.6033	.6040	.6047	.6053	.6059	.6064	.6069	.6073	.6077	.6081

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	trt
A	14.2900	3	31
B	13.6967	3	19
C B	13.3233	3	32
C D	13.1133	3	35
E D	12.7800	3	30
E F	12.6000	3	20
G F	12.2033	3	23
G	12.0433	3	18
G	11.9200	3	36
H	11.3367	3	34
I H	10.9200	3	27
I	10.5433	3	15
I	10.4900	3	24
I J	10.4367	3	28
K J	9.9733	3	22
K	9.7600	3	16
K L	9.5500	3	26
M L	9.1700	3	14
M N	8.7700	3	29
N	8.3867	3	17
N	8.3767	3	33
O	7.5900	3	21
O	7.1433	3	25
P	6.3433	3	13
Q	5.2067	3	7
Q	4.7867	3	8

Means with the same letter are not significantly different.

Duncan Grouping		Mean	N	trt
	R	4.1467	3	11
	R	3.8733	3	12
	R	3.8100	3	3
	S	3.1600	3	4
	T	2.4967	3	6
U	T	2.3167	3	2
U	T	2.1433	3	10
U	T	2.1333	3	5
U	V	1.9333	3	9
	V	1.5933	3	1

Appendix 3. Statistical analysis (continue)

Dependent Variable: wetwtculturesystems7d

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	15218.56277	3804.64069	25.86	<.0001
Error	10	1471.37480	147.13748		
Corrected Total	14	16689.93757			

R-Square	Coeff Var	Root MSE	wetwtculturesystems7d Mean
0.911841	4.424034	12.13002	274.1847

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culturesystems	4	15218.56277	3804.64069	25.86	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culturesystems	4	15218.56277	3804.64069	25.86	<.0001

Duncan's Multiple Range Test for wetwtculturesystems7d

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	147.1375

Number of Means	2	3	4	5
Critical Range	22.07	23.06	23.65	24.02

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	culturesystems
A	324.233	3	horizont
B	290.810	3	spraying
C	267.260	3	vertical
C	259.453	3	static
D	229.167	3	Layer

Appendix 3. Statistical analysis (continue)

Dependent Variable: wetwtculturesystems14d

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	69357.13413	17339.28353	10.51	0.0013
Error	10	16492.12527	1649.21253		
Corrected Total	14	85849.25940			

R-Square	Coeff Var	Root MSE	wetwtculturesystems14d Mean
0.807894	9.581111	40.61050	423.8600

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culturesystems	4	69357.13413	17339.28353	10.51	0.0013

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culturesystems	4	69357.13413	17339.28353	10.51	0.0013

Duncan's Multiple Range Test for wetwtculturesystems14d

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	1649.213

Number of Means	2	3	4	5
Critical Range	73.88	77.21	79.16	80.41

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	culturesystems
A	524.15	3	horizont
B A	468.42	3	spraying
B C	419.23	3	vertical
D C	378.49	3	static
D	329.01	3	Layer

Appendix 3. Statistical analysis (continue)

Dependent Variable: wetwtculturesystems21d

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	230626.5691	57656.6423	9.07	0.0023
Error	10	63547.6189	6354.7619		
Corrected Total	14	294174.1880			

R-Square	Coeff Var	Root MSE	wetwtculturesystems21d Mean
0.783980	10.88902	79.71676	732.0840

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culturesystems	4	230626.5691	57656.6423	9.07	0.0023

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culturesystems	4	230626.5691	57656.6423	9.07	0.0023

Duncan's Multiple Range Test for wetwtculturesystems21d

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	6354.762

Number of Means	2	3	4	5
Critical Range	145.0	151.6	155.4	157.8

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	culturesystems
A	954.34	3	horizont
B	777.70	3	spraying
C B	662.96	3	vertical
C B	653.53	3	static
C	611.89	3	Layer

Appendix 3. Statistical analysis (continue)

Dependent Variable: wetwtculturesystems28d

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	329015.6691	82253.9173	6.47	0.0077
Error	10	127113.7833	12711.3783		
Corrected Total	14	456129.4524			

R-Square	Coeff Var	Root MSE	wetwtculturesystems28d Mean
0.721321	13.74215	112.7447	820.4300

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culturesystems	4	329015.6691	82253.9173	6.47	0.0077

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culturesystems	4	329015.6691	82253.9173	6.47	0.0077

