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A Nutritional and Morphological Study of Two Cyanobacteria Free Azolla Ferns

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This is to certify that the thesis prepared by Michael Paul Bonner entitled A Nutritional and Morphological Study on Two Species of Cyanobacteria Free Azolla Ferns has been approved by his committee as satisfactory completion of the thesis requirement for the degree of Master of Science in Biology.

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A NUTRITIONAL AND MORPHOLOGICAL STUDY OF TWO CYANOBACTERIA FREE AZOLLA FERNS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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ABSTRACT

The symbiotic association between the aquatic water fern Azolla and its symbiont, Anabaena azollae Straus. has been extensively studied in the past ten years. The cyanobacteria has received much of the attention due to its ability to fix atmospheric nitrogen while the fern free of the cyanobiont has been somewhat neglected. The purpose of this research was to determine the nutritional requirements of the cyanobacteria free (CBF) ferns and study the morphology of the leaves and branches of these CBF ferns.

Azolla filiculoides and Azolla pinnata were freed of the cyanobiont using the surface sterilization technique adopted from Duckett, et al.(1975) and the antibiotic treatments of Peters and Mayne(1974a). The size of leaf seven, both length and width, along with leaf spacing and branch spacing along the stem, in CBF ferns were compared to that in the intact ferns. Growth studies on medium containing three levels of the nutrients nitrogen, phosphorus and potassium were conducted.

The CBF ferns were found to have shorter leaves with no difference in width. The leaf spacing and branch spacing in the CBF ferns were more compact than the intact association. This is the first reported quantitative data to show a

definite difference in the morphology of the CBF ferns. The nutrient studies showed an interaction effect between nitrogen and phosphorus in the A. pinnata CBF ferns while the A. filiculoides CBF ferns showed a nitrogen, phosphorus, potassium interaction. The A. pinnata CBF ferns grew best on a medium containing twice the concentration of phosphorus, one times the concentration of nitrogen and three times the concentration of potassium found in our I+ medium. The A. filiculoides CBF ferns grew best on concentrations of twice phosphorus, half nitrogen and three times potassium found in our I+ medium.

The results of this research shows the importance of phosphorus to the growth of the CBF ferns. This has been demonstrated in the intact fern association by Subudhi and Watanabe(1981). The results of the morphological studies indicate that either the cyanobiont, by its presence or through secretion of some substance affects the intact ferns growth and that this change can not be completely removed by the addition of specific nutrients to the medium.

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INTRODUCTION

The water fern Azolla and its symbiont Anabaena azollae have received much attention recently due to the ability of the association to fix atmospheric nitrogen(Watanabe, et al.,1977; Lumpkin and Plucknett,1980; Peters, et al.,1980a; Talley and Rains, 1980b). Azolla belongs to the order Salviniales of which there are two genera, Azolla and Salvinia (Moore,1969; Bold,1980). The genus Azolla contains two sections or subgenera into which the six extant species are grouped(Moore, 1969; Becking, 1979; Lumpkin and Plucknett, 1980). Section I, the Euazolla is characterized by the presence of three floats on the megaspore(Becking, 1979; Lumpkin and Plucknett, 1980).This section contains the four species A. filiculoides Lamarck, A. caroliniana Willd., A. microphylla Kalfuss and A. mexicana Presl. Section II, the Rhizosperma has two species and is characterized by the presence of nine megaspore floats. The two species of this subgenera are A. pinnata R. Brown which has two morphologically distinct forms, (A. pinnata var. pinnata R. Br. and A. pinnata var. imbricata Roxb. Bonap.) and A. nilotica Decaisne(Becking, 1979). The oldest living species are A. filiculoides of the Euazolla and A. pinnata

of Rhizosperma both of which date back to the Pleistocene era(Hills and Gopal 1967 in Lumpkin and Plucknett, 1980). Azolla is widely distributed throughout tropical and temperate fresh waters(Talley and Rains, 1980a). Species native to the United States are A. caroliniana in the East, A. filiculoides in the West(Bold,1980; Lumpkin and Plucknett,1980), and A. mexicana in the upper plains and Southwest(Lumpkin and Plucknett, 1980).

