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นางสาวรัศมี หวะสุวรรณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CLASSIFICATION AND NITROGEN FIXATION EFFICIENCY ANALYSIS OF *AZOLLA* SPECIES IN RICE FIELDS

Rudsamee Wasuwan

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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CLASSIFICATION AND NITROGEN FIXATION EFFICIENCY ANALYSIS OF *AZOLLA* SPECIES IN RICE FIELDS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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CLASSIFICATION/ NITROGEN FIXATION/ AZOLLA/ RICE

The classification of *Azolla* (Azollaceae) has been quite complicated and continued for a long time. Because most taxonomies of *Azolla* (Azollaceae) focus primarily on reproductive structures which are rarely present in nature and unclear in some species, this study aimed to classify *Azolla* species by observing their morphology through stereo microscope, SEM and DNA analysis. Three isolated *Azolla* (AZO1, AZO2 and AZO3) were collected from the two ponds: one in the university's farm and the other in the organic farm, Suranaree University of Technology. The morphological study which was based on diameters of vegetative, epidermal trichomes and float number in megasporocarp found that the three species of *Azolla* were further classified as follows: AZ01 as *A. microphylla*, AZO2 as *A. cristata* and AZO3 as *A. filiculoides*. Then, DNA sequences (18S rDNA and ITS region) were investigated. The alignment sequencing (18S rDNA) indicated that AZO1 had 99.2% homology with *Azolla* sp. Qiu 02051, AZO2 had 99.6% homology with *A. filiculoides* and AZO3 had 99.4% homology with *A. filiculoides*. However, 18S rDNA could not be used to classify deep down to species level for AZO2, so the ITS region was used for more specific results. From the sequencing of ITS region, it was indicated that AZO1 had 99.3% homology with *A. microphylla*, AZO2 had 99.0%

homology with *A. mexicana* and AZO3 had 99.2% homology with *A. filiculoides*. The results indicated that the ITS region identification corresponded with the morphological study. so the molecular method using ITS region was needed. Furthermore, the application of *Azolla* species as biofertilizer in the rice field has been evaluated by comparing with the chemical fertilizer (12-8-8 kg $N\text{-}P_2O_5\text{-}K_2O$/rai or 0.075-0.05-0.05t $N\text{-}P_2O_5\text{-}K_2O$ / ha). The highest grain yield (4.97 t/ha or 795.2 kg/rai) obtained from the rice field incorporated with AZO1 (*A. microphylla*), did not significantly differ from the chemical fertilizer. Nevertheless, the grain yield (16.72%) obtained from AZO1 was higher than that from chemical fertilizer (12.28%). Therefore, *A. microphylla* could be used as biofertilizer with the same result as chemical fertilizer.

School of Biotechnology

Academic Year 2008

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รัศมี หวะสุวรรณ : การจำแนกสายพันธุ์และการวิเคราะห์หาประสิทธิภาพการตรึงไนโตรเจนของแหนแดงในนาข้าว (CLASSIFICATION AND NITROGRN FIXATION EFFICIENCY ANALYSIS OF AZOLLA SPECIES IN RICE FIELDS) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.โชคชัย วนภู, 86 หน้า

การจัดจำแนกสายพันธุ์ของแหนแดงมีมาอย่างยาวนานและค่อนข้างมีความซับซ้อน โดยส่วนมากจะเน้นการศึกษาลักษณะโครงสร้างของสปอร์ซึ่งหาได้ยากในธรรมชาติ วัตถุประสงค์ของการศึกษาในครั้งนี้เพื่อจำแนกสายพันธุ์โดยศึกษาทั้งลักษณะทางสัณฐานวิทยาและการวิเคราะห์ดีเอ็นเอ โดยคัดเลือกแหนแดงได้จากบ่อภายในฟาร์มและสวนเกษตรอินทรีย์ มหาวิทยาลัยเทคโนโลยีสุรนารีทั้งหมด 3 สายพันธุ์ (AZO1 AZO2 และ AZO3) โดยศึกษาลักษณะทางสัณฐานวิทยาและการวิเคราะห์ดีเอ็นเอ ในส่วนของสปอร์เพศเมีย (megaspore) จะพบทุ่นลอย (float) ซึ่งเป็นโครงสร้างที่มีความสำคัญที่ใช้ในการจำแนกร่วมกับการศึกษาขนาดของใบและลักษณะของขนใบ (trichome) ผลของการจำแนกโดยศึกษาจากลักษณะทางสัณฐานวิทยาพบว่าแหนแดงทั้ง 3 สายพันธุ์อยู่ใน section *Azolla* AZO1 คือสายพันธุ์ *A. microphylla*, AZO2 คือสายพันธุ์ *A. cristata* และ AZO3 คือ สายพันธุ์ *A. filiculoides* สำหรับการวิเคราะห์ลำดับดีเอ็นเอ ใช้ดีเอ็นเอที่สกัดจากรากของแหนแดงเป็นดีเอ็นเอต้นแบบในการเพิ่มปริมาณดีเอ็นเอร่วมกับ 18S rDNA และ ITS region จากนั้นนำชิ้นส่วนของดีเอ็นเอที่ได้ไปวิเคราะห์หาลำดับเบส โดยนำมาเปรียบเทียบข้อมูลลำดับเบสของแหนแดงใน GenBank พบว่าการใช้ 18S rDNA ให้ผลลำดับเบสของ AZO1 มีความใกล้เคียงกับ *Azolla* sp. Qiu 02051 99.2% AZO2 มีความใกล้เคียงกับ *A. filiculoides* 99.6% และ AZO3 มีความใกล้เคียงกับ *A. filiculoides* 99.4% ผลจากการใช้ 18S rDNA ไม่สามารถจำแนก AZO2 ในระดับสปีชีส์ได้ ดังนั้น จึงนำ ITS region มาใช้เพื่อให้ได้ผลการจำแนกที่จำเพาะเจาะจงขึ้น จากการใช้ ITS ให้ผลลำดับเบสของ AZO1 มีความใกล้เคียงกับ *A. microphylla* 99.3% AZO2 ใกล้เคียงกับ *A. mexicana* 99.0% และ AZO3 มีความใกล้เคียงกับ *A. filiculoides* 99.2% ดังนั้นการใช้วิธีวิเคราะห์ดีเอ็นเอโดยการใช้ ITS region จึงเป็นวิธีที่สามารถจำแนกสายพันธุ์ของแหนแดงได้อย่างชัดเจน

สำหรับการใช้แหนแดงเพื่อเป็นปุ๋ยชีวภาพในนาข้าว เปรียบเทียบผลของการใช้แหนแดงแต่ละสายพันธุ์กับการใช้ปุ๋ยเคมี (12-8-8 กก. N-P_2O_5-K_2O/ไร่ หรือ 0.075-0.05-0.05 ตัน N-P_2O_5-K_2O/เฮกแทร์) ผลผลิตของเมล็ดสูงที่สุดคือ 4.97 ตัน/เฮกแทร์ (795.2 กก./ไร่) ซึ่งพบในแปลงข้าวที่ปลูกร่วมกับการไถกลบแหนแดงสายพันธุ์ AZO1 (*A. microphylla*) พบว่าไม่มีความแตกต่างอย่างมีนัยสำคัญกับการใช้ปุ๋ยเคมี โดยผลผลิตที่ได้จากการใช้แหนแดงสายพันธุ์นี้มีผลผลิตที่เพิ่มสูงขึ้นกว่าการใช้ปุ๋ยเคมีอย่างมีนัยสำคัญ จากการทดลองนี้สรุปได้ว่าแหนแดงสายพันธุ์

A. microphylla เป็นแหนแดงสายพันธุ์ที่ดีที่สุดที่จะใช้เป็นปุ๋ยในนาข้าวโดยเพิ่มผลผลิตได้เทียบเท่ากับการใช้ปุ๋ยเคมี

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2551 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

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Rudsamee Wasuwan

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

ADP	=	adenosine diphosphate
ARA	=	acethylene reduction assay
ATP	=	adenosine triphosphate
°C	=	degree celcius
cm	=	centrimeter
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxynucleosine 5'phosphate
Exch. K	=	exchange potassium
Extr. P	=	extract phosphorus
g	=	gram
GDH	=	glutamate dehydrogenase
GS	=	glutamine synthetase
h	=	hour
ha	=	hectare
ITS	=	internal transcribed spacer
kg	=	kilogram
l	=	liter
µl	=	microliter
m	=	meter
M	=	molarity

LIST OF ABBREVIATIONS (Continued)

Min	=	minute
ml	=	milliliter
OM	=	organic matter
PCR	=	polymerase chain reaction
ppm	=	part per million
RBCD	=	randomized complete block design
rpm	=	round per minute
rRNA	=	ribosomal ribonucleic acid
s	=	second
sect.	=	section
SEM	=	scanning electron microscope
sp.	=	species
SSU	=	small sub unit
subsp.	=	subspecies
t	=	ton
wt.	=	weigh

CHAPTER I

INTRODUCTION

1.1 Significant of this study

Azolla is a free-floating water fern which in symbiotic association with the cyanobacterium-*Anabaena azollae* contributes substantial amount of biologically fixed nitrogen to the rice crop. The nitrogen fixing water fern, *Azolla* has been used as a biofertilizer for rice in India, China, Vietnam, Thailand, Philippines, Korea, Sri Lanka, Bangladesh, Pakistan, Nepal, Burma, Indonesia, Brazil and West Africa. The technology of utilizing the nitrogen fixing water fern *Azolla* has been attempted by these countries but the full potential of this biological system has not been exploited. The *Azolla-Anabaena* association is a living floating nitrogen manufacturing factory, which is utilizing the energy from photosynthesis to fix atmospheric nitrogen at the rate of 150-200 kg / hectare / year. *Azolla* is used as biofertilizer for rice crop and it could contribute 40-60 kg N /ha/ crop (Kannaiyan, 1979).

The important factor in using *Azolla* as biofertilizer for rice crop is its quick decomposition in soil and efficient availability of its nitrogen to rice plants. In tropical rice soils, the applied *Azolla* mineralizes rapidly and its nitrogen is made available to the rice crop in very short period.

All species of *Azolla* are aquatic ferns distributed throughout the temperate and tropical regions. Classification of this genus varies widely but the most commonly

accepted system is that of Svenson (1944), who recognized seven species in two sections (sects. *Azolla* and *Rhizosperma*) on the basis of morphological discontinuities.

Difficulties in the identification and consequently the classification of *Azolla* stem from the phenotypic similarity among all seven species, particularly when sterile. Most taxonomic system focus primarity on reproductive characters of *Azolla*, but these structures are rarely present in nature and are often difficult to induce in culture.

Long history of agricultural use, *Azolla* taxonomy is controverted and knowledge on the subject is still limited, which probably mean is that a new global revision of the taxon is required. The whole taxonomy of pteridophyta shows modern systematics can not just use traditional morphological characters but has to appeal in a compulse way to different complementary areas such as electron microscopy, phytochemistry and DNA analysis.

Therefore, this study aims to classify *Azolla* species according to morphology by using scanning electron microscope. Moreover, DNA analyses on each species were employed. Application of *Azolla* species was also tested as biofertilizer in rice fields

1.2 Research objectives

1.2.1 To classify *Azolla* according to morphology and DNA analysis

1.2.2 To study *Azolla* species as biofertilizer in rice field

1.3 Scope and limitation of the study

Azolla species were classified on the basis morphology of reproductive and vegetative structures. Morphology of all *Azolla* species was considered under stereo microscope and electron microscope. DNA analysis of *Azolla* species was performed.

The *Azolla* species were applied in rice field and N_2 fixation efficiency in each species, growth and rice yield- were determined.

1.4 Expected results

1.4.1 *Azolla* species can be clearly classified by using both morphology and DNA analysis.

1.4.2 Application of *Azolla* to rice field can increase rice growth, yield and soil nutrients.

CHAPTER II

LITERATURE REVIEW

2.1 *Azolla*

Azolla is a genus of small aquatic fern that is native to Asia, Africa and America. *Azolla* species live in lakes, swamps, streams and other small bodies of water, and have been dispersed by man and natural means to various parts of the world. Some are strictly tropical or sub-tropical in nature, while other grow and thrive in either temperature or tropical climates.

Azolla has been of interest to botanists for years because of its symbiotic relationship with a nitrogen-fixing cyanobacterium, *Anabaena*. Recently it has come under scrutiny in developed countries as a potential green manure for flooded crops particularly rice. The most remarkable feature of *Azolla* is its symbiotic relationship with the cyanobacterium *Anabaena azollae*. The delicated fern provides nutrient and a protective leaf cavity for the *Anabaena*, which in turn provides nitrogen for the fern. Under suitable field condition *Azolla* can double in weight every 3-5 days and fix atmospheric nitrogen at a rate exceeding that of the legume/*Rhizobium* symbiotic relationship.*Azolla* can accumulate 2-4 or more kilograms (kg) of nitrogen/hectare/day (equivalent to 10-20 kg of ammonium sulfate) and since the *Azolla*_anabaena symbiosis grows in aquatic condition, it can provide a potential nitrogen source for flooded crops such as rice.

The nitrogen fertilizer fixed by *Azolla* becomes available to the rice after the *Azolla* mat is incorporated into the soil and its nitrogen begins to be released through decomposition.

2.2 Reproductive of *Azolla*

2.2.1 Sexual reproduction

Azolla usually reproduces vegetatively by fragmentation of the abscission layer, at the base of each branch. Sexual reproduction is not very common and seems to be influenced by environmental factors, namely several stress condition. Sporocarps are visible in pairs (in *A. nilotica* in four) in the place of the first leaf lower lobe of a sporophyte branch. They occur in pairs of either microsporocarps, megasporocarps or one of each. Mature microsporocarps are globular and endclose numerous stalked microsporangia, each one with 32 or 64 microspores, divided into 3 to 10 complex structures, called massulae. In subgenus *Azolla* each massulae shows characteristic arrow like projections these called glochidia. In subgenus *Rhizosperma* glochidia are filiform internal or do not exist. At maturation microsporangia releases massulae, which may be dragged underwater. Microspores germinate inside the massulae and flagellated antherozoid move through the gelatinized massulae to fertilize oospore within the archegonia (Braun-Howland and Nierzwicki-Bauer, 1990).

Megasporocarps are much smaller than microsporocarps and have two distinct parts (Figure 2.1). The upper is filled by the so called floats, 3 in subgenus *Azolla* and 9 in subgenus *Rhizosperma*. In its lower portion each megasporocarp contains only one megaspore. This germinates under water and a prothallus is formed producing archegonia. Antherozoids will fertilize the only egg cell existing in each archegonium and the zygote formation occurs within the megaspore apparatus below

the water surface and will give rise to a new sporophyte (Braun-Howland and Nierzwicki-Bauer, 1990).

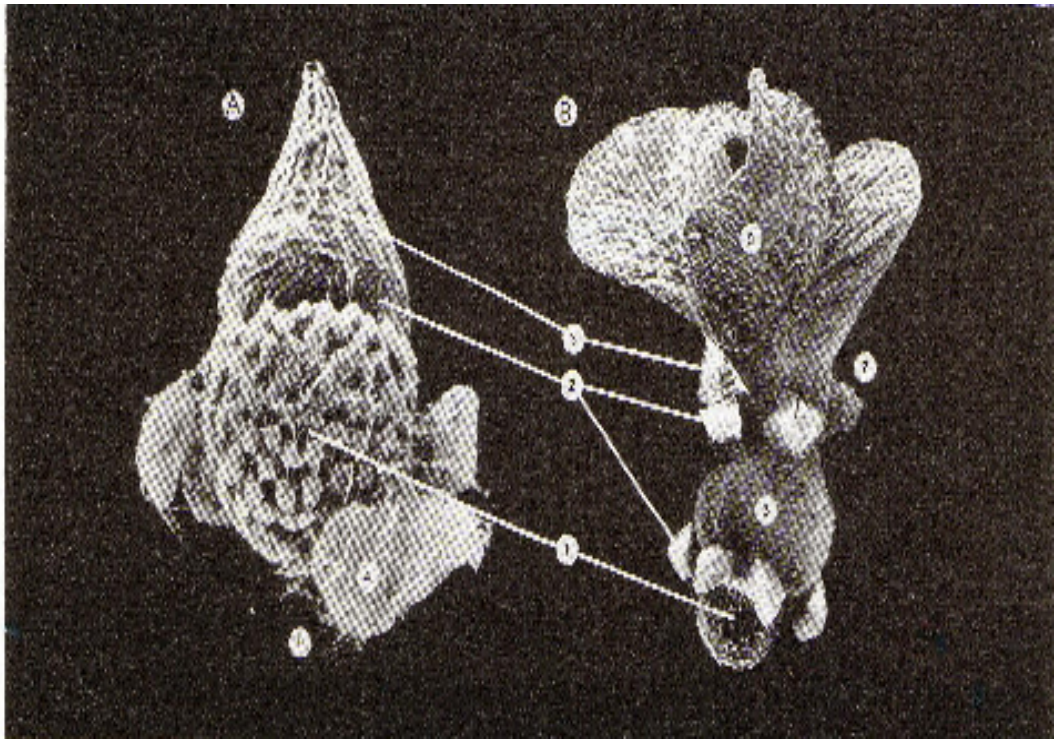

Figure 2.1 A. megasporocarp of *A. filiculoides* covered with massulae (83x)B.Young plant of *A. pinnata* emerging from a megasporocarp (35x) 1. Megaspore; 2. Float; 3. Indusium; 4. Massulae; 5. Glochidium; 6. Prothallus; 7.Root; 8. Leaf (Adapted from van Hove, 1989)

2.2.2 Vegetative reproduction

Vegetative reproduction of *Azolla* is an extremely efficient process. The sporophyte of the fern reproduces by fragmentation via an abscission layer that forms at the base of each branch. Since roots develop in fixed sequence along with leaves on each branch, the separating fragments are autonomous. They then drift away from the

parent plant and become independent. The plant is almost fully grown in 15 to 20 days (Lumpkin and Plucknett, 1982). Under favorable conditions populations of some species can double in weight in 3 to 5 days.