Duncan's Multiple Range Test for wetwtculturesystems28d

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	12711.38

Number of Means	2	3	4	5
Critical Range	205.1	214.3	219.8	223.2

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	culturesystems
A	1073.46	3	horizont
B A	877.90	3	spraying
B C	783.03	3	static
B C	726.62	3	vertical
C	641.14	3	Layer

Appendix 3. Statistical analysis (continue)

Dependent Variable: drywtculturesystems7d

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	24.35956000	6.08989000	25.79	<.0001
Error	10	2.36120000	0.23612000		
Corrected Total	14	26.72076000			

R-Square	Coeff Var	Root MSE	drywtculturesystems7d Mean
0.911634	4.431167	0.485922	10.96600

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culturesystems	4	24.35956000	6.08989000	25.79	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culturesystems	4	24.35956000	6.08989000	25.79	<.0001

Duncan's Multiple Range Test for drywtculturesystems7d

Alpha	0.05			
Error Degrees of Freedom	10			
Error Mean Square	0.23612			
Number of Means	2	3	4	5
Critical Range	.8840	.9238	.9472	.9622

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	culturesystems
A	12.9700	3	horizont
B	11.6300	3	spraying
C	10.6867	3	vertical
C	10.3767	3	static
D	9.1667	3	Layer

Appendix 3. Statistical analysis (continue)

Dependent Variable: drywtculturesystems14d

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	111.0342400	27.7585600	10.53	0.0013
Error	10	26.3623333	2.6362333		
Corrected Total	14	137.3965733			

R-Square	Coeff Var	Root MSE	drywtculturesystems14d Mean
0.808130	9.576409	1.623648	16.95467

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culturesystems	4	111.0342400	27.7585600	10.53	0.0013

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culturesystems	4	111.0342400	27.7585600	10.53	0.0013

Duncan's Multiple Range Test for drywtculturesystems14d

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	2.636233

Number of Means	2	3	4	5
Critical Range	2.954	3.087	3.165	3.215

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	culturesystems
A	20.967	3	horizont
B A	18.740	3	spraying
B C	16.767	3	vertical
D C	15.140	3	static
D	13.160	3	Layer

Appendix 3. Statistical analysis (continue)

Dependent Variable: drywttculturesystems21d

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	368.9242000	92.2310500	9.07	0.0023
Error	10	101.6473333	10.1647333		
Corrected Total	14	470.5715333			

R-Square	Coeff Var	Root MSE	drywttculturesystems21d Mean
0.783992	10.88748	3.188218	29.28333

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culturesystems	4	368.9242000	92.2310500	9.07	0.0023

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culturesystems	4	368.9242000	92.2310500	9.07	0.0023

Duncan's Multiple Range Test for drywttculturesystems21d

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	10.16473

Number of Means	2	3	4	5
Critical Range	5.800	6.061	6.215	6.313

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	culturesystems
A	38.173	3	horizont
B	31.107	3	spraying
C B	26.517	3	vertical
C B	26.143	3	static
C	24.477	3	Layer

Appendix 3. Statistical analysis (continue)

Dependent Variable: drywtculturesystems28d

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	526.4789333	131.6197333	6.48	0.0077
Error	10	203.1788000	20.3178800		
Corrected Total	14	729.6577333			

R-Square	Coeff Var	Root MSE	drywtculturesystems28d Mean
0.721542	13.73551	4.507536	32.81667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culturesystems	4	526.4789333	131.6197333	6.48	0.0077

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culturesystems	4	526.4789333	131.6197333	6.48	0.0077

Duncan's Multiple Range Test for drywtculturesystems28d

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	20.31788

Number of Means	2	3	4	5
Critical Range	8.200	8.569	8.787	8.925

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	culturesystems
A	42.940	3	horizont
B A	35.113	3	spraying
B C	31.320	3	static
B C	29.063	3	vertical
C	25.647	3	Layer

Appendix 3. Statistical analysis (continue)

Dependent Variable: productionrateculturesystems7d

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.49704000	0.12426000	26.14	<.0001
Error	10	0.04753333	0.00475333		
Corrected Total	14	0.54457333			

R-Square	Coeff Var	Root MSE	productionrateculturesystems7d
0.912715	10.37278	0.068944	0.664667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	4	0.49704000	0.12426000	26.14	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	4	0.49704000	0.12426000	26.14	<.0001

Duncan's Multiple Range Test for productionrateculturesystems7d

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.004753

Number of Means	2	3	4	5
Critical Range	.1254	.1311	.1344	.1365

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	trt
A	0.95000	3	horizont
B	0.76000	3	spraying
C	0.62667	3	vertical
C	0.58000	3	static
D	0.40667	3	Layer