All of the currently living species of Azolla are known to contain the heterocystous cyanobiont Anabaena azollae Stras. which was first noted by Strasburger in 1873 in A. filiculoides (Lumpkin and Plucknett,1980). The age of this symbiosis is not known since the cyanobiont has not been recognized in fossil form (Becking, 1979). The ferns life cycle is outlined in Figure 1. The cyanobiont is maintained during the life cycle through infection of the macrosporocarp(Becking, 1978), and is located under the indusium cap of the macrosporocarps(Ashton and Walmsley, 1976). When the zygote is formed the cyanobiont becomes closely associated with the apical meristem of the fern. As the meristem forms the leaf primordia, the filaments of Anabaena become entrapped in the developing cavity in the dorsal lobe where they remain. This cavity provides the cyanobacteria with the microenvironment conducive to nitrogen fixation as well as affording protection from the atmosphere(Ashton and Walmsley, 1976). Studies have

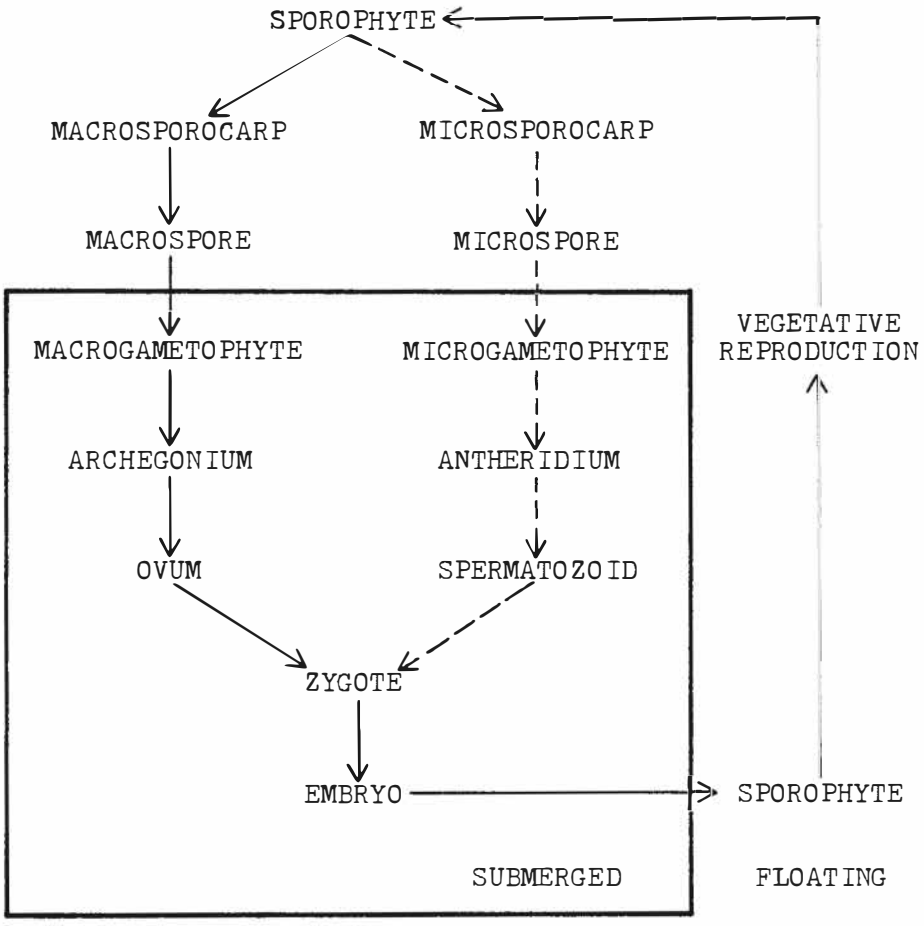

Figure 1. Life cycle of Azolla. From Becking, 1978; and Lumpkin and Plucknett, 1980. Solid lines indicate how the cyanobiont is passed on in the association.

indicated that it is only the cyanobacterium, Anabaena azollae, that fixes the atmospheric nitrogen(Peters and Mayne, 1974b).

It has been repored that this symbiosis rivals that of the Rhizobium-legume symbiosis in its rate of atmospheric nitrogen-fixation(Watanabe, et al., 1977; Lumpkin and Plucknett, 1980). This ability to fix nitrogen has been responsible for the interest in utilizing the Azolla fern as a nitrogen fertilizer for agricultural food crops. Azolla has been used as a fertilizer crop in Vietnam for several centuries and it is currently being grown in mainland China, parts of Thailand and Indonesia. Further research is also being carried on in India, the Republic of China, and the Philippines (Talley and Rains, 1980; Lumpkin and Plucknett, 1980). The main use of Azolla has been as a nitrogen fetilizer for rice crops, but it has also been used as a food source for animals in some countries.