2.3 Classification of *Azolla*

2.3.1 Morphology

Azolla is a genus of aquatic ferns, mainly found in tropical and warm temperate regions. It can be observed in quiet water, ponds, ditches, canals and paddy fields. Such areas may be seasonally covered by a mat of *Azolla* associated with other free-floating plant such as species of *Lemma, Pistia*, *Salvinia, Trapa, Wolffia* and mud rooting species of *Cerathophyllum, Ludwigia, Neptunia and Polygonum.*

The genus has been established in1783 by Lamarck and has been placed in the Salviniaceae along with the genus *Salvinia* (Svenson, 1944). Since then, it has been placed in the Marsileaceae by R. Brown, in 1810 (Svenson, 1944). The first to consider *Azolla* in the monotypic family Azollaceae was Wettstein, in 1903 but only with Reed, in 1954, such proposal has begun to be accepted and followed (Ashton and Walmsley, 1984). The species of *Azolla* are divided into two subgenera: *Azolla* and *Rhizosperma* (Ashton and Walmsley, 1984). This separation is based in the floats number and in the massulae glochidia. All species with three floats in the megasporocarp and arrow-shaped glochidia belong to the subgenus *Azolla.* The specific classification usually accepted recognizes five species integrated in the subgenus *Azolla* which are: *A. caroliniana, A. filiculoides, A. mexicana, A. microphylla and A. rubra.* Subgenus *Rhizosperma* includes the species with nine floats in the megasporocarp and absent glochidia or with glochidia inside the massulae. It comprises two species: *A. nilotica* and *A. pinnata* with latter having three

subspecies: *A. pinnata* subsp. *africana*, *A. pinnata* subsp. *asiatica* and *A. pinnata* subsp. *pinnata*. Table 2.1 presents the last classification proposed by Saunders and Fowler (1993) and followed by Diniz and Carrapico (1994).

Table 2.1 Synopsis of the classification of *Azolla*. (Saunders and Fowler, 1993)

Division	Pteridophyta		
Class	Filicopsida		
Order	Salviniales		
Family	Azollaceae		
Genus	*Azolla*		
Subgenus	*Azolla*		*Tetrasporocarpia*
Sections	*Azolla*	*Rhizosperma*	
Species	*A. caroliniana*	*A. pinnata*	*A. nilotica*
	A. filiculoides		
	A. mexicana		
	A. microphylla		
	A. rubra		
Subspecies		*A. pinnata subsp. africana*	
		A. pinnata subsp. asiatica	
		A. pinnata subsp. pinnata	

Species classification within the section *Azolla* is based upon details of the megasporocarp structure, including scanning electron microscope. Perkin et al., (1985) found that scanning electron microscopy raveals distinctive perine or ganization which are constant within taxa and consistently different between taxa. Other features such as

the nature of the collar, the column, float retention mechanism and distribution and type of leaf hairs (Figure 2.2) might also be important taxonomic characteristics. However many accessions do not develop sporocarps in culture collections, making their taxonomic assignment doubtful. Different studies on the megasporocarp structure have also failed to give a clear species classification within section *Azolla* (Dunham and Fowler, 1987).

The whole taxonomy of pteridophyta shows us that modern systematics can not just use trational morphological characters but has to appeal in a compulse way to different complementary areas such as scanning electronic microscopy, phytochemistry and DNA analysis.

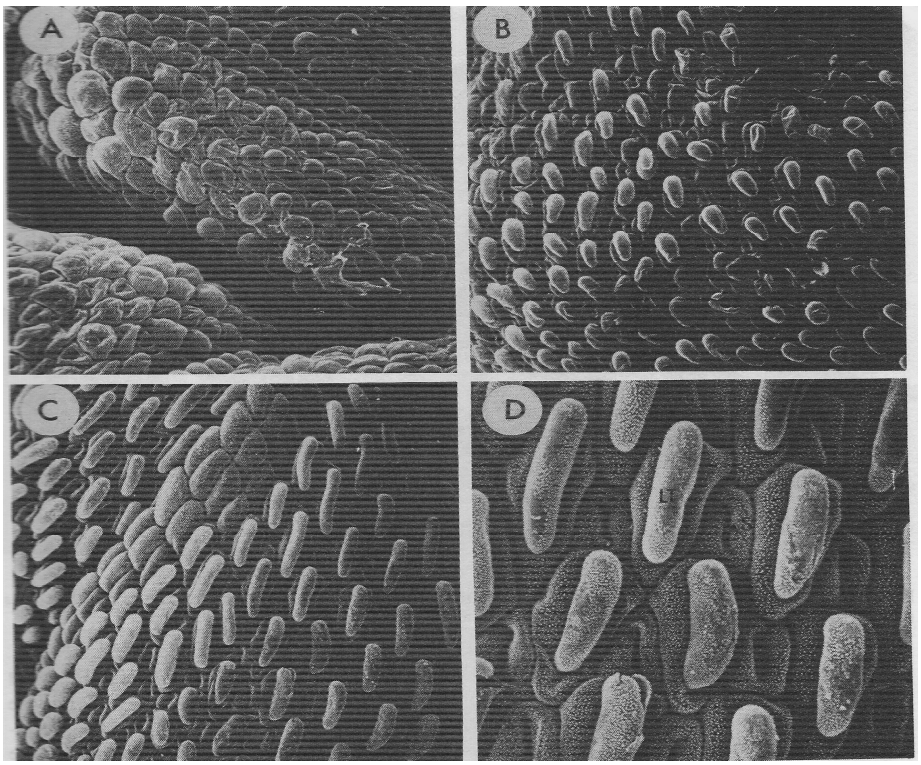

Figure 2.2 Scanning electron micrographs of the *Azolla* leaf surface. Pictures show structural differences of the epidermal trichomes (hairs). Such differences can be used as taxonomic criteria. (A) Micrograph of the *A. filiculoides* leaf surface (magnification X235); (B) leaf trichomes of *A. mexicana* (magnification X 135); (C) leaf trichomes of *A.pinnata*; (D) close up of (C) showing epidermis of a leaf with trichomes and *B.stomata*. LT= leaf trichome, S= stomata. (Martin, 1976)

2.3.2 DNA analysis

For classification with DNA analysis, results using isozyme and restriction fragment length polymorphism (RFLP) analysis have given more consistent results (Zimmerman et al., 1991). Isozyme analysis of 9 enzymes showing 17 polymorphic loci indicated that *A. filiculoides* and *A. rubra* were both chemically unique and recognizable as species, but principal component analysis did not indicate that *A. microphylla, A. caroliniana* and *A. mexicana* were distinct species (Zimmerman et al., 1989). In 1991, Zimmerman et al. screened 30 heterologous DNA clones and a partial genetic library of *A. mexicana* to find DNA probes which would reveal RFLP differences. RFLP analysis confirmed the overall conclusions of the earlier isozyme analysis, and could also differentiate among accession that were enzymatically closely relate.

Amplified PCR products or bands (200 to 4000 bp) were revealed by electrophoresis. These bands are called random amplified polymorphic DNA or RAPD. About 486 RAPDs markers were detected by only 22 primers (10 nucleotides long, 70% G+C content and selected from Operon Random Primer Kit) and were scored as present or absent in each accession. They were suggested that able to detect relatively more polymorphism by random amplification than was detected using isozymes and a limited number of RFLP probes. However because different accession were included in the different analyses, a direct comparison of the efficiency of the different methods awaits a further study

Present studies, DNA sequence data from three noncoding regions, derived from the plastid genome and internal transcribed spacers of the nuclear rRNA genes. Clasidic analyses of these data confirm the division of *Azolla* into two major clades,

corresponding to the traditional classification. Moreover, the monophyly of *A. pinnata* plus *A. nilotica* (sect. *Rhizosperma*) contradicts newer classification of the family, in which these species were placed in different subgenera. In section *Azolla*, DNA sequence data support several past reports in suggesting that *A. rubra* and *A. filiculoides* are distinct species and that *A. caroliniana* is distinct from both *A. microphylla* and *A. mexicana* (Jill et al., 2006).

2.4 *Azolla-Anabaena* symbiosis

2.4.1 Properties of *Anabaena* cells

Anabaena azollae grows as a filament or trichome of cells. Growth leads to elongation of the trichome and to an increase through intercalary cell division in the number of its constituent cells. Reproduction occurs by breakage of the trichome into shorter chains of cells called hormogonia. The trichome is unbranched and uniseriate (one cell in width) since intercalary cell division is confined to a plane at right angles to its long axis (Stanier and Cohen-Bazire, 1977). There are three types of cells: vegetative cells (primary site of photosynthesis), heterocysts (site of N_2-fixation) and akinetes (thick-walled resting spores formed from vegetative cells) (Figure 2.3).

In the vegetative cell the protoplast is always surrounded by a bilayer wall. The cell membrane (plasmalemma) is usually simple in structure (Stanier and Cohen-Bazire, 1977). Much of the protoplast is occupied by the component of the photosynthetic apparatus which is located in a series of membranous sacs or thylakoids (the site of the lipophilic pigment such as chlorophyll *a* and carotenoids as well as centers of the photochemical reaction). *Anabaena* like other cyanobacteria, synthesizes glycogen as a principal nonnitrogenous organic reserve material (Fogg et al., 1973). Cyanobacteria also synthesize cyanophycin, a highly branched polypeptide,

composed of only two amino acids; a polyaspartic acid core bearing arginyl residues attached to all free carboxyl groups of the core (Simon, 1971).

Besides their characteristic shape and size heterocysts can readily be distinguished from the vegetative cells by three structural properties: (1) they are weakly pigmented; (2) they have a thick outer envelope; and (3) near each junction with a vegetative cell they contain a polar granule (Stanier and Cohen-Bazire, 1977). The ability to form heterocysts is associated with a physiological property, the capacity of nitrogen fixation under aerobic conditions (Iyengar and Desikachary, 1953). The heterocyst envelope is composed of three layers and a laminated inner layer (Lang, 1965). As the two inner layers are formed the initially rounded cell pole in contact with the adjacent vegetative cell change into a short, narrow tubular neck around which the two inner envelope layers are thickened.

The heterocysts do not contain the glycolypids that are the major neutral lipids of the membrane of vegetative cell (Walsby and Nichols, 1969). Heterocyst differentiation is subject to inductive control however, not every cell in the trichome is component to give rise to heterocysts (Wilcox, 1970).

Differentiation of akinetes usually occurs adjacent to heterocysts and is accompanied by the formation of thick often pigmented envelopes around the walls of vegetative cells (Clark and Jensen, 1969). The cells as a rule enlarge considerably and may become considerably longer and wider than vegetative cells (Clark and Jensen, 1969).

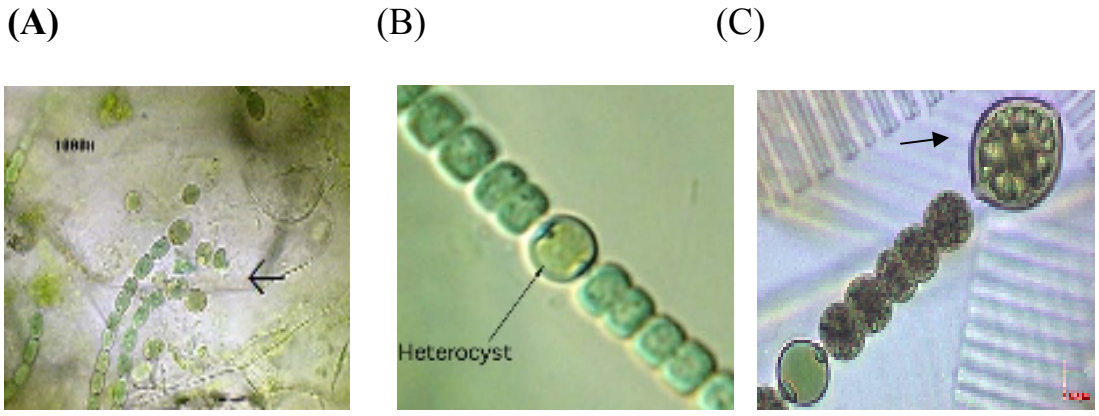

Figure 2.3 Cell structure of *Anabaena* sp.; (A) vegetative and heterocyst cell (B) and (C) akinete cell (www, 2007)

2.4.2 Symbiosis

The aquatic fern of the genus *Azolla* is a small-leaf floating plant, which contains an endosymbiotic community living in the dorsal lobe cavity of the pteridophyte leaf (Figure 2.4). This community composed of two types of prokaryotic organisms: one species of nitrogen-fixing filamentous cyanobacteria, *Anabaena azollae* (Ashton and Walmsley, 1976), and a variety of bacteria that identified as *Arthrobacter* sp. and associate with other showing the presence of nitrogenase (Costa et al., 1994). In this association, it is assumed that an exchange of metabolites, namely fixed nitrogen compounds from the cyanobiont to the host.

Diazotrophic cyanobacteria were first reported in 1889. They were an ancient group of prokaryotic microorganism exhibiting the general characteristic of gram negative bacteria (Rasmussen and Svenning, 1998). Cyanobacteria are phototrophic microbes that perform oxygenic photosynthesis similar to that of higher plants. Many species are capable to fix nitrogen despite the fact that nitrogenase, the key enzyme in this process is highly oxygen sensitive (Fay, 1992). Some

cyanobacteria fix nitrogen in special cell, called heterocysts. A thick envelope composed that functions to limit gas permeation, moreover, lack oxygen-envolving photosystem II activity to protect nitrogenase from the effect of O_2. Fixed nitrogen in the heterocysts is transported to vegetative cells in the filament, while vegetative cells supply carbon and reductant to heterocysts (Khudyakov and Wolk, 1997).

Filaments of *A. azollae* are localized in a cavity of the dorsal lobe of the fern's leaves, where special conditions stimulate high heterocyst frequency and vegetative cell differentiation during leaf development (Carrapico and Tavares, 1989).The existence of the two symbionts inside the *Azolla* leaf cavity and its relationship with the fern, namely the metabolites flow between the host and the symbionts. This association is maintained during all the life cycle of the pteridophyte. The *Anabaena* apical colony is associated with shoot apex lacks heterocysts and, therefore, is unable to fix nitrogen. In mature leaves, the *Anabaena* filaments grow and differentiate to heterocyst, which are the site of N_2 fixation. The prokaryotic colony, cyanobacteria and bacteria, are present in the sexual structures (sporocarps) of the fern (Carrapico, 1991). The cyanobacterium is transferred from the sporophyte to the next generation via the megasporocarp. A colony of the symbiotic cyanobacteria is formed near the shoot apex and thus enables symbiosis to be established within the developing leaf cavities (Watanabe and Van Hove, 1996).

A colony of cyanobactaria is always present on the apical meristem in the dorsal lobes (see figure2.3) and under the developing indusium (cap) of both the microsporocarps and megasporocarps (Shen, 1960). *A. azollae* persists in the mature megasporocarps and infects sporelings unlike the colonies entrapped within the microsporocarps. The role of akinetes is difficult to access. When an akinete

germinates its contents divide and give rise to a hormogonium. The spore membrane becomes mucilaginous, swell and then ruptures releasing its content (Shen, 1960).

Anabaena in the cavity develops in synchrony with cavity formation in the *Azolla* leaves. The filaments are undifferentiated in the plant apex but their cells divide as leaf primordial developed (Peter et al., 1978). The depression in the adaxial surface becomes occupied by *Anabaena* from the apical colony, and after cavities have been formed the hair cells developed (Neumuller and Bergman, 1981). Filaments of the apical colony are unable to fix nitrogen, but those isolated into leaf cavities progressively differentiate heterocysts and being to fix nitrogen (Kaplan and Peter, 1981). The frequency of the heterocysts increased from zero at the stem apex to as high as 33% in the 15^{th} leaf (Hill, 1975). Kaplan et al. (1982) have presented information on the total phycobiliprotein content (found also in heterocysts) as a function of leaf cavity and endophyte age. They have also determined adsorption and fluorescence spectra of the phycobiliproteins in individual vegetative cells and heterocysts from leaf cavities of different developmental stages in *A. caroliniana* and *A. pinnata*. Nitrogen fixation by the microsymbiont is highest in mature leaves and declines as the leaves begin senescence.

The presence of bacteria in the megasporocarps in association with the cyanobacteria also suggested a behaviour pattern similar to the cyanosymbionts (Carrapico, 1991). The presence of *Anabaena* throughout the life cycle of the fern favours the obligatory nature of the symbiosis and suggested a parallel phylogenetic evolution of both partners (Watanabe and Van Hove, 1996).

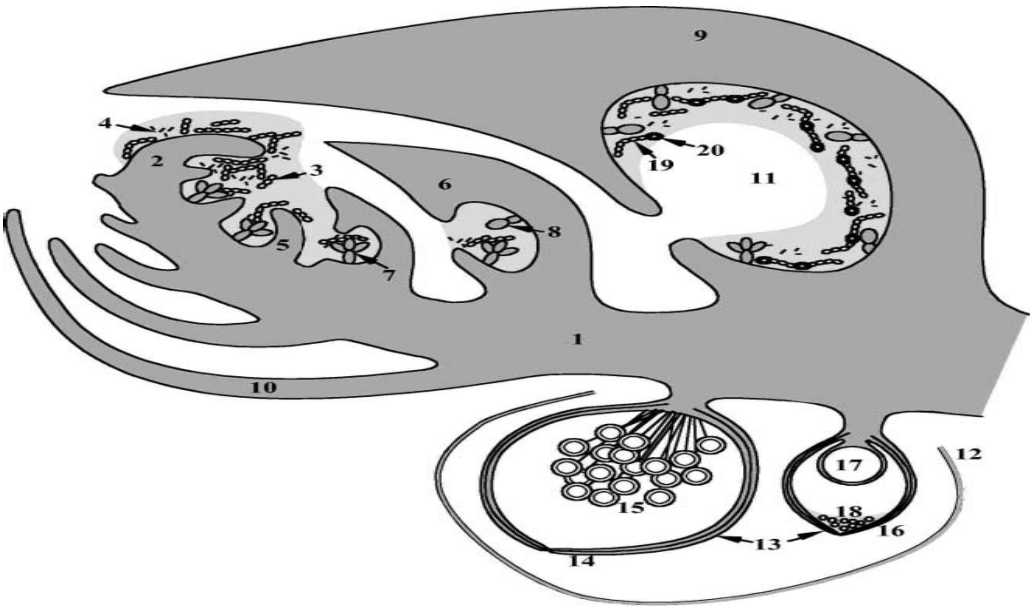

Figure 2.4 Morphology of *Azolla* stem and sporocarps (longitudinal section). 1. Stem; 2. Stem apex; 3. Apical *Anabaena* colony without heterocysts; 4. Other bacteria; 5. Leaf primordium; 6. Young leaf; 7. Pluricellular branched hair; 8. Bicellular single hair; 9. Upper leaf lobe; 10. Lower leaf lobe; 11. Leaf cavity (showing a central gaseous region and a peripheral mucilaginous region); 12.Involucre; 13. Indusia; 14. Microsporocarp; 15. Microsporangia; 16. Megasporocarp;17. Megasporangium; 18. Akinetes of *Anabaena*; 19. Vegetative cell of *Anabaena*; 20. Heterocyst. (Adapted from van Hove, 1989)

2.5 Nitrogen fixation and Assimilation

Nitrogen is extremely important in agriculture because it is a constituent of essential molecules such as chlorophyll, proteins and nucleic acids. Eventhough nitrogen makes up approximately 80 percent of the elements presenting the Earth's atmosphere, the plants and other eukaryotes are unable to directly utilize atmospheric nitrogen to meet their biological requirement. Biological nitrogen, the enzyme

conversion of N_2 gas to ammonia, is one of the important source of nitrogen in plants and nitrogenase is the key enzyme which is responsible for this conversion.