Appendix 3. Statistical analysis (continue)

Dependent Variable: productionrateculturesystems14d

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.57322667	0.14330667	10.49	0.0013
Error	10	0.13666667	0.01366667		
Corrected Total	14	0.70989333			

R-Square	Coeff Var	Root MSE	productionrateculturesystems14d Mean
0.807483	15.36869	0.116905	0.760667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	4	0.57322667	0.14330667	10.49	0.0013

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	4	0.57322667	0.14330667	10.49	0.0013

Duncan's Multiple Range Test for productionrateculturesystems14d

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.013667

Number of Means	2	3	4	5
Critical Range	.2127	.2222	.2279	.2315

Means with the same letter are not significantly different.

Duncan Grouping		Mean	N	trt
	A	1.05000	3	horizont
B	A	0.89000	3	spraying
B	C	0.74333	3	vertical
D	C	0.63000	3	static
D		0.49000	3	Layer

Appendix 3. Statistical analysis (continue)

Dependent Variable: productionrateculturesystems21d

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.83882667	0.20970667	9.14	0.0023
Error	10	0.22953333	0.02295333		
Corrected Total	14	1.06836000			

R-Square	Coeff Var	Root MSE	productionrateculturesystems21d Mean
0.785154	13.84859	0.151504	1.094000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	4	0.83882667	0.20970667	9.14	0.0023

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	4	0.83882667	0.20970667	9.14	0.0023

Duncan's Multiple Range Test for productionrateculturesystems21d

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.022953

Number of Means	2	3	4	5
Critical Range	.2756	.2880	.2953	.3000

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	trt
A	1.5167	3	horizont
B	1.1833	3	spraying
C B	0.9633	3	vertical
C B	0.9433	3	static
C	0.8633	3	Layer

Appendix 3. Statistical analysis (continue)

Dependent Variable: productionrateculturesystems28d

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.67989333	0.16997333	6.57	0.0073
Error	10	0.25866667	0.02586667		
Corrected Total	14	0.93856000			

R-Square	Coeff Var	Root MSE	productionrateculturesystems28d Mean
0.724401	17.00118	0.160831	0.946000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culturesystems	4	0.67989333	0.16997333	6.57	0.0073

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culturesystems	4	0.67989333	0.16997333	6.57	0.0073

Duncan's Multiple Range Test for productionrateculturesystems28d

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.025867

Number of Means	2	3	4	5
Critical Range	.2926	.3058	.3135	.3185

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	culturesystems
A	1.3100	3	horizont
B A	1.0267	3	spraying
B C	0.8933	3	static
B C	0.8133	3	vertical
C	0.6867	3	Layer

Appendix 3. Statistical analysis (continue)

Dependent Variable: frondsizeculturesystems14d

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.01382667	0.00345667	3.65	0.0440
Error	10	0.00946667	0.00094667		
Corrected Total	14	0.02329333			

R-Square	Coeff Var	Root MSE	frondsizeculturesystems14d Mean
0.593589	7.553506	0.030768	0.407333

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culturesystems	4	0.01382667	0.00345667	3.65	0.0440

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culturesystems	4	0.01382667	0.00345667	3.65	0.0440

Duncan's Multiple Range Test for frondsizeculturesystems14d

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.000947

Number of Means	2	3	4	5
Critical Range	.05597	.05849	.05998	.06092

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	culturesystems
A	0.44333	3	spraying
A	0.43000	3	layer
B A	0.41333	3	horizont
B A	0.39333	3	vertical
B	0.35667	3	static

Appendix 3. Statistical analysis (continue)

Dependent Variable: frondsizeculturesystems21d

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.01356000	0.00339000	7.37	0.0049
Error	10	0.00460000	0.00046000		
Corrected Total	14	0.01816000			

R-Square	Coeff Var	Root MSE	frondsizeculturesystems21d Mean
0.746696	5.892201	0.021448	0.364000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
culturesystems	4	0.01356000	0.00339000	7.37	0.0049

Source	DF	Type III SS	Mean Square	F Value	Pr > F
culturesystems	4	0.01356000	0.00339000	7.37	0.0049

Duncan's Multiple Range Test for frondsizeculturesystems21d

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	0.00046

Number of Means	2	3	4	5
Critical Range	.03902	.04077	.04181	.04247

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	culturesystems
A	0.41000	3	layer
B A	0.38000	3	horizont
B C	0.36667	3	vertical
C	0.33667	3	static
C	0.32667	3	spraying

BIOGRAPHY

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