1.1 AGRICULTURAL SIGNIFICANCE OF AZOLLA

The potential use of Azolla as a photosynthetic nitrogen fertilizer producer is great. With the increase in the cost of production of nitrogen fertilizers this fern may provide an alternative means to incorporate nitrogen into the soil. Several field studies have been conducted to determine the amount of nitrogen that Azolla can incorporate. From these studies it has been shown that temperature is the single

most influential factor affecting the nitrogen fixation rate of Azolla(Talley and Rains, 1980b). Talley and Rains(1980a) reported that the highest rate of nitrogen-fixation occurred in fallow season growth studies when the morning and afternoon surface temperatures are between 25-30C and the midday Azolla surface temperatures ranged between 30-40C. These conditions occurred during the month of April, in California, and resulted in the fastest growth and nitrogen fixation rates.

Several reseachers(Becking, 1979) have estimated the potential annual nitrogen-fixation rates for several species of Azolla, but these estimates are based on unlimited continuous growth in open water under optimal conditions. Since the growth of Azolla under field conditions is discontinuous and not always under optimal conditions these estimates tend to be higher than the actual rates(Becking, 1979). Becking revised his values based on this information and also included heterotropic dark dinitrogen-fixation to arrive at a value of 103-162 Kg N/ha/yr. Talley et al.(1977) reported maximum yields of 24-45 Kg N/ha/dry wt. for A. mexicana and 58-105 Kg N/ha/dry wt. for A. filiculoides. They also reported that A. filiculoides and A. mexicana have a nitrogen fixation rate of 1.2 Kg N/ha/day between 10-35 days after field inoculation which agrees with that reported by Watanabe et al.(1977) of 1.1 Kg N/ha/day. Watanabe et al.(1980) have reported a daily

average nitrogen accumulation rate of 1.4 Kg N/ha in a field experiment in which 22 consecutive crops grown during 335 days had a total nitrogen accumulation of 465 Kg N/ha. This rate was comparable with that reported by Nutman(1976) for legumes. Talley and Rains(1980a) reported that incorporation of 40 Kg N/ha of dried A. filiculoides into the soil during spring field preparation resulted in an increase in rice yields equivalent to that obtained with an equal amount of ammonium sulfate. However, when 93 Kg N/ha of dry A. filiculoides was incorporated into the soil this resulted in a rice yield 30% less than that obtained with the same amount of ammonium sulfate. Talley and Rains(1980a), noting that Watanabe, et al., 1977 had reported that Azolla incorporated into the soil released its nitrogen slowly, suggested that the decreased yield of the Azolla may be due to a loss through denitrification or the fact that the release is so slow that it is not all available for use in a single season. Using this information and that from Singh(1977) and Watanabe et al.(1977) Talley and Rains calculated that 228 Kg N/ha of Azolla would be necessary to produce rice yields equivalent to those now achieved by use of 160 Kg N/ha of ammonium sulfate.

In a field study Talley and Rains(1980a) reported that the production of 93 Kg N/ha as Azolla nitrogen was obtained by the application of 30 Kg P/ha and 0.15 Kg Fe/ha. This

level of phosphorus(P) is only 10% over that recommended for California rice crops(Talley and Rains, 1980a). The use of this level of phosphorus with the fallow season growth of Azolla would still leave some of the P available for the summer rice crop as well as enhance the N content of the soil. They have also reported that the yield of a single crop of A. filiculoides grown over 46 days (93 Kg N/ha) would supply 43% of the current California rice fertilizer requirement (Talley and Rains, 1980a). This shows the importance that Azolla can play in use as a green manure, but Azolla can also be used as a crop grown simultaneously with rice.

1.2 AZOLLA USE IN RICE PRODUCTION

Azolla has been used for several centuries to fertilize the standing rice crops in Asia (Lumpkin and Plucknett, 1980). Research on the effects of Azolla on rice yields has shown a very wide range. Watanabe, et al.(1977) showed in pot experiments that approximately 50% of the available nitrogen in the Azolla is utilized by the rice and that the nitrogen release is quite slow. Talley and Rains(1980b) report that half the nitrogen requirement of rice can be supplied by A. filiculoides. They have also reported the highest increase in rice yield, 216%, by incorporating one 60 kg N/ha layer of A. filiculoides into the soil and then growing another layer of Azolla as a dual crop with the rice. Singh(1977)

reported a 6% increase in rice yield when A. pinnata was grown with the rice but not incorporated, while if the Azolla was incorporated into the soil he reported rice yield increases of 9-38%. He also reported that the incorporation of one layer of Azolla of 12 tons fresh weight/ha produced the same increase in rice production as an application of 30 kg N/ha.