Biological N_2 fixation

$$N_2 \xrightarrow{e^-,\ H^+,\ \text{nitrogenase}} NH_3$$

On global basis the symbiotic may reduce 20 million tonnes of atmospheric nitrogen to ammonia per annum which accounts for about 70-80% of all biologically fixed nitrogen per year and one third of total nitrogen input needed for world agriculture. Nitrogenase requires ATP and electron carrier like Flavodoxin and Ferredoxin for its activity. The nitrogenase enzyme consists of two proteins, the Fe-protein and the MoFe-protein. Fe-protein is involved in the coupling of ATP hydrolysis to electron transfer. MoFe-protein serves as e^- redox group. Electrons are transferred from Fe-protein to MoFe-protein and then to N_2. The ammonia formed by the enzyme nitrogenase is assimilated through the joint action of glutamine synthetase and glutamate synthase. Glutamate acts as acceptor. The synthesis and activity of nitrogenase is regulated by several factor such as nitrogenous compounds, O_2, Mo and MgATP: ADP ratio. Nitrogenous compounds cause modification of Fe-protein thereby prevents the transfer of e^- to MoFe-protein. Though oxygen inhibits the activity of nitrogenase various physiological mechanisms are operating in different organisms to protect the nitrogenase from oxygen damage. In Mo deficient conditions Mo-Fe-protein becomes inactive. The concentration of ATP: ADP also affects the e^- transfer in the nitrogenase system. Hence the complete understanding of the biological nitrogen fixation will help in evolving better nitrogen fixing organisms.

Although *Azolla* also extracts nitrogen from its aquatic environment, the cyanobiont is capable of meeting the entire nitrogen requirement of the association. The nitrogen-fixation capacity of the *Azolla-Anabaena* association has been

demonstated indirectly (acetylene reduction-gas chromatography and assay of H_2 production) and directly (by use of $^{15}N_2$) (Moore, 1969). In comparison to the legume-*Rhizobium* symbiosis, nitrogenase activity by *Anabaena* does not cease when the association is grown for long periods with adequate or excessive amounts of combined N in the medium (Peters et al., 1978). Okoronkwo and Van Hove (1987) have studied the *Azolla-Anabaena* nitrogenase activity in the presence of combined nitrogen and concluded that in comparison to other symbioses, the *Azolla-Anabaena* relationship is tolerant to repression by combined N. The results implied that nitrogenase in *Anabaena azollae* is not inactivated, but only diluted out, in the presence of NH_4^+ nitrogen.

It has been shown that *Anabaena*, immediately after isolation from *Azolla*, fixes nitrogen at a rate at least equal to free-living cyanobacteria (Peters et al., 1980). The conclusion was drawn that ammonium was released by the symbiont and assimilated by the host tissue in the association.

Acetylene reduction rates for Azolla measured by various workers have been reviewed by Watanabe (1978), Becking (1979) and Lumpkin and Plucknett (1980). The acetylene reduction/ gas chromatograph technique is a useful and relatively inexpensive indicator of nitrogenase activity (Watanabe et al., 1977) but should not used for estimating potential nitrogenase fixation in the absence of conversion factors determined with $^{15}N_2$. The variability of the $C_2 H_2$: N_2 conversion ratio has been reported to range from 1.6 to 3.4 (Peter et al., 1977; Watanabe et al., 1977).

Rates of nitrogen accumulation by *A. filiculoides* calculated from the results of a cooperative experiment conducted in China (Lumpkin and Plucknett, 1982). The researchers were found to be as high as 10.5 kg N/ ha/day. The average fresh weighed 36.3 t/ha and had a doubling time of 4.8 days. This rate of nitrogen accumulation can

be considered high though not excessive. Other reports of N accumulation are considerably lower presumably because of lower mat weights, average accumulation calculated over a longer period and less than optimal growth conditions. Talley and Rains (1980) reported a rate of 2.7 kg N/ha/day under field conditions and calculated a daily average of 1.4 kg N/ ha from a year-round plot experiment.

Meek et al. (1985) have confirmed that symbiotic *Anabaena* releases a substantial portion of its newly fixed nitrogen as ammonium. The nitrogen assimilation is conducted by the glutamine synthetase-glutamate synthase (GS/GOGAT) pathway with little or no synthesis of glutamate dehydrogenase (GDH). The little biosynthetic activity of GDH in the isolated *Anabaena* indicated that all of GDH activity is associated with the hair cells of the cavity. This suggestion has been confirmed by other workers (Uheda, 1986). It was also shown that N_2-fixation and NH_4^+ assimilation were not tightly coupled as they are in free-living cyanobacteria (Stewart and Rowell, 1975; Walk et al., 1976). The release of ammonium supports the idea that GS, the initial enzyme of the primary ammonium assimilatory pathway is repressed in symbiotic Anabaena (Orr and Haselkorn, 1982).

A study with ^{15}N (Peter et al., 1980) revealed that the isolated symbiont exudes up to 50% of its fixed N into the incubation medium as ammonia; this observation led to studies on the enzymes in the *Anabaena* and *Azolla* that are involved in ammonia assimilation. They found that both organisms are capable of assimilating ammonia via the enzymes glutamine synthetase, glutamate synthase and glutamate dehydrogenase. The fern was estimated to account for 90% of the total glutamine synthetase activity and 80% of the glutamate dehydrogenase activity exhibited by the symbiosis (Ray et al., 1978).

Yoneyama et al. (1987) have shown that *Anabaena* has higher ^{15}N abundance than the host *Azolla* plants and that the contribution of N_2-fixation to the N nutrition of *Azolla* is as high as 99%.

The enzyme nitrogenase which is believed to occur in heterocysts of the *Anabaena* symbiont is capable of reducing N_2 and other substrates such as acetylene. Under argon or various partial pressures of N_2, H_2 evolution occurs (Peters et al., 1976; Newton, 1976) but is partially oxidized by unidirectional hydrogenase activity (Peters et al., 1981). Acetylene concentrations of only 0.1 to 0.15 atmospheres will saturate the nitrogenase enzyme and essentially eliminate N_2 but are proportionally related to the concentration of N_2.

Azolla and its symbiotic *Anabaena* are both photosynthetic organisms and their pigmentation is complementary. The association and individual partners exhibit Calvin cycle intermediates of photosynthetic CO_2 fixation. Sucrose is a major fixation product in the *Azolla* but it is not detectable as a labeled product in the isolated endophyte (Peters, 1984). The *Anabaena* endosymbionts contribute as little as 2% of the association's total photosynthetic capability.

Enhancement of nitrogenase activity by sugar supply has been demonstrated in the cultured cyanobiont isolated from *A. caroliniana* (Newton and Herman, 1979). Relatively low concentration of fructose (up to 1mM) and higher concentration of sucrose (up to 20 mM) were found to support nitrogenase activity (Tel-Or and Sadovsky, 1982). In contrast, Rozen et al. (1986) have found that the *A. azollae* isolated from *A. filiculoides* takes up and utilizes fructose in the light for mixotropic growth. Fructose was favored by the endophyte as a substrate over sucrose and glucose. The *A. azollae* cells grown in the presence of 8 mM fructose accumulated glycogen within 2 to 3 d followed by reduction of glycogen content during the fourth

day. In fructose-grown cells the glucose-6-phosphate dehydrogenase (G6PDH) activity was increased five-to sixfold, the frequency of heterocysts was increased from 6 to 18% and the acetylene reduction activity was increased threefold as compared with control cells. The turnover of sugar stored derivative glycogen and the events leading to heterocyst differentiation. Probably sudden changes in the C/N ratio and/or presence of specific metabolites of carbohydrate metabolism trigger the differentiation (Rozen et al, 1986). Peters (1984) postulated that there is a transition from photoautotrophic metabolism in generative *Anabaena* filaments to a photoheterotropic or mixotropic mode of metabolism with increasing differentiation of heterocysts and an exogenous carbon source, sucrose being utilized to maintain levels of reducing power.

The close relationship between photosynthesis and nitrogen fixation in *Azolla-Anabaena* symbiosis is well documented (Peters et al., 1980). Photosynthesis is the ultimate source of ATP, reductant and carbon skeletons used in N_2 fixation. Dark, aerobic nitrogenase-catalyzed reduction of substrates is dependent upon endogenous reserves of photosynthate and dark rates are always less than one half of those obtained in the light (Peters, 1984). A number of studies strongly indicate that reductant from endogenous reserves of photosynthate and photophosphorylation are the primary driving forces for nitrogenase activity in the light and that dark, respiratory-driven activities may be ATP limited (Peters et al., 1980). Interactions between photosynthesis and nitrogenase activity have been demonstrated by comparing action spectra for nitrogenase catalyzed C_2H_2 reduction in the *A. caroliniana-Anabaena* association and isolated endophyte (Tyagi et al., 1981).

2.6 Composition of *Azolla*

The dry weight of *Azolla* has been reported to range from 4.8 to 7.7% (Peters and Calvert, 1982). In most instances dry weight is calculated by multiplying fresh weight times a dry weight subsample. Unfortunately a standardized method for removing surface water from *Azolla* has not been developed and has led to considerable variation in the results of fresh weight determinations and the resultant dry weight. It is uniformly agreed that *Azolla* samples should be washed prior to weighing. After washing, samples have been either drained blotted and/or centrifuged before oven drying and weighing.

The dry weight nitrogen content of *Azolla* has been reported to be as high as 6.5% for aseptic cultures (Peters et al., 1980) but will probably be closer to 3.5-4.0%. The nitrogen content of *Azolla* is strongly influenced by its environment. Under normal field conditions phosphorus availability and temperature will probably have the greatest influence. The application of phosphorus four or five days before the incorporation of a phosphorus-stressed *Azolla* mat can increase the nitrogen content of the biomass by 50% or more and result in a lower C:N ratio.

The C:N ratio affect the decomposition rate of soil incorporated *Azolla* (Watanabe et al., 1977). Ratio of 7:1 to 18:1 have been reported for *Azolla* (Peters et al., 1980). Field experiments conducted in China by Lumpkin and Plucknett (1982) yielded C:N ratios ranging from 8.3:1 to 13.5:1 for various species of *Azolla* (Lumpkin et al., 1982). The dry weight carbon contents of these samples ranged from 41.5 to 45.3% and were comparable to values reported elsewhere (Peters and Calvert, 1982).

The dry weight protein content of *Azolla* ranges from 13 to 30% (Newton and Calvins, 1976; Buckingham et al., 1978; Singh, 1979). Percentage ranges for other

reported constituents are cellulose 5.6-15.2; hemicellulose 9.76-17.9; lignin 9.3-34.8; ash 9.7-23.8; crude fat 4.4-6.3; acid detergent fiber 26.6; neutral detergent fiber 39.2 (Buckingham et al., 1978).

2.7 *Azolla* as biofertilizer

2.7.1 Effect of *Azolla* on soils

Azolla has a high nitrogen content and a favorable carbon: nitrogen ratio (about 8-17:1) which allow it to decompose faster than *Astragalus sinicus* at similar C: N ratios (Lin Xin-xiong et al., 1980; Shi Shu-lian et al., 1980). When incorporated into the soil *Azolla* helps to increase humus and organic nitrogen levels in the soil. There is also some improvement in soil chemical and physical properties which will result in less energy required for tillage. Over a period of several years with continued *Azolla* cultivation, humus and organic nitrogen will accumulate. Also, *Azolla* may help to improve the water-holding capacity and cation exchange capacity of the soil.

There are several general principles relating to soil improvement with *Azolla*: (1) soil incorporation is more effective than allowing *Azolla* to die on the surface and decompose naturally (Figure 2.5) (2) incorporating *Azolla* several times is better than a single incorporation (3) thorough mixing with the soil is better than simply turning under the *Azolla* and (4) poorer soils benefit more than good soils. *Azolla* starts to decompose in 5-10 days after incorporation and begins to release its nitrogen. The rate of decomposition is significantly affected by the quantity, variety and stage of maturity of the *Azolla.* In addition decomposition is affected by the soil environments especially in regard to composition, microorganisms and temperature.

Figure 2.5 Incorporation of intercropped *Azolla* in rice field of Rwanda. (Van Hove, 1989)

Within limits, ammonia-N levels increase as *Azolla* incorporations and yields increase and as *Azolla* is used over a period of years. Several mats of *Azolla* plowed in before rice planting can supply all of the crop's nitrogen requirements. However, if the *Azolla* crop was insufficient, topdressing of supplemental N may be required to obtain good grain filling since soil N levels tend to decline as the rice crop grows and matures. When *Azolla* is grown with rice, early rice growth may be retarded somewhat by competition between *Azolla* and rice for nitrogen that is cycling between the soil and paddy water. After the first incorporation of intercropped *Azolla*, soil N

levels begin to rise and continue to remain high as *Azolla* N is released through decomposition. The dual monocropped/ intercropped system lead to high soil N levels with time when compared to the other two systems.

Azolla releases its nitrogen more slowly than chemical fertilizers hence it is often referred to as a slow-release fertilizer. Only about 10-40% of the *Azolla* N is released and absorbed by the rice during the first crop season and thus the following crop may still obtain some benefits. Such residual benefits of *Azolla* are important in planning crop management practices.

2.7.2 Effect of *Azolla* on rice yields

Azolla can have a significant effect on rice yields and most research have focused on this topic. Yield increases from 10% or less to more than 100% have been recorded when *Azolla* was used as a green manure. Most field researchs have been done in China cultivation of *A. pinnata* var. *imbricata* increased the yield of rice by 600-750 kg/ha above the yield of control plots (Zhejiang Acad. Ag. Sci, 1975). These results were somewhat low because the experimental procedure was not adjusted to compensate for competition between *Azolla* and rice. Moore (1969) cited rice yield increases of 14, 17, 22 and 40 percent with cultivation of *Azolla*.

The effect of *Azolla* on rice yield varies according to the species of *Azolla* used, methods of cultivation, time and methods of incorporation. For example, Singh (1977) obtained 6% rice yield increase when *A. pinnata* was grown with rice without incorporated and increase ranging from 9-38% when *Azolla* was incoporated into the soil. Watanabe (1977) reported 13% increase in grain yield with incorporated *A. pinnata* but recently has observed considerably higher yields, possibly due to a cumulative effect of slow nitrogen release from successive incorporations of *Azolla*. In California, Talley et al. (1977) achieved: (1) rice yield increases of 112% over the

control by incorporating one 60 kg N/ha basal green manure crop of *A. filiculoides* into the paddy soil and (2) 216% increase (over 4 metric tons) by first incorporating one basal leyer (as in (1) above) and then growing *Azolla* as an intercrop with rice. An *Azolla*-rice experiment conducted at 8 locations in Thailand during the wet season of 1979 resulted in rice yield increases of 13-75% (mean of 38%) over the zero N control when *Azolla* was grown and incorporated before and with the rice (Sawatdee et al., 1979). An *Azolla*-rice experiment in Taiwan during the winter of 1980-81 produced rice yield increases of 45.6 and 33.9% over the zero N control with the species *A. pinnata* and *A. filiculoides*, respectively.

2.7.3 Effect on components of yield

Azolla green manuring has the same effect on rice yields as most other green manures. Increases in straw and grain yields are generally the same as those expected from N fertilizer application as are the number of tillers per hill, number of panicles per hill, number of filled grains per panicle and grain size. However in comparison to N fertilization, *Azolla* produces much higher protein content. Of course, like other sources of N, an excessive N supply or poorly timed incorporation of *Azolla* can result in delayed tillering, delayed flowering and a longer maturation period. Excessive levels of *Azolla* green manure are conducive to sheath blight and lodging.

CHAPTER III

MATERIALS AND METHODS

3.1 *Azolla* species and culture condition

Three *Azolla* isolated were selected from ponds at university farm and organic farm in Suranaree University of Technology, Thailand (Table 3.1). A handful of *Azolla* were rinsed in clear water to remove foreign material such as fragments of plants, insect, soil colloids, etc. For morphological classification and DNA analysis studied, *Azolla* were cultured with about 15 cm. height and 17 cm. in diameter soil pots containing 1 L of N-free medium solution (Table 3.2) under greenhouse condition. It is better to make up a nutrient solution of know composition this greatly reduces the risk of proliferating organisms other than *Azolla*. For apply in rice fields, *Azolla* were increased on a larger scale these were grown in 2 m^2 of cement containers, the bottom covered with 2 kg of rice field soil and filled with about 5 cm of water and placed under sunlight. To promote the vegetative development of *Azolla* plant, 0.20 g m^{-2} of P_2O_5 were applied to the containers at seven-day interval (van Hove, 1989).

Table 3.1 Isolation of *Azolla* species used in this study.

Species	Source
AZO1	Organic Farm, Suranaree University of Technology, Thailand
AZO2	University Farm, Suranaree University of Technology, Thailand
AZO3	University Farm, Suranaree University of Technology, Thailand

Table 3.2 The stock solution of N-free medium. (van Hove, 1989)

Stock solution	Salts	Concentration (g/l)
1	$CaCl_2$	55.50
2	$MgSO_4.7H_2O$	49.50
3	KCl	37.50
4	KH_2PO_4	13.60
5	FeEDTA.Na	30.00
6	H_3BO_3	2.86
	$MnCl_2.4H_2O$	1.81
	$ZnSO_4.7H_2O$	0.22
	$CoCl_2.6H_2O$	0.10
	$CuSO_4.5H_2O$	0.08
	MoO_3	0.02

For 2.5 l culture medium; 100 ml of solution 1, 2, 3, 4 and 10 ml of solution 5 and 6; adjust to 2.5 l with RO water

3.2 Morphological classification

3.2.1 Observation of vegetative and reproductive structures using SEM

The SEM procedures were carried out as follows: first, *Azolla* leaves and sporocarps were fixed with 2.0% glutaraldehyde in 0.1M phosphate buffer pH 6.8 for 1 h or overnight, then washed 3 times with phosphate buffer and allowed to sit for 10-15 min. These were followed by post-fixing in 1% osmium tetraoxide in distilled water for 30 min and washed 3 times with distilled water. After 10-15 min, the samples were dehydrated with the ethanol series 30, 50, 70, 90, 95 and 100%(2 times).

Dehydrated samples were then further dried using a critical point dryer (CPD, Samdri-PVT-3B, Japan) and fixed on stub. Dry samples were finally coated with gold-palladium using an ion sputter (JFC-1100E, Japan) and evaluated under 85X, 100X, 120X, 200X and 800X scanning electron microscope (JSM-6400, JEOL, Japan) and photographed.

3.2.2 Observation of *Azolla-Anabaena* symbiosis

To determine *Anabaena azollae* in cavity of *Azolla* leaf, the surface and freshly cut longitudinal and cross sections of leaves were pre-fixed by 2.5% glutaraldedyde in 0.1 M phosphate buffer, pH 6.8. The sample was post-fixed with OsO_4 in 0.1 M phosphate buffer (pH 6.8), then dehydrated in series concentration of ethanol. The sample was embedded in Spurr's resin. Two μM thick was sectioned with ultramicrotome (RMC, MTXultramicrotrome, USA). The sections were stained with toluidine blue for 1 min, rinsed with water, and then stained with basic fuchsin for 30s, rinsed with water again. These were investigated under 100X light microscope (Nikon, Japan).

3.3 Classification with DNA analysis

3.3.1 DNA extraction

DNA was extracted from *Azolla* roots by the method of Dellaporta et al, (1983). Plant roots were frozen at -20˚C and grind to a fine powder with liquid nitrogen in a prechilled mortar and pestle. The DNA extraction was performed by adding 720 μl of extraction buffer (1M Tris-HCl pH 8.0, 0.5M EDTA, 5M NaCl and 10% SDS). The aliquot as transferred into the microfuge tube, incubated at 65˚C, vortex every 5 min for 15-20 min, then 5M NaOAC were added and gently mixed. Microfuge tubes containing those aliquots were incubated on ice and shaken for 20

min prior to be centrifuged at 14000 rpm for 15 min. The supernatant was transferred from the aqueous phase into a new tube and extracted with equal volume of phenol. The upper phase was further extracted with chloroform: isoamylalcohol (24:1). The supernatant was precipitated with cold isopropanol. Pellet was washed with cold ethanol before dried and resuspened in TE buffer. Then, DNA was incubated with RNAse at 55˚C for 10 min, stored at -20˚C.

3.3.2 PCR amplification

DNA was amplified by using universal primer NS1, NS2 and ITS1, ITS4 (White et al., 1990). The PCR reactions were performed in a final volume of 50µl, contain 10-50 ng of DNA template, 10mM Tris-HCl, 1.5 mM $MgCl_2$, 200µM of dNTPs, 10 pmol of each primers and 2.5 U of *Taq* polymerase (Promega, U. S. A.).The reactions of NS primers were performed in a Thermal cycler (Px2 Thermal cycler, Thermal Electron Corporation, U. S. A) by heating at 95˚C for 5 min and then amplification were performed with 40 cycles at 95˚C for 30 s, 45˚C for 30 s and 72˚C for 1 min, followed by elongation at 72˚C for 10 min. For ITS primers, the amplification reactions were performed as followed: heating at 95˚C for 2 min, and then amplification were performed with 35 cycles at 95˚C for 30s, 50-60˚C (vary from DNA template) for 30s and 72˚C for 45s. The extension step, amplification ended at 72˚C for 10 min. PCR products were separated by electrophoresis on 1% of agarose gel and stained with ethidium bromide prior to observe on Gel documentation and analysis system (GDS8000, Ultra violet product, UK).

Table 3.3 Primer used for PCR and sequencing.

Primer	5'-seguence-3'
NS1 (F)	GTA GTC ATA TGC TTG TCT C
NS2 (R)	GGC TGC TGG CAC CAG ACT TGC
ITS1 (F)	TCC GTA GGT GAA CCT GCG G
ITS4 (R)	TCC TCC GCT TAT TGA TAT GC

(F) and (R) denote forward and reverse primers, respectively.

3.3.3 DNA sequence analysis

PCR products of each *Azolla* specie were purified with PCR purification Kit (QIAquick® PCR purification Kit (50), QIAGEN, Germany). The nucleotide sequences were determined by Macrogen's sequencing service (Macrogen Inc., Korea). The sequence data was compared to gene libraries (Genbank) with BLAST program. Multiple sequence alignments of translated gene sequence were carried out with the program CLUSTALW (version 1.83). Genetic distances were estimated by using the Maximum parsimony with bootstrap method employed by PHYLIP (Felstein, 1993). The tree was displayed with the TREEVIEW program (Page, 1996).

3.4 Application of *Azolla* species as biofertilizer in rice field

Rice field experiments were performed at Suranaree University of Technology, Thailand. Rice seedlings (*Oryza sativa*) of Khao Dok Mali 105 (KDML105) rice variety were planted during August 2006 to December 2006. The experiment was set out followed a Randomized Complete Block Design (RCBD). Rice seedling of 30 days old were transplanted in experimental field with 2 m x 4 m and 25 cm x 25 cm

row to row distance with three replications in each treatment. There were 5 treatments in this experiment consist of

(T1) AZO1

(T2) AZO2

(T3) AZO3

(T4) with chemical fertilizer (12-8-8 kg N-P_2O_5-K_2O/ rai / or 0.075-0.05-0.05 t N- P_2O_5-K_2O / ha)

(T5) without *Azolla* and fertilizer and

Fresh *Azolla* plants were applied at the rate of 250 g m^{-2} into 8 m^{-2} plots. All plots were constantly stagnant with the water level. All *Azolla* were grown on flooded field for 4 weeks and incorporated in the soil before rice transplanting. Fresh weight, dry weight and doubling time of *Azolla* species were investigated. After cultivated for 45 days, the *Azolla* species were supplied in treatment T1, T2, and T3 again. Every rice field plots were offered 0-8-8 kg P_2O_5-K_2O /rai or 0-0.05-0.05 t N- P_2O_5-K_2O / ha). The data of rice growth were collected in 3 stages there were tillering, flowering and harvesting stages and analyzed by SPSS programme. Four parameters were investigated as followed.

1. Height of rice by measure from soil to the end of leaf or the end of panicle
2. Number of tillers per hill
3. Number of grain per panicle
4. Dried weight of grain

Grains were counted to determine percentage of filled grains. The hills were taken from area where grain yield was determined weight per area at maturity. The

harvest plants within two rows of the edge of each plot were not sampled to avoid the border effect. Doubling time of *Azolla* was calculated by the following formula.

Doubling time = ln2/ RGR, where RGR (Relative growth rate) = (lnW2 - lnW1) (t^{-1}), W1 = initial weight, W2 = final weight and t = number of days of growth (Mitchell, 1974).

3.5 Measurement of nitrogen fixation

All species cultured with N-free medium solution under greenhouse condition were detected nitrogenase activity. The nitrogen fixing potential of *Azolla* species was measured by Acetylene Reduction Assay. At 21 days of cultivation, 2-3 g of *Azolla* fronds were put in 20 ml of N-free medium solution in 125-ml erlenmeyer flasks and incubated at 2-3 klux at 28±2 °C for 2 hr. They plugged with septum then 10% of space air volumes were replaced with acetylene. One ml of gas mixture was withdrawn and analyzed by gas chromatography (AutoSystem XL, Perkin Elmer,U.S.A.) with capillary PE-Alumina column. After measurement of ARA, the *Azolla* were dried in the oven at 70 °C for 24 hr and dried weight was determined. The ARA values were expressed per gram dry weight of *Azolla*. All values presented are means of triplicate measurements, nitrogenase activity was calculated using equation. Nitrogenase activity was expressed as μmole C_2H_4/ g dwt/h.

$$\text{Nitrogenase activity} = \frac{\text{Ethylene produced}}{\text{Time (h) x number of plant}}$$

3.6 Measurement of total nitrogen

Moist samples from field experiment were dried at 70 °C for 24 hr. Samples were ground with a grinder. Ground materials were weighed in approximately 1 g of the sample, was placed into Kjeldahl digestion tubes, along with 15 ml conc. H_2SO_4. Five grams of K_2SO_4 and catalyst usually $CuSO_4$ ratio 1:10 were added for 2 hr. Digestion tubes were placed in Digester (J. P. Selecta, Spain). Samples were distilled with Distillation unit (Vapodest 30, Gerhardt, Germany). The total nitrogen was determined by titration with a standard solution (0.1N HCl) and N content was calculated using equation. Blanks were carried through the same procedure simultaneously with the experimental determination.

$$\% N = \frac{\text{volume of HCl x Normality of HCl x 14 x 100 t ha}^{-1}}{\text{Weight of sample (g) x 1000}}$$

3.7 Soil analysis

The soil samples from field experiment were collected both before and after rice plantation. Then soil samples were air dry for 4-5 day before analysis. The soil sample were crumbled mechanically and passed through a sieve with 2 mm. The soil analysis was carried out by Department of Land Resources and Environment, Faculty of Agriculture Khon Kaen University, Thailand.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Morphological study of *Azolla*

4.1.1 The morphological structure of *Azolla*

All *Azolla* species were classified from pond at SUT farm. For morphological classification, *Azolla* were cultured with N-free medium solution under greenhouse condition. For preliminary studied, after 14 days of culture, *Azolla* were observed morphology of vegetative structure under stereo microscope as shown in Figure 4.1 The elongate plants of AZO1, AZO2 and AZO3 were about up to 1.5 cm long, 1.5 cm and 2.5 cm, respectively. This observation following conspectus of classification and tentative identification key (Lumpkin and Plucknett, 1982) that reported section *Azolla* (*A. caroliniana*, *A. mexicana*, *A. microphylla*, *A. filiculoides* and *A. rubra*) had immature fronds 1-3 cm in diameter, growing horizontally, composed of two or more flabelliform main rhizomes with lateral branches.

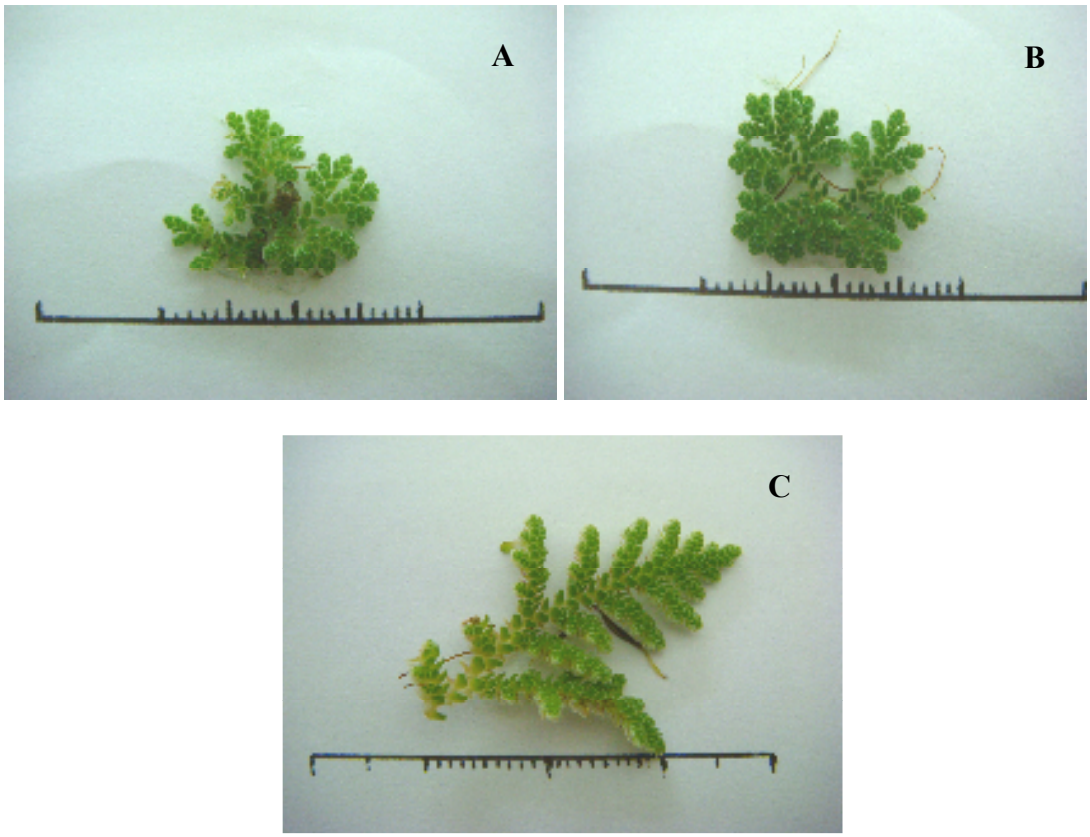

Figure 4.1 Morphology of vegetative structure under stereo microscope; A) AZO1, B) AZO2 and C) AZO3 Magnification = x 0.67

Since, Martin (1976) suggested that feature such as type of leaf hair might also be important taxonomic characteristic. Thus, trichomes (leaf epidermal hairs) from each vegetative structure were examined. The difference of epidermal trichomes structure was observed from leaf surface under SEM (Figure 4.2). The hair cells morphology of AZO3 are spherical- like, about 30-40 µm in diameter whilst AZO1 and AZO2 are ovoidal in shape, about 50 µm in length by 20 µm in width. Every species are considered as vertical rows of single cell stomata and trichomes of one or more cells.

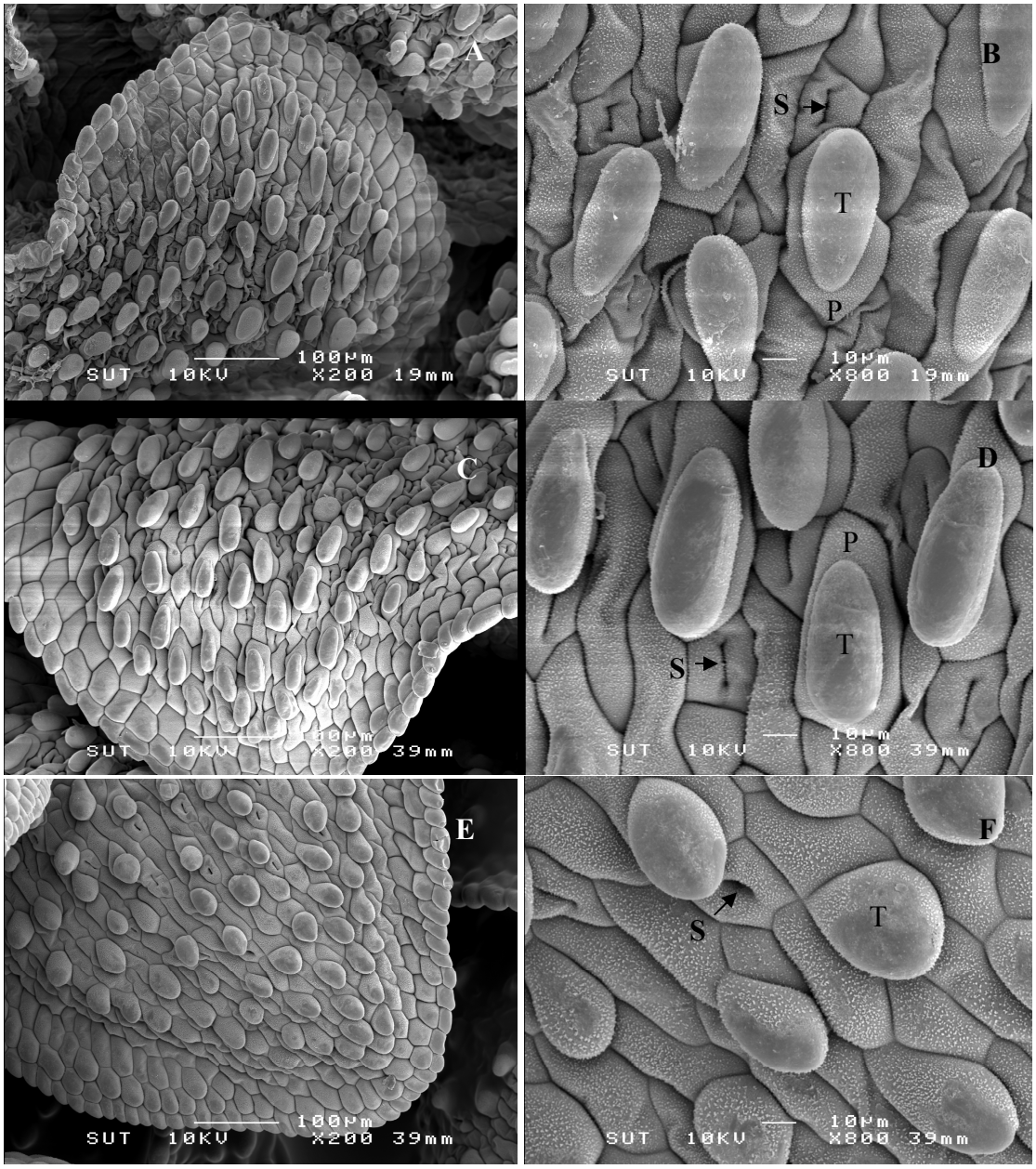

Figure 4.2 Scanning electron micrographs of epidermal trichomes. (A-B) Micrograph of the AZO1 leaf surface and leaf trichomes; (C-D) leaf surface and leaf trichomes of AZO2; (E-F) leaf surface and leaf trichomes of AZO3.T= apical trichome cell, P=pedicel cell and S=stomata. A, C and E magnification = x200; B, D and F magnification = x800

Following to the tentative indentification key of Lumpkin and Plucknett (1980) the leaf trichomes are the one part that use for indication of *Azolla* species. The trichomes structure consisted of 2 cells; pedicel cell and apical cell. From figure 4.2A and B, AZO1 presented 1 pedicel cell and 1 apical cell. The pictures of AZO2 (Figure 4.2C and D) indicated a broad pedicel cell ½ or more of trichome height and apical cell perpendicular to the lobe. Figure 4.2 E and F, AZO3 showed the single trichome cell, clearly discernable from epidermal layer. However, the precise identification of the members of section *Azolla* (*A. caroliniana*, *A. filiculoides*, *A. mexicana*, *A. microphylla* and *A. rubra*) is somewhat complex due to similarity in vegetative characters. Thus, the morphology of sporocarps was demonstrated.