Watanabe, et al.(1977) reported that the incorporation of phosphorus along with Azolla resulted in a significantly greater yield of rice than treatments without phosphorus. They also reported that this increase in yield was greater in the straw weight than in the grain yield. This data conflicts with that of Talley, et al.(1977) which shows a greater increase in the rice grain than in the straw grain when Azolla is used as a green manure or as a dual crop.

1.3 AZOLLA AS A FOOD CROP

Not only has Azolla been used as a fertilizer or green manure, it has also been used as a food crop(Moore, 1969; Buckingham, et al., 1978; Lumpkin and Plucknett, 1980). Moore(1969) has noted that Azolla has been used as a feed for pigs and ducks in Indochina and in Vietnam for cattle, poultry and fish. Buckingham, et al.(1978) reported that A. filiculoides had a low nutritive value when used as the sole source of protein for growing rats. They assumed this to be true for other simple-stomached animals also, because of the

high neutral detergent fiber of Azolla and the deficiency of the essential amino acids, methionine, lysine and histidine. The entrapment of nitrogenous compounds in the insoluble lignin or cellulose complexes of the cell wall may also account for its low nutritive value. They noted that other tests showed the cell walls to be readily digestible by ruminants and that the digestion coefficient was considerably greater than that of alfalfa (77% vs. 55.6%). Therefore they suggested that ruminants may very well be the ones to most effectively utilize both the high protein and energy content of Azolla.

Subudhi and Singh(1978) used fresh green A. pinnata in chick nutrition experiments and found that Azolla could replace 20-25% of the commercial feed in their diet. With this information they calculated that 100 birds would need nine kg of fresh Azolla per day to replace 20% of the commercial food. In so doing the authors concluded that a money savings could be realized without any ill effects on the chicks.

The information in the preceding sections indicates that the use of Azolla may have more possibilities than just as a fertilizer. It should be pointed out that the growth and maintenance of Azolla is labor intensive, this is an advantage in many societies where rice is a major staple food since there is usually a large labor force but small amounts of money for the purchase of nitrogen fertilizer.

Finally the nutrition studies indicate that Azolla can be used as a protein source for ruminants and chicks, and in so doing increase the protein supply in these societies. The importance of good food sources at a low price to these societies is their very survival and the use of Azolla in their agriculture can contribute significantly to their improved health.

1.4 MORPHOLOGY OF THE INTACT ASSOCIATION

The free-floating fresh water fern Azolla has a triangular or polygonal plant body(Konar and Kapoor, 1972). The bilobed leaves have a dorsal lobe containing a cavity in which the cyanobiont Anabaena azollae Stras. is located. The ventral lobe, which floats on the water surface, supports the plant. True roots with root hairs arise from the stem and hang down into the water. The following sections will deal with each of these structures in more detail.

1.4.1 ROOTS

The roots of Azolla arise from the ventral side of the stem(Rao, 1935; Konar and Kapoor, 1972). The roots which attain a length of 3-4 cm when mature, have a large number of root hairs that are covered by a sheath and cap when immature(Shen, 1960; Konar and Kapoor, 1972). The roots are also chlorophyllous when young(Konar and Kapoor, 1972). The

root initial cell is larger than its surrounding cells and has a prominent nucleus. This cell divides periclinally to form two unequal cells(Rao, 1935; Konar and Kapoor, 1972). The outer, smaller cell from this division becomes one of the root sheath initial cells while the larger cell divides periclinally to form the other sheath initial cell and the root apical cell. The root sheath initial cells produce the root sheath through anticlinal divisions(Konar and Kapoor, 1972).

The root apical cell divides by three oblique divisions and produces a four-sided apical cell composed of one terminal cutting face and three lateral cutting faces(Rao, 1935; Konar and Kapoor, 1972). The apical cell at the terminal cutting face then produces the root cap initial cell, but not before producing several lateral cells(Shen, 1960; Konar and Kapoor, 1972; Nitayangkua, et al., 1980). The apical initial "only gives rise to this cap initial in the early initiation of the root primordium" and the root cap is produced by