4.1.2 Classification with reproductive structure

The reproductive structures (sporocarps) were classified under stereo- and scanning electron microscope. Sporocarps of each species are borne in pair on the ventral surface of the frond and are located at the vertices of frond branches. The pair may be mixed with a female megasporocarp, which contains a single megasporangium, and a male microsporocarp, which contains numerous microsporangia (Figure 4.3C, 4.4C and 4.5D), or the pair may be of the same sex. Microsporocarp (Figure 4.3B, 4.4B and 4.5B) is larger than megasporocarp (Figure 4.3D and 4.4D). In this examination, microsporocarps were more abundant than megasporocarps and while number of a pair was more commonly of the same type (Figure 4.3-4.6).

Figure 4.3 Sporocarps formation under x4.5 stereo microscope in AZO1; (A) sporophyte with megasporocarps and microsporocarps; (B) detached microsporocarps; (C) microsporangia; (D) megaspore

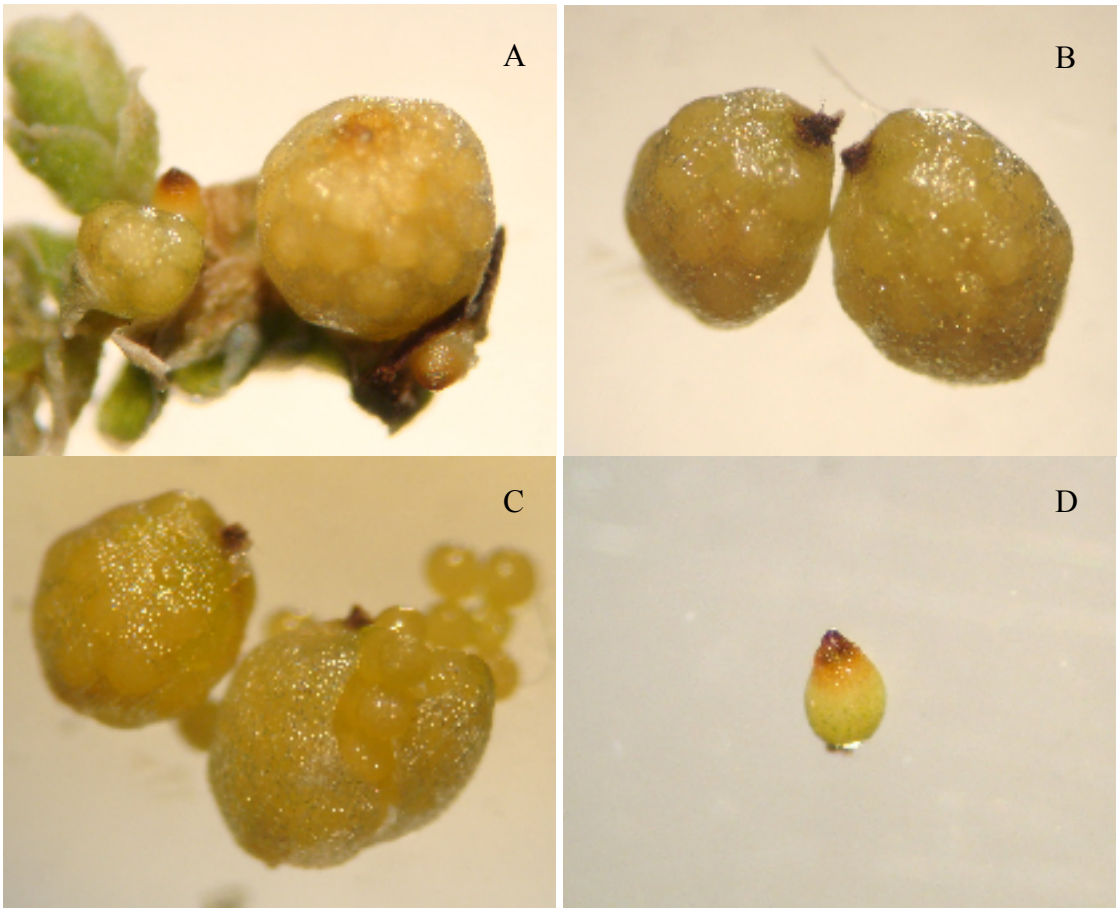

Figure 4.4 Sporocarps formation under x4.5 stereo microscope in AZO2; (A) sporophyte with megasporocarps and microsporocarps; (B) detached microsporocarps; (C) microsporangia; (D) megaspore

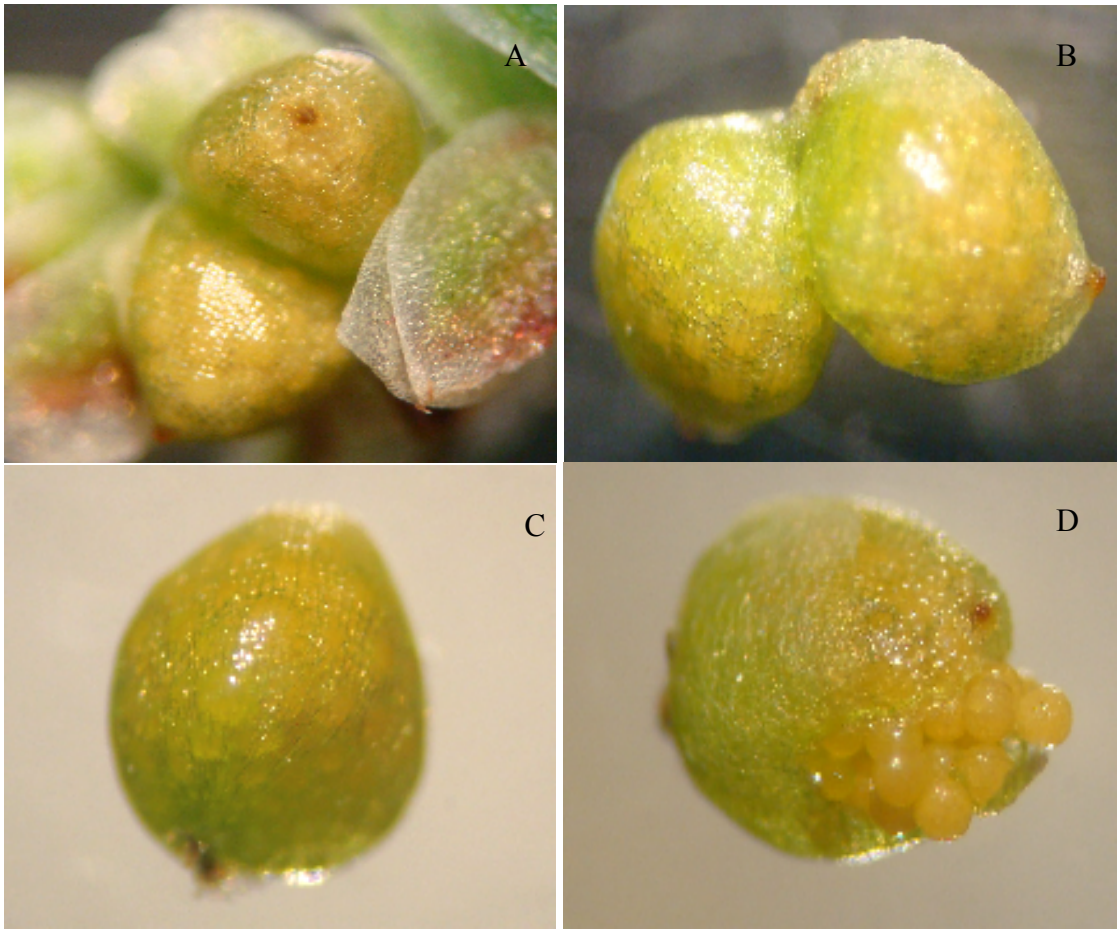

Figure 4.5 Sporocarps formation under x4.5 stereo microscope in AZO3; (A) sporophyte with microsporocarps; (B-C) detached microsporocarps; (D) microsporangia

For each megasporocarp is developed as a single megasporangium. As the single megaspore matures, the periplasmodium divides into four parts. One of the four masses, termed the perispore, encloses the megaspore. This mass differentiates a spore covering is called the perine including the grindle (Figure 4.6 and 4.7).The floats are also part of the megaspore apparatus that species separation has been based primarily on float number. Both AZO1 and AZO2 have three floats (AZO3's megaspore is rarely found).

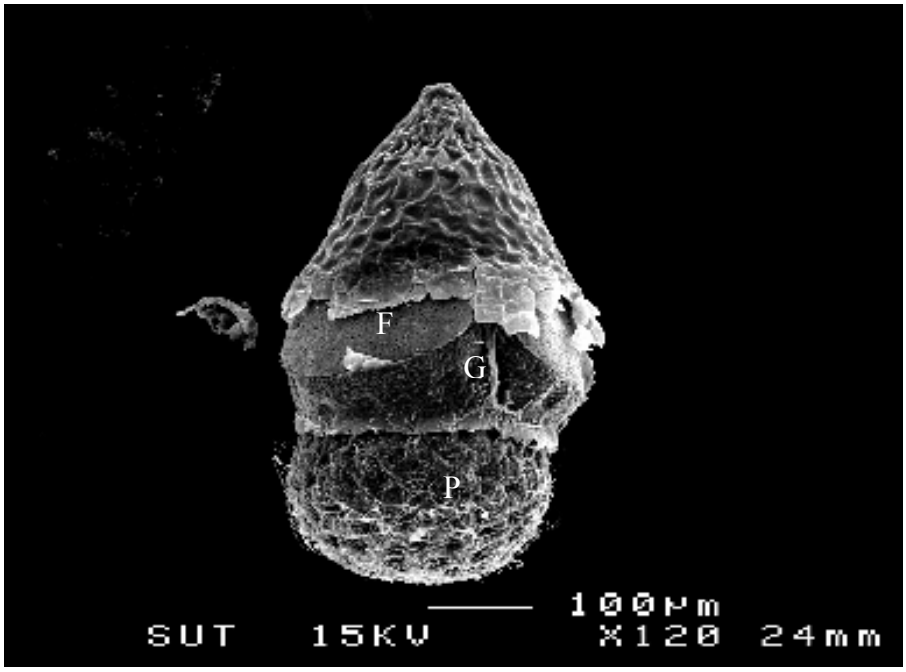

Figure 4.6 A megasporocarp of AZO1 with the proximal half of its indusium removed to reveal the perispore or purine (P) covering the megaspore. Several additional structures of the megaspore apparatus can be seen, including the girdle (G) and float (F). Magnification = x120; scale bar = 100 µm.

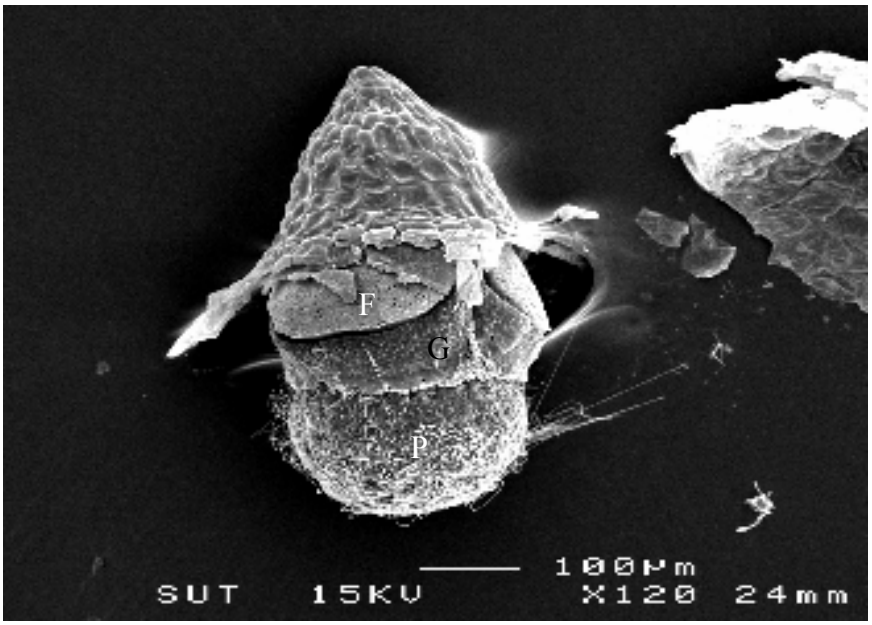

Figure 4.7 A megasporocarp of AZO2 with the proximal half of its indusium removed to reveal the perispore or purine (P) covering the megaspore. Several additional structures of the megaspore apparatus can be seen, including the girdle (G) and float (F). Magnification = x120; scale bar = 100 µm.

Microsporocarps which lead to the formation of the male prothalli of the gametophytic generation, are large spherical to oblate bodies in AZO1 and AZO3, about 1 mm in diameter. The microsporocarps of AZO2 were considered ovoidal shape, about 1.1 mm in length by 0.9 mm in diameter at their widest point and are fusiform to ovoid in shape (Figure 4.8).

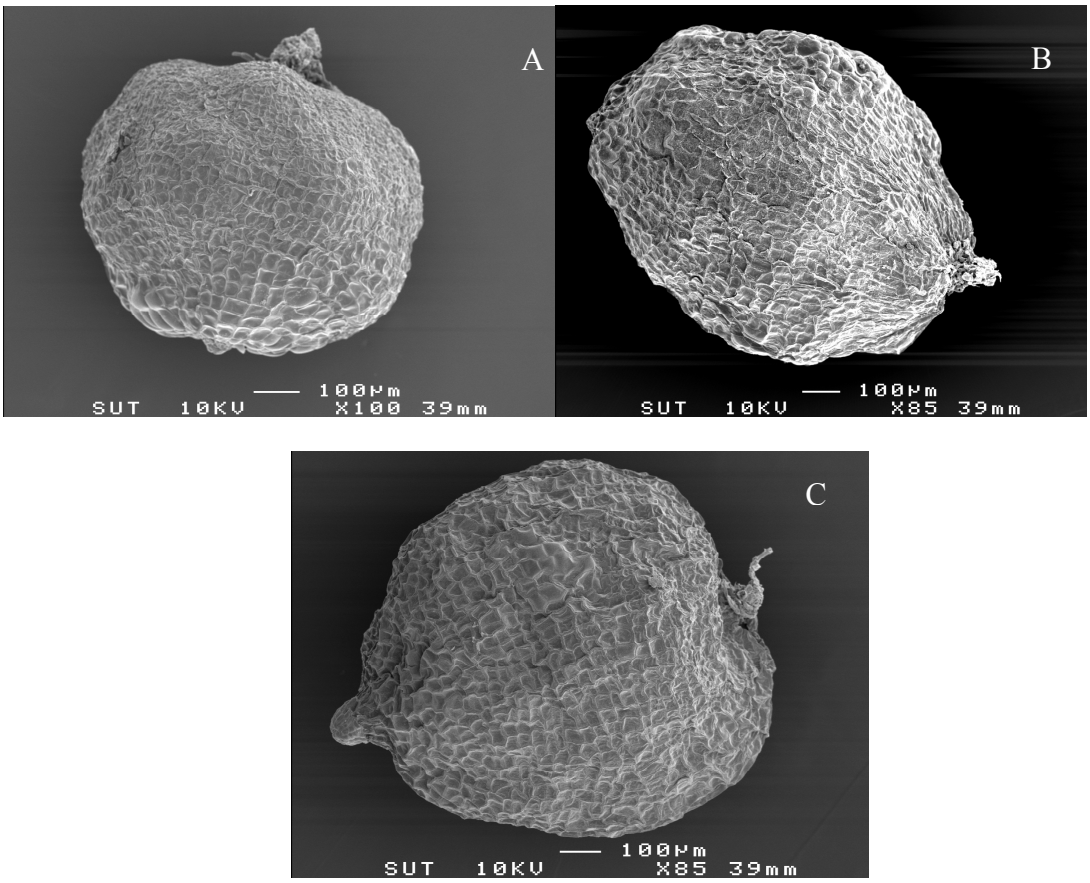

Figure 4.8 A single mature microsporocarp as seen in lateral view; A) AZO1 (100x); B) AZO2 (85x) and C) AZO3 (100x). Scale bar = 100 µm

From the results of morphology (vegetative and reproductive structures), it can be concluded that *Azolla* species belong to section *Azolla*. AZO1 presented immature fronds 1.5 cm in diameter, 1 pedicel cell and 1 apical cell and had three floats these indicated that AZO1 was *A. microphylla*. AZO2 presented immature fronds 1.5 cm in diameter, broad pedicel cell ½ or more of trichome height and apical cell perpendicular to the lobe and three floats. This diagnostic character was only partially effective in that those accessions with broad pedicel cells were always members of *A. mexicana* or *A. caroliniana* and *A. microphylla* which called *A. cristata*

(Dunham and Fowler, 1987). AZO3 presented immature fronds 2.5 cm in diameter, single trichome cell, clearly discernable from epidermal layer was *A. filiculoides* which this specie had distinct vegetative structure when compared between the other species.

Dunham and Fowler (1987) have proposed tentative taxonomic conclusion based on critical assessment of vegetative and reproductive characters using light microscope, thin sectioning, SEM. They found that apart leaf trichomes and possibly root anatomy, vegetative features provide little assistance in taxonomic separation which was the same as this study. They confirmed that, despite variations observed, features of megaspore apparatus are the most reliable means of separating taxa within *Azolla*. The six main megaspore types are recognized as belonging to *A. filiculoides*, *A. mexicana*, *A. microphylla* and *Azolla* sp. within section *Euazolla* and *A. pinnata* and *A. nilotica* in section *Rhizosperma*. The precise identification of the member of section *Azolla* is somewhat complex due to similarity in vegetative characters. But in *Rhizosperma*, it is easy since members are distinct from each other. A megasporocarp is less obvious because of that sporocarp's smaller size. They are hardly visible to the naked eye, bear three or nine special structures named floats, whose exact function is not know. With the hope of resolving the complex taxonomical problems, attention has been focused on DNA analysis.

4.1.3 Morphological study of *Azolla-Anabaena* endosymbiont

To determine the cyanobacterial, *Anabaena azollae* localization in cavity leaf the surfaces and freshly cut longitudinal and cross sections of *Azolla* leaf were examined by light microscope. In mature stage the cavities are ellipsoid in shape. The interior of the cavity is lined by an outer and an inner envelope, creating a narrow

space close to the periphery of the cavity, where the filamentous cyanobacteria, *A. azollae* is located. In this narrow space all the endosymbionts are embedded at the cavity cell as shown in Figure 4.9 Two cell types except akinete cell were observed in cyanobacterial filaments found in association with AZO1, AZO2 and AZO3 (Figure 4.10). Filaments composed almost entirely of vegetative cells. Vegetative cells comprising a single filament were similar in size and shape however, these characteristis varied considerably among different filaments. Heterocysts were morphologically distinct from vegetative cells because of their increased size and rounder shape.

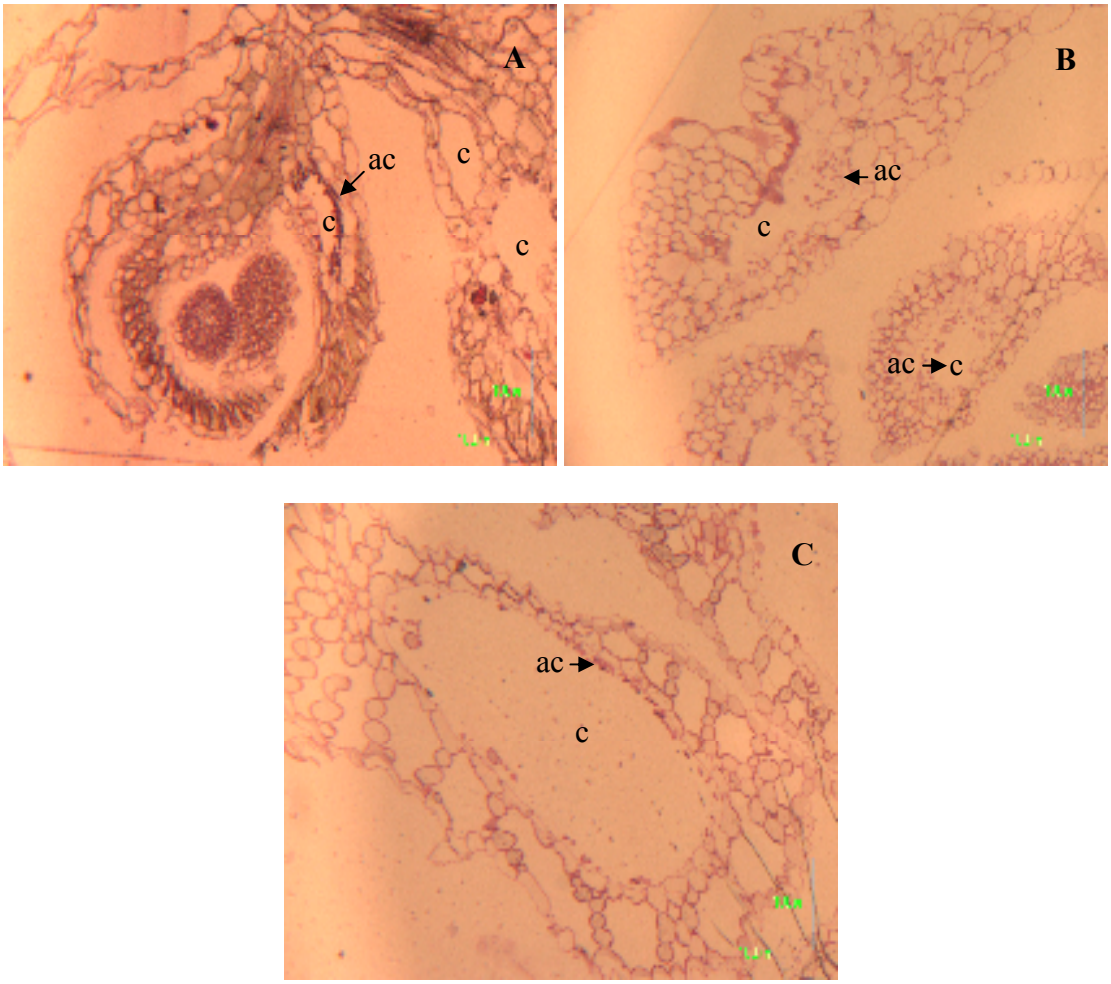

Figure 4.9 Longitudinal section through the dorsal lobes of successive leaves of AZO1 (A); AZO2 (B); AZO3 (C). *Anabeana azollae* cells are present inside cavities. c = cavity, ac = Anabaena cells. Magnification = x400

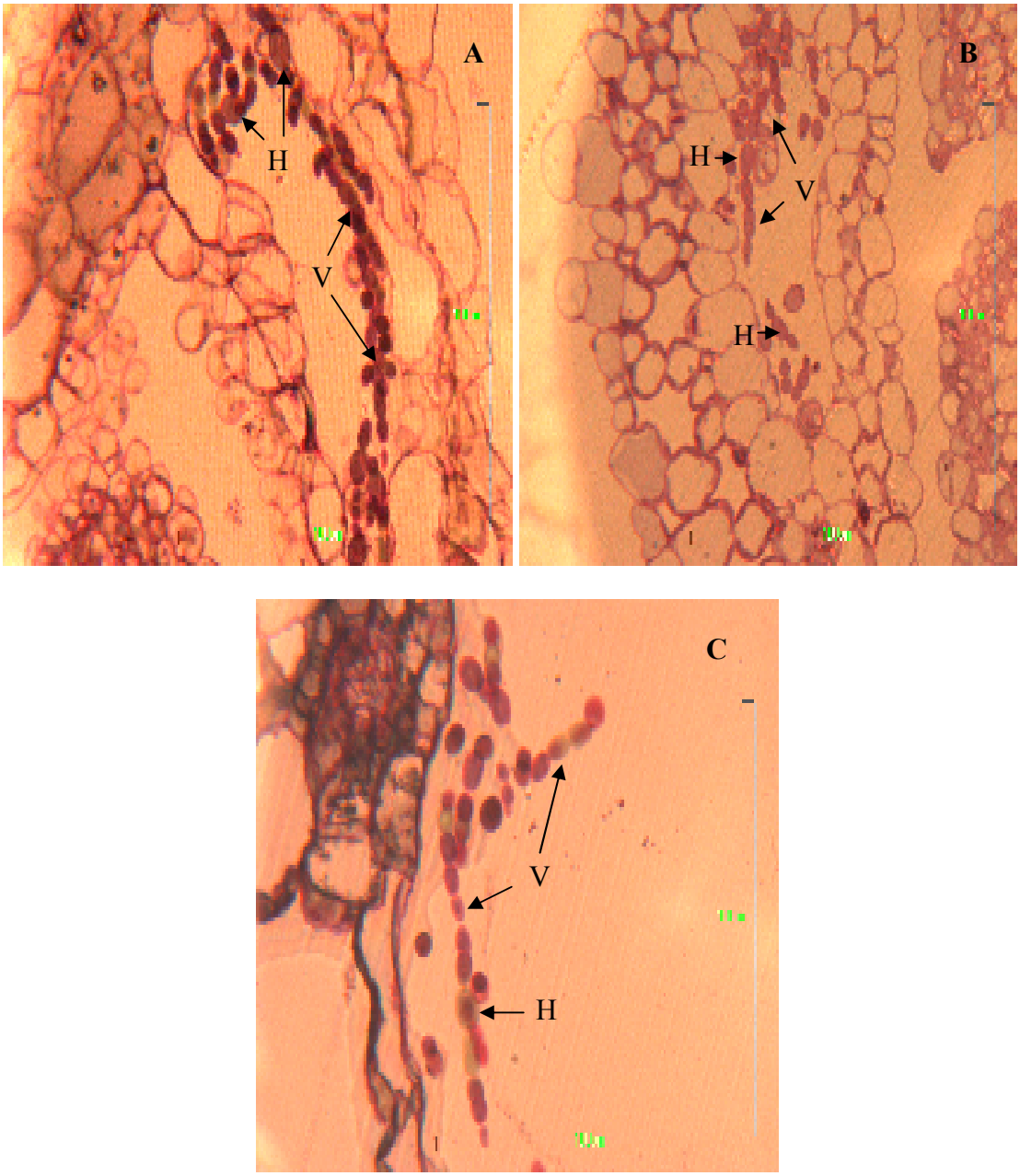

Figure 4.10 Light micrographs of cyanobacterial filaments symbiosis from AZO1, AZO2 and AZO3 are shown in A, B and C, respectively. V= vegetative cell; H= heterocyst cell. Magnification =x1000

4.2 Classification with DNA analysis

4.2.1 Genetic analysis

The DNA extraction from the root was done according to the method of Dellaporta et al., (1983). DNA extracted from *Azolla* root was used as a template for PCR reactions. The PCR amplification was generated from NS1-NS2 region (conserve region) and ITS1-ITS4 region (variable region). The result of NS1-NS2 (18S rDNA) primer showed that the size of PCR products is approximate 600 bp. (Figure 4.11).

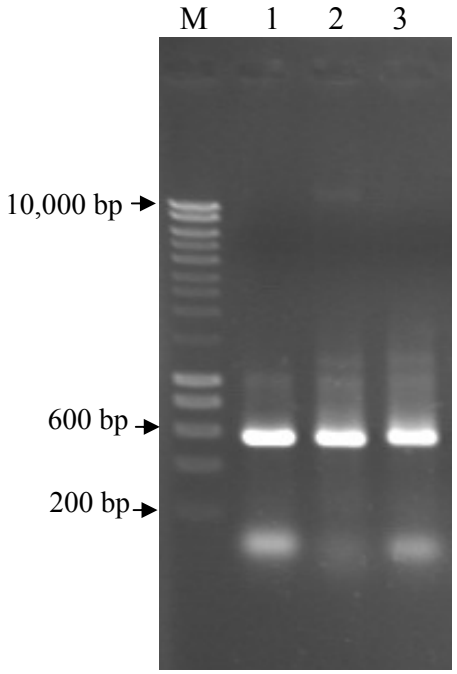

Figure 4.11 Agarose gel electrophoresis of PCR amplified fragment of *Azolla* species using NS primers; M = HyperLadder marker, Lane 1 = AZO3, Lane 2 = AZO1 Lane 3 = AZO2

Azolla species were genetically classified using Internal Transcribed Spacer (ITS) region. Genomic DNA from roots of *Azolla* species were amplified by PCR using universal primer ITS1-ITS4 (White et al., 1990) these several derived from fungi and useful for other eukaryote. The PCR product was generated in approximate size of 700, 900 and 800 bp of AZO1, AZO3 and AZO2 (Figure 4.12).

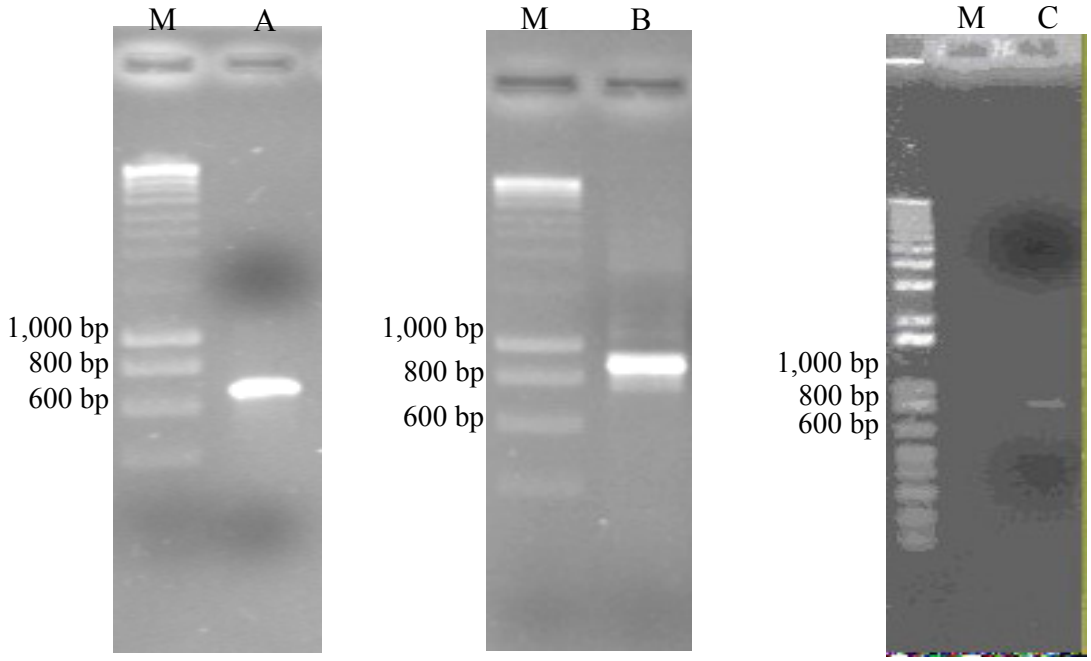

Figure 4.12 Agarose gel electrophoresis of PCR amplified fragment of *Azolla* species; A = AZO1, B = AZO3, C = AZO2 and M= HyperladderI

4.2.2 DNA sequence analysis

The partial sequence of *Azolla* species by used 18S rDNA region were obtained from the sequence analysis, and compared with other sequences of *Azolla* from Genbank database. When examined by BLAST similarity analysis, the sequences from AZO1, AZO2 and AZO3 were closely related with *Azolla* sp.Qui 02051 (Accession no. DQ629421), *A. filiculoides* (Accession no. AY612717) and *A. filiculoides* (Accession no. AY612717)**,** respectively (Figure 4.13-4.15).

DQ629421; *Azolla* sp. Qiu 02051 18S ribosomal RNA gene, partial sequence
Length=1582
Score = 893 bits (990), Expect = 0.0
Identities = 507/511 (99.2%), Gaps = 3/511 (0%)
Strand=Plus/Plus

```
AZO1   16   AACACTTTTGT-CTGTGAA-CTGCGAATGGCTCATTAAATCAGTTATAGTTTCTTTGATG  73
            ||| ||||||| ||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct    1   AACTCTTTTGTACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTCTTTGATG  60

AZO1   74   GTACCTTGCTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCACCAACTCCCGA  133
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   61   GTACCTTGCTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCACCAACTCCCGA  120

AZO1  134   CTTCTGGAAGGGACGCATTTATTAGATAAAAGGCCGATGCGGGCTTGCCCGGTATTGCGG  193
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   CTTCTGGAAGGGACGCATTTATTAGATAAAAGGCCGATGCGGGCTTGCCCGGTATTGCGG  180

AZO1  194   TGAATCATGATAACTTCCCGAATCGCACGGCCTTTGCGCCGGCGATGCTTCATTCAAATT  253
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   TGAATCATGATAACTTCCCGAATCGCACGGCCTTTGCGCCGGCGATGCTTCATTCAAATT  240

AZO1  254   TCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTGGTGACGGGTGACGG  313
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   TCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTGGTGACGGGTGACGG  300

AZO1  314   AGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGG  373
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   AGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGG  360

AZO1  374   CAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATAACAATA  433
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   CAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATAACAATA  420

AZO1  434   CTGGGCTTTTACAAGTCCGGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGGATC  493
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   CTGGGCTTTTACAAGTCCGGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGGATC  480

AZO1  494   CATTGGAGGGCAAGTCTGGTGCNCAGCAGCC  524
            |||||||||||||||||||| ||||||||
Sbjct  481   CATTGGAGGGCAAGTCTGGTGC-CAGCAGCC  510
```

Figure 4.13 Alignment sequence of AZO1 which was performed in the GenBank data library by using Basic Local Alignment Search Tool program (BLAST, http://www.ncbi.nlm.nih.gov/BLAST)

AY612717; *Azolla filiculoides* 18S ribosomal RNA gene, partial sequence

```
Length=1713
Score = 915 bits (1014), Expect = 0.0
Identities = 512/514 (99.6%), Gaps = 1/514 (0%)
Strand=Plus/Plus

AZO2   14   ATAACCTACTTTTGTACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTCTTT  73
            |||| || ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25   ATAAACT-CTTTTGTACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTCTTT  83

AZO2   74   GATGGTACCTTGCTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCACCAACTC  133
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  84   GATGGTACCTTGCTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCACCAACTC  143

AZO2  134   CCGACTTCTGGAAGGGACGCATTTATTAGATAAAAGGCCGATGCGGGCTTGCCCGGTATT  193
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 144   CCGACTTCTGGAAGGGACGCATTTATTAGATAAAAGGCCGATGCGGGCTTGCCCGGTATT  203

AZO2  194   GCGGTGAATCATGATAACTTCCCGAATCGCACGGCCTTTGCGCCGGCGATGCTTCATTCA  253
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 204   GCGGTGAATCATGATAACTTCCCGAATCGCACGGCCTTTGCGCCGGCGATGCTTCATTCA  263

AZO2  254   AATTTCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTGGTGACGGGTG  313
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 264   AATTTCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTGGTGACGGGTG  323

AZO2  314   ACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGG  373
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 324   ACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGG  383

AZO2  374   AAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATAAC  433
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 384   AAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATAAC  443

AZO2  434   AATACTGGGCTTTTACAAGTCCGGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAG  493
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 444   AATACTGGGCTTTTACAAGTCCGGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAG  503

AZO2  494   GATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCC  527
            |||||||||||||||||||||||||||||||||
Sbjct 504   GATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCC  537
```

Figure 4.14 Alignment sequence of AZO2 which was performed in the GenBank data library by using Basic Local Alignment Search Tool program (BLAST, http://www.ncbi.nlm.nih.gov/BLAST)

AY612717; *Azolla filiculoides* 18S ribosomal RNA gene, partial sequence
Length=1713
Score = 904 bits (1002), Expect = 0.0
Identities = 509/512 (99.4%), Gaps = 3/512 (0%)
Strand=Plus/Plus

```
AZO3   16   AACTCTTTTGTACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTCTTTGATG  75
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  28   AACTCTTTTGTACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTCTTTGATG  87

AZO3   76   GTACCTTGCTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCACCAACTCCCGA  135
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  88   GTACCTTGCTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCACCAACTCCCGA  147

AZO3  136   CTTCTGGAAGGGACGCATTTATTAGATAAAAGGCCGATGCGGGCTTGCCCGGTATTGCGG  195
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 148   CTTCTGGAAGGGACGCATTTATTAGATAAAAGGCCGATGCGGGCTTGCCCGGTATTGCGG  207

AZO3  196   TGAATCATGATAACTTCCCGAATCGCACGGCCTTTGCGCCGGCGATGCTTCATTCAAATT  255
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 208   TGAATCATGATAACTTCCCGAATCGCACGGCCTTTGCGCCGGCGATGCTTCATTCAAATT  267

AZO3  256   TCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTGGTGACGGGTGACGG  315
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 268   TCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTGGTGACGGGTGACGG  327

AZO3  316   AGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGG  375
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 328   AGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGG  387

AZO3  376   CAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATAACAATA  435
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 388   CAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATAACAATA  447

AZO3  436   CTGGGCTTTTACAAGTCCGGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGGATC  495
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 448   CTGGGCTTTTACAAGTCCGGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGGATC  507

AZO3  496   CATTGGAGGGCAAGTCTGG-GCCAAAGCAGCC  526
            |||||||||||||||||| |||  |||||||
Sbjct 508   CATTGGAGGGCAAGTCTGGTGCC--AGCAGCC  537
```

Figure 4.15 Alignment sequence of AZO3 which was performed in the GenBank data library by using Basic Local Alignment Search Tool program (BLAST, http://www.ncbi.nlm.nih.gov/BLAST)

The partial sequence of *Azolla* species by used ITS region primers were obtained from the sequence analysis, and compared with other sequences of *Azolla* from Genbank database. When examined by BLAST similarity analysis, the sequences from AZO1, AZO2 and AZO3 were closely related with *A. microphylla* (Accession no. DQ066481), *A. mexicana* (Accession no. DQ066479) and *A. filiculoides* (Accession no. DQ066494). The results showed that 99.3%, 99.0% and 99.2% respectively similarity of internal transcribed spacer of nuclear ribosomal RNA gene sequence with *Azolla* species in GenBank (Figure 4.16-4.19).

DQ066481; *Azolla microphylla* voucher Reid & Peters 77 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence Length=827 Score = 1598 bits (806), Expect = 0.0 Identities = 819/824 (99.3%), Gaps = 0/824 (0%) Strand=Plus/Minus

```
AZO1   11   CGCCTGATCTGAGGTCCGTTTGATAGTTTCGGTCTAGGCAACACCTAGAACGATTAGCCC  70
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  824  CGCCTGATCTGAGGTCCGTTTGATAGTTTCGGTCTAGGCAACACCTAGAACGATTAGCCC 765

AZO1   71   GACTCGATAGAAGCATCCTTAAACGGGAAACACGTCGCAACAGTTGCAATCTCGGAGCCT 130
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  764  GACTCGATAGATGCATCCTTAAACGGGAAACACGTCGCAACAGTTGCAATCTCGGAGCCT 705

AZO1   131  TGATGGGCTACGGAAGCAAACCACCCTTGCGACGCATCGCCACACGGAGCAATCGATGCA 190
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  704  TGATGGGCTACGGAAGCAAACCACCCTTGCGACGCATCGCCACACGGAGCAATCGATGCA 645

AZO1   191  TTTCAGCCGACCGCTAGTGCCAAATACTCACATCATGTGCAAGAAAGGCTCCAACGGACA 250
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  644  TTTCAGCCGACCGCTAGTGCCAAATACTCACATCATGTGCAAGAAAGGCTCCAACGGACA 585

AZO1   251  ACCATCTCCGCCACTCCATCACACCAAATGGTGGGGGAGTGGCGTGGGGGTCGTGAGGAC 310
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  584  ACCATCTCCGCCACTCCATCACACCAAATGGTGGGGGAGTGGCGTGGGGGTCGTGAGGAC 525

AZO1   311  GCTCAGGCAGACGTGCCCTTGGACAAGCCTCGGGCGCAAGTTGCGTTCAAAGACTCGATG 370
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  524  GCTCAGGCAGACGTGCCCTTGGACAAGCCTCGGGCGCAAGTTGCGTTCAAAGACTCGATG 465

AZO1   371  ATTCGCGGAATTCTGCAATTCACATTACGTATCGCATTTCGCTGCGTTCTTCATCGTTGC 430
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  464  ATTCGCGGAATTCTGCAATTCACATTACGTATCGCATTTCGCTGCGTTCTTCATCGTTGC 405

AZO1   431  AAGAGCCAAGATATCCGTTGCTGAGAGTCGTTTGTGATTGTATATCCTCCCATAAGGAGG 490
            |||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  404  AAGAGCCAAGATATCCGTTGCTGAGAGTCGTTTGTGATTGTATATCCTCCCATAGGGAGG 345

AZO1   491  GCGTCTCAGTCAATAAGTTTTATGGGTTGCCTCCCGCTCCCTCCGTACCCCACGAGGGGG 550
            |||||||||||||||||||||||||||||||||| |||||||||||||||| ||||||
Sbjct  344  GCGTCTCAGTCAATAAGTTTTATGGGTTGCCTCCCACTCCCTCCGTACCCCACTAGGGGG 285
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AZO1   551  GTTAGGAGGGGCAAGAGATGGTTTGACATAGCGGCCCCGTGCCAATACGACACGGAGGAG 610
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  284  GTTAGGAGGGGCAAGAGATGGTTTGACATAGCGGCCCCGTGCCAATACGACACGGAGGAG 225

AZO1   611  GGGGGTCGACCGCACGATCCGCACAATGAGAAGCGTCCCTCTCATCGTGCATATCGCACT 670
            |||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||
Sbjct  224  GGGGGTCGACCGCACGATCCGCACAATGASAAGCGTCCCTCTCATCGTGCATATCGCACT 165

AZO1   671  CTCGGTGGGACAACCCCTACAACCCACACAAGACCCACAACGTGCCCCGCATCTGGCACA 730
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  164  CTCGGTGGGACAACCCCTACAACCCACACAAGACCCACAACGTGCCCCGCATCTGGCACA 105

AZO1   731  GGAGTCGAGCGGGGCGGACACGCCTCCGAGCGCGTCGCCTCAAAATACAAGCACGGTTCG 790
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  104  GGAGTCGAGCGGGGCGGACACGCCTCCGAGCGCGTCGCCTCAAAATACAAGCACGGTTCG  45

AZO1   791  CGTGGTTGGTTTGGATGTGTGTCAATGATCCTTCCGCAGGTTCA  834
            |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  44   CGTGGTTGGTTTGGATGTGTGTCAATGATCCTTCCGCAGGTTCA  1
```

Figure 4.16 Alignment sequence of AZO1 which was performed in the GenBank data library by using Basic Local Alignment Search Tool program (BLAST, http://www.ncbi.nlm.nih.gov/BLAST

DQ066479; *Azolla mexicana* voucher Reid & Peters 69 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence Length=827 Score = 1418 bits (1572), Expect = 0.0 Identities = 801/809 (99.0%), Gaps = 3/809 (0%) Strand=Plus/Minus

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AZO2   19   AGGTC-GTTTGA-AGTTTCGGTCTAGGCAACACCTAGAACGATTAGCCCGACTCGATAGA  76
            ||||| |||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  813  AGGTCCGTTTGATAGTTTCGGTCTAGGCAACACCTAGAACGATTAGCCCGACTCGATAGA  754

AZO2   77   AGCATCCTTAAACGGGAAACACGTCGCAACAGTTGCAATCTCGGAGCCTTGATGGGCTAC  136
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  753  AGCATCCTTAAACGGGAAACACGTCGCAACAGTTGCAATCTCGGAGCCTTGATGGGCTAC  694

AZO2   137  GGAAGCAAACCACCCTTGCGACGCATCGCCACACGGAGCAATCGATGCATTTCAGCCGAC  196
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  693  GGAAGCAAACCACCCTTGCGACGCATCGCCACACGGAGCAATCGATGCATTTCAGCCGAC  634

AZO2   197  CGCTAGTGCCAAATACTCACATCATGTGCAAGAAAGGCTCCAACGGACAACCATCTCCGC  256
            |||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||
Sbjct  633  CGCTAGTGCCAAATACTCWCATCATGTGCAAGAAAGGCTCCAACGGACAACCATCTCCGC  574

AZO2   257  CACTCCATCACACCAAATGGTGGGGGAGTGGCGTGGGGGTCGTGAGGACGCTCAGGCAGA  316
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  573  CACTCCATCACACCAAATGGTGGGGGAGTGGCGTGGGGGTCGTGAGGACGCTCAGGCAGA  514

AZO2   317  CGTGCCCTTGGACAAGCCTCGGGCGCAAGTTGCGTTCAAAGACTCGATGATTCGCGGAAT  376
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  513  CGTGCCCTTGGACAAGCCTCGGGCGCAAGTTGCGTTCAAAGACTCGATGATTCGCGGAAT  454

AZO2   377  TCTGCAATTCACATTACGTATCGCATTTCGCTGCGTTCTTCATCGTTGCAAGAGCCAAGA  436
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  453  TCTGCAATTCACATTACGTATCGCATTTCGCTGCGTTCTTCATCGTTGCAAGAGCCAAGA  394
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AZO2   437  TATCCGTTGCTGAGAGTCGTTTGTGATTGTATATCCTCCCATAAAGAGGGCGTCTCAGTC  496
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  393  TATCCGTTGCTGAGAGTCGTTTGTGATTGTATATCCTCCCATAAGGAGGGCGTCTCAGTC  334

AZO2   497  AATAAGTTTTATGGGTTGCCTCCCGCTCCCTCCGTACCCCACCAGGGGGGTTAGGAGGGG  556
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  333  AATAAGTTTTATGGGTTGCCTCCCGCTCCCTCCGTACCCCACGAGGGGGGTTAGGAGGGG  274

AZO2   557  CAAGAGATGGTTTGACATAGCGGCCCCGTGCCAATACGACACGGAGGAGGGGGGTCGACC  616
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  273  CAAGAGATGGTTTGACATAGCGGCCCCGTGCCAATACGACACGGAGGAGGGGGGTCGACC  214

AZO2   617  GCACGATCCGCACAATGAGAAGCGTCCCTCTCATCGTGCATATCGCACTCTCGGTGGGAC  676
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  213  GCACGATCCGCACAATGASAAGCGTCCCTCTCATCGTGCATATCGCACTCTCGGTGGGAC  154

AZO2   677  AACCCCTACAACCCACACAAGACCCACAACGTGCCCCGCATCTGGCACAGGAGTCGAGCG  736
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  153  AACCCCTACAACCCACACAAGACCCACAACGTGCCCCGCATCTGGCACAGGAGTCGAGCG  94

AZO2   737  GGGCGGACACGCCTCCGAGCGCGTCGCCTCAAAATACAAGCACAGTTCGCGTGGTTGGTT  796
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  93   GGGCGGACACGCCTCCGAGCGCGTCGCCTCAAAATACAAGCACGGTTCGCGTGGTTGGTT  34

AZO2   797  TGGATGTGTGTCAATGATCCTTCCCGCAG  825
            |||||||||||||||||||| ||||||
Sbjct  33   TGGATGTGTGTCAATGATCCTT-CCGCAG  6
```

Figure 4.17 Alignment sequence of AZO2 which was performed in the GenBank data library by using Basic Local Alignment Search Tool program (BLAST, http://www.ncbi.nlm.nih.gov/BLAST

DQ066494; *Azolla filiculoides* voucher Reid & Peters 68 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene,complete sequence; and internal transcribed spacer 2, partial sequence Length=841, Score= 1542 bits(778), Expect= 0.0Identities 794/800(99.2%), Gaps= 0/800 (0%), Strand= Plus/Plus

```
AZO3  23  ACGCGAACCTTGCTTGTATTTTGAGGCGACGCGCTCGGAGGCGTGTCCGCCCCGCTTGAC 82
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 42  ACGCGAACCTTGCTTGTATTTTGAGGCGACGCGCTCGGAGGCGTGTCCGCCCCGCTTGAC 101

AZO3  83  TCCTGTGCTAGATCCGGGGCACGTCGTGGGTCATGTGTGGGTTGTAGGGGTTGTCCCACC 142
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 102 TCCTGTGCTAGATCCGGGGCACGTCGTGGGTCATGTGTGGGTTGTAGGGGTTGTCCCACC 161

AZO3  143 GAGAGTGCGATATGTATGATGACAGGGACGCCTGTCTTTGTGCGCATCGTGCGGTCGACC 202
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 162 GAGAGTGCGATATGTATGATGACAGGGACGCCTGTCTTTGTGCGCATCGTGCGGTCGACC 221

AZO3  203 CCCCTCCTCCGTGTCGTATCGGCACGGGGCCGCTATGTCAAACCATCTCTTGCCCCTCCT 262
          |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct 222 CCCCTCCTCCGTGTCGTATCGGCACGGGGCCGMTATGTCAAACCATCTCTTGCCCCTCCT 281

AZO3  263 AACCCCCCTCGTGGGGTAAGGAGGGAGCGGGAGGCAACCCATAAACGCATTGACTGAGAC 322
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 282 AACCCCCCTCGTGGGGTAAGGAGGGAGCGGGAGGCAACCCATAAACGCATTGACTGAGAC 341
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AZO3  323 GCCCTCCCTATGGGAGGATACATATTAACAAACGACTCTCACCAACGGATATCTTGGCTC 382
          |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct 342 GCCCTCCCTATGGGAGGATACATATTAACAAACGACTCTCAGCAACGGATATCTTGGCTC 401

AZO3  383 TTGCAACGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCCGCGA 442
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 402 TTGCAACGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATTCCGCGA 461

AZO3  443 ATCATCTAGTCTTTGAACGCAACTTGCGCCCGAGGCTTGTCCAAGGGCACGTCTGCCTGA 502
          |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 462 ATCATCGAGTCTTTGAACGCAACTTGCGCCCGAGGCTTGTCCAAGGGCACGTCTGCCTGA 521

AZO3  503 GCGTCCTCACGACCCCCACGCCGCTCCCCCTCCACTCGGTGGGATGGAGTATCGGAGATG 562
          |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct 522 GCGTCCTCACGACCCCCACGCCGCTCCCCCTCCACTCGGTGGGATGGAGTAGCGGAGATG 581

AZO3  563 GTTGTCCGTTGGAGCCCTTCTAGCGGCTCTAGCGGTCGGCTGAAATGCATCGATTGCTCC 622
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 582 GTTGTCCGTTGGAGCCCTTCTAGCGGCTCTAGCGGTCGGCTGAAATGCATCGATTGCTCC 641

AZO3  623 GTGTGGCGATGCGTCGCAAGGGTGGTTTGCTTCCTCGGCCCGCAAGGGATCCCAGATCGC 682
          |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct 642 GTGTGGCGATGCGTCGCAAGGGTGGTTTGCTTCCTCGGCCCGCAAGGGATCCGAGATCGC 701

AZO3  683 AACTGTTGCGGCGTGTTACCCGATCAAGGATGCTTATATCGAGTCGGGCTAATCGATCTG 742
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 702 AACTGTTGCGGCGTGTTACCCGATCAAGGATGCTTATATCGAGTCGGGCTAATCGATCTG 761

AZO3  743 GGCATTACGTCCATTTGCTTCGTGCAGTTTGGCGTTATGCGTAGACCGAAACCATCGAAC 802
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 762 GGCATTACGTCCATTTGCTTCGTGCAGTTTGGCGTTATGCGTAGACCGAAACCATCGAAC 821

AZO3  803 GGACCTCACATCAGGCGAGA 822
          |||||||| ||||||||||
Sbjct 822 GGACCTCAGATCAGGCGAGA 841
```

Figure 4.18 Alignment sequence of AZO3 which was performed in the GenBank data library by using Basic Local Alignment Search Tool program (BLAST, http://www.ncbi.nlm.nih.gov/BLAST

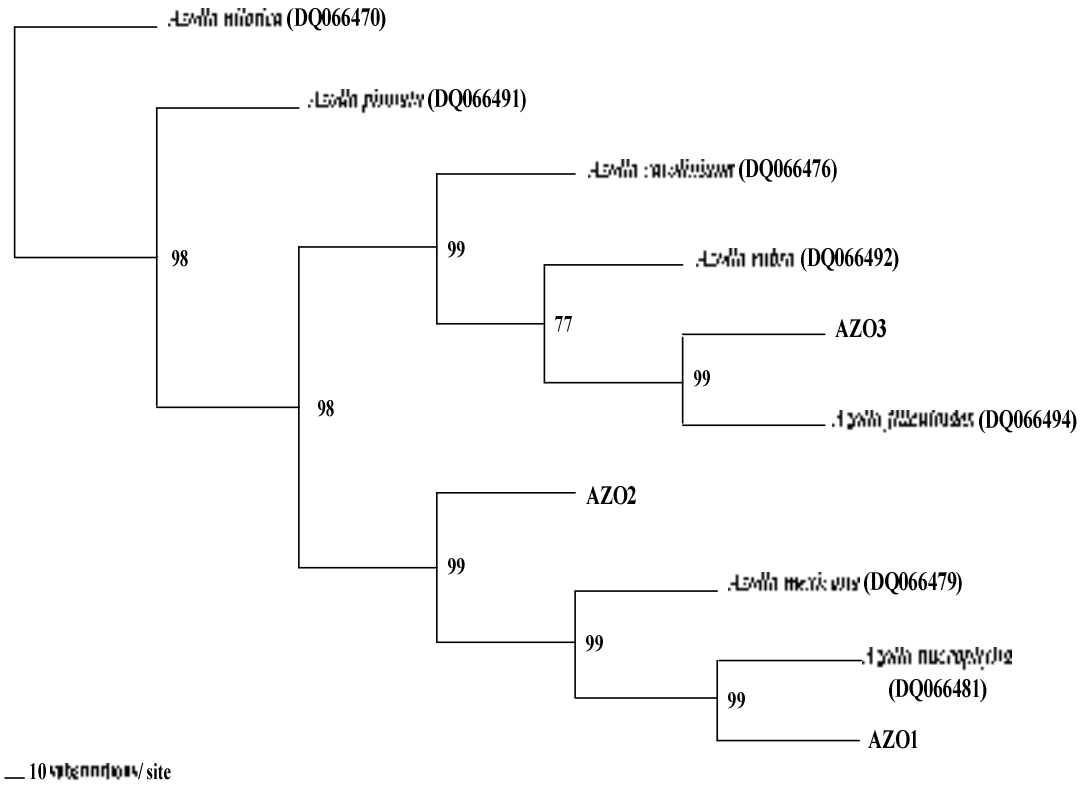


Figure 4.19 Phylogenetic relationship of *Azolla* species by used ITS region primer. Branch lengths are proportional to the numbers of nucleotide and amino acid changes, and the numerals given on the branches are the frequencies with which a given branch appeared in 100 bootstrap replications. Reference sequences were retrieved from GenBank under the accession numbers in parentheses.

Three different morphological species (AZO1, AZO2 and AZO3) were analyzed sequence of *Azolla* by used 18S rDNA region and ITS region. Alignment of the partial SSU rRNA (18S rDNA) fragment generated using NS1-2 primers corresponding to one species; *A. filiculoides*. Almost of them gave up to 99% homology with responding the sequence from database. Whereas, the results of

sequencing when using ITS region corresponding to three species; *A. microphylla, A. mexicana* and *A. filiculoides*. These provided 99.3, 99.0 and 99.2% homology with the sequence from database. (Table 4.1) Thus the sequencing from ITS gave highest species due to ITS region has typically been most useful for molecular systematics at the species level and even within species. Because of its higher degree of variation than other regions of rDNA (SSU).

Table 4.1 Analysis of 18SrRNA and Internal transcribe spacer of nuclear ribosomal RNA (rRNA) of *Azolla* species.

Species	Primer	% homology	Closest organism	Accession no.
AZO1	18SrRNA	99.2	*Azolla* sp. Qiu 02051	DQ629421
AZO2		99.6	*Azolla filiculoides*	AY612717
AZO3		99.4	*Azolla filiculoides*	AY612717
AZO1	ITS	99.3	*Azolla microphylla*	DQ066481
AZO2		99.0	*Azolla mexicana*	DQ066479
AZO3		99.2	*Azolla filiculoides*	DQ066494

4.2.3 Comparison of *Azolla* species DNA sequencing and morphological analysis.

Part of classification, *Azolla* have been amplified and sequenced using universal primer (NS1-NS2) and non-coding internal transcribed spacers (ITS) of the DNA from *Azolla* root that used to classified species. In addition the standard ITS1-ITS4 primers were used in this study. The results of sequencing (18S rDNA) in this study indicated the classification of AZO1 (99.2% homology with *Azolla* sp. Qiu

02051), AZO2 (99.6% homology with *A. filiculoides*) and AZO3 (99.4% homology with *A. filiculoides*). Whereas, the results of sequencing when using ITS region indicated that AZO1 (99.3% homology with *A. microphylla*), AZO2 (99.0% homology with *A. mexicana*) and AZO3 (99.2% homoloy with *A. filiculoides*). In the part of morphological analysis showed the classification of AZO1 was *A. microphylla*, AZO2 was *A. cristata* (*A. caroliniana, A. microphylla and A. mexicana* are considered the same morphology) and AZO3 was *A. filiculoides*.

The comparative results from two techniques were summarized in Table 4.2 The *Azolla* contain divergent rDNA seguence which make it difficult to distinquish closely related *Azolla* by sequence comparisons. However the result of ITS region gave accuracy classification of AZO2 (99.0% homology with *A. mexicana*) when compared with morphological classification. Thus, the sequencing from ITS region gave highest species because of its higher degree of variation than other region of rDNA (SSU). The result of molecular is also clearly distinguish *Azolla* speices, these indicated that *A. microphylla*, *A. mexicana* and *A. filiculoides* were classified from this study.

Table 4.2 Comparison analysis of DNA sequence from *Azolla* and morphological classification.

Isolate	**Morphological classification**	**DNA sequence analysis**	
		18S rDNA	**ITS region**
AZO1	*A. microphylla*	*Azolla* sp. Qiu 02051	*A. microphylla*
AZO2	*A. cristata*	*A. microphylla*	*A. mexicana*
AZO3	*A. filiculoides*	*A. filiculoides*	*A. filiculoides*

4.3 Soil characterization

There were slight decrease in organic matter contents, total nitrogen and potassium in the most treatments (Table 4.3). The maximum of organic matter content was found to be 1.39% in treatment T2. When compared between initial and post harvest soil in each treatment this results showed the close to values. Rice was applied organic matter and total nitrogen from *Azolla* for rice growth these cause why most treatment were decreased. In the other hand, the most phosphorus values in each treatment were increased because of phosphorus fertilizer that applied for *Azolla* growth.

Table 4.3 Effect of *Azolla* and chemical fertilizer on organic matter, N, P and K in initial and post harvest soil in rice field.

Treatment	pH		OM (%)		Total N (%)		Extr.P (ppm)		Exch.K (ppm)	
	initial	post	initial	post	initial	post	initial	post	initial	post
T1 (AZO1)	7.85	7.32	1.26	1.15	0.06	0.05	14	17	149	88
T2 (AZO2)		7.15		1.39		0.05		31		113
T3 (AZO3)		7.32		1.20		0.05		20		84
T4 (fertilizer)		6.90		1.09		0.04		17		79
T5 (No *Azolla*)		6.50		1.17		0.04		11		94

4.4 Application of *Azolla* species as biofertilizer in rice field

The experiment was conducted during August 2006 to December 2006 using RCBD with three replications. Fresh *Azolla* plants were inoculated at the rate of 250 g m^2 into 2m x 4m plots. All plots were stagnant with a water level. The treatments consisted of 1) rice with AZO1, 2) rice with AZO2, 3) rice with AZO3, 4) rice with

chemical fertilizer (12-8-8 kg N-P_2O_5-K_2O/ rai) and 5) rice without *Azolla* and fertilizer. Statistical analyses demonstrated the difference between treatments. All results of growth parameter of KDML 105 rice (Plant height, Number of filled grain/ panicle and 100 grain weight (g)) and grain yield (t ha^{-1}) were summarized in Table 4.4 and 4.5, respectively.

Table 4.4 Effect of *Azolla* and chemical fertilizer application on different growth parameters of KDML 105 rice

Treatment	Plant height (cm)	Number of tillers/ hill	Number filled grain/ panicle	100 grain wt. (g)
T1 (AZO1)	106b	11.1a	239a	2.46b
T2 (AZO2)	101b	10.9a	240a	2.40a
T3 (AZO3)	96ab	11.2a	237a	2.34a
T4 (fertilizer)	103b	11.1a	252a	2.46b
T5 (No *Azolla*)	81a	9.3a	217a	2.36a

AZO1 showed a significantly highest plant height as 105.95 cm while there was no significant difference between treatment of *Azolla* and chemical fertilizer except non-applied *Azolla* treatment. For number of tiller/ hill and number of filled grain/ panicle did not differ in all of treatments. The highest 100-grain weight was detected as 2.46 g in both treatment of AZO1 and chemical fertilizer treatment.

Table 4.5 Effect of *Azolla* species on the grain yields of KDML 105 rice.

Treatment	Grain yield (t ha^{-1})	% increasing over control
T1 (AZO1)	4.97c	16.72
T2 (AZO2)	4.49ab	7.92
T3 (AZO3)	4.15a	0.29
T4 (fertilizer)	4.72bc	12.28
T5 (No Azolla)	4.14a	-

Number between common letter in each column showed no significance different at 95% DMRT.

The increase of grain yield in each treatment (%) was compared with grain yield of no N-fertilizer treatment. Grain yield of KDML 105 rice was influenced significantly by different treatments of *Azolla* species (Table 4.5). The grain yield ranged from 4.14 to 4.97 t ha^{-1} over the treatments. The highest grain yield was observed in T1 and it was similar to T4, they gave significantly higher yield over the control. The grain yield of T3 (4.15 t ha^{-1}), however did not differ significantly with that control. The percent increase in grain yield due to different treatments over control ranged from 16.72 to 0.29. The grain yield result from *A. microphylla* (16.72) showed more increasing yield than fertilizer (12.28) and non-applied. From this experiment *Azolla* (especially *A. microphylla*) as the best basic manure in rice culture can greatly increase the yield to equivalent chemical fertilizer. According to the statistics on effectively of fertilizer experiments in *Azolla* in Kwangtong Province (Lui Xi lian et al., 1981), applying *Azolla* of 22,500 - 37,000 kg per hectare, the yield applied *Azolla* is 587-795 kg more than non-applying one. The average rate of increasing yield is 9.6-13.0%.

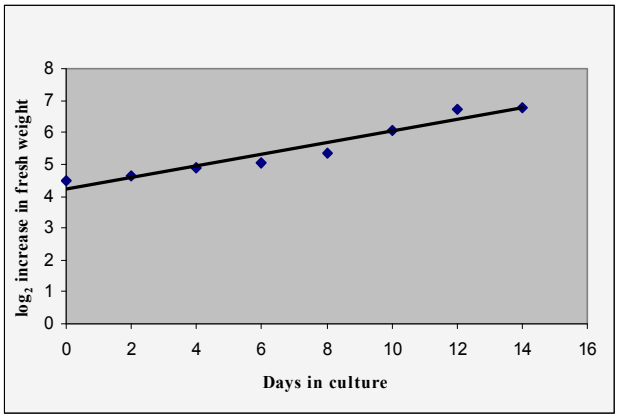
Another region where *Azolla* was used is Egypt. From 1977 to 1980, soil microbiologists of the Agricultural Research Centre, introduced three *Azolla* species: *A. pinnata*, *A. caroliniana* and *A. filiculoides* for green manuring of rice (Yanni et al., 1994). Two main procedures for *Azolla* application were used. Either the fern was grown on flooded field for 2-3 weeks and incorporated in the soil by ploughing two weeks before rice planting or transplanting, or a dual culture method was used in which *Azolla* was added during the week after rice transplanting and the propagated *Azolla* incorporate in the soil after a temporary water drainage. The environmental and climatic conditions in Egypt indicate that the second method was more appropriate (Yanni et al., 1994). The result was better when *Azolla* was incorporated in soil than was left floating during the all the rice growth season. With this technique the researchers could save half of the recommended amounts of chemical nitrogen fertilizer (urea) and minimize the hazard effect of nitrate and nitrite ions in water resources (Yanni et al., 1994).

From the result of Gevrek (2000), showed the effect of *A. mexicana* combined with inorganic fertilizer on rice yield under Aegean conditions. This research found statistically important according to grain yield among years, combination and varieties. The highest yield was 4.4 t ha^{-1} Toag92 rice. The result concluded that applying *Azolla* with 200 g. m^2 fresh weight in rice might save about 1/3 of mineral fertilize in these study conditions. Hossain et al. (2001), compared the effect of N (80 kg N ha^{-1}) with that of incorporated two layers of *Azolla* plus 40 kg N ha^{-1} on the yield of BR 26 rice. The highest grain of 3.95 t ha^{-1} with the highest total dry matter (grain + straw) was recorded for incorporation of two layers of *Azolla* (grown from 0.2 kg m^{-2} inoculum).

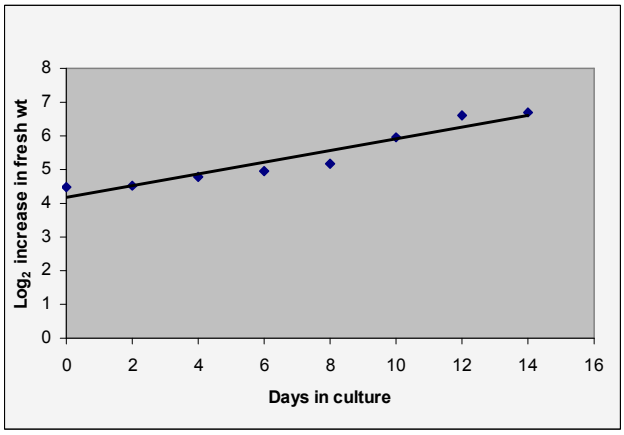
4.5 Response of *Azolla*

Azolla fresh weight, dry weight, Acethylene Reduction Activity (ARA), doubling time and N yield were determined at 21 days after application. The results were summarized in Table 4.6. The result showed that fresh weight was significantly difference in AZO1 and AZO2 while there was no significant difference in AZO3. The highest fresh weight was detected as 1174 g/m^2 in AZO1. For dry weight, the highest dry weight observed in AZO1 while the result showed that there was a significant difference. The doubling time of AZO1 and AZO2 did not differ significantly under field condition, whereas AZO3 showed poor biomass and longer doubling time (Figure 4.20). The result of AZO1 showed lowest doubling time as 3.8 days under field condition. Gopalaswamy and Kannaiyan (1998) also reported doubling time of 5.8 days at 14 days incubation in IRRI medium for *A. microphylla*.

(a)



(b)



(c)

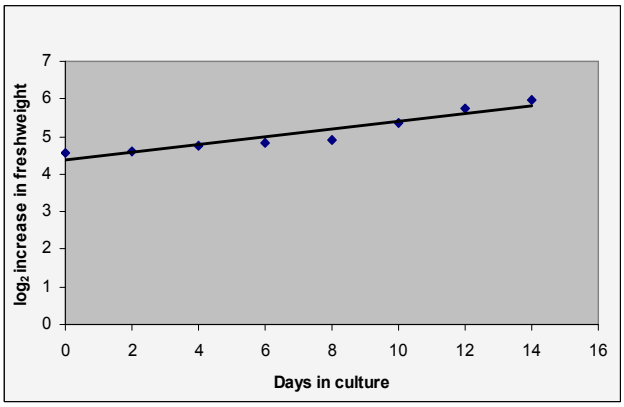


Figure 4.20 Growth of AZO1 (a), AZO2 (b) and AZO3 (c) cultured on N-free medium. *Azolla* plant grown in a nitrogen-free medium under natural light and temperature conditions were used as the starting material.

Results from Table 4.6 indicated that there were no differences in ARA. Maximum ARA of 13.58 µmole C_2H_4/g dw /h was recorded in AZO2. Lowest value was recorded in sample from AZO3 but did not exhibit any significant differences. The total nitrogen of *Azolla* ranged from 2.94-3.62% with the highest recorded by AZO1 followed by AZO3 and AZO2, respectively. Nitrogen is the main factor of increasing rice yield by culturing *Azolla* in paddy fields (Lui Chung-chu, 1979). The content of nitrogen in fresh *Azolla* is generally about 0.25% (vary with *Azolla* species, climate and propagating technique).

Table 4.6 Fresh weight, dry weight, acetylene reduction activity, growth rate and Total N in different species in rice field.

Azolla	**fresh weight**	**dry weight**	**doubling time**	**ARA**	**%Total N**
	(g/ m^2)	**(g/ m^2)**	**(days)**	**µmole C_2H_4/g dw/h**	
AZO1	1774b	98.66b	3.8a	13.12a	3.62b
AZO2	1568b	89.74a	3.9a	13.58a	2.94a
AZO3	633a	80.80a	6.6b	11.26a	3.26ab

Same letter in the same column showed no significance different at 95% DMRT.

The heat and pest caused the low biomass productivity and high doubling time in AZO3. *Azolla* species have different levels of tolerance to temperature extremes and as a result some can survive within a very wide temperature range while others can survive only within a very narrow temperature range. *A. filiculoides* is quite tolerant to low temperature, whereas *A. mexicana*, *A. microphylla* and *A. caroliniana* posses considerable tolerance to high temperature (Lumpkin and Plucknett, 1982). The pests, water snails, rice field craps and larvae of Lepidoptera were found to interfere its

cultivation. Furadan (carbofuran) was found to be the least toxic for *Azolla* growth and was used for pest control in this experiment. For larvae of Lepidoptera was eliminated with monocrotophos 56% W.P. sprayed to the *Azolla* plots every week.

Data from three species showed that AZO1 and AZO2 are adapted to tropical conditions of Thailand. From the result of Boonkerd and co-worker (1986) showed the selection of *Azolla* under field condition at different location in Thailand. Field test at Khon Kaen Rice experimental station showed no differences in *Azolla* growth (doubling time) and ARA. There were significantly difference in *Azolla* mass and doubling time in Chiangmai's field study. From this research, that the performances of selected *Azolla* including two exotic species *A. caroliniana* and *A. microphylla* (Choonlunchanon et al., 1988) in three field sites were satisfactory these were relatively less infected by fungal disease and insects as compared to the *A. pinnata*. Lumpkin and Bartholomew (1986) who reported that *A. microphylla* was resistant to fungal attack and heat stress.

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BIOGRAPHY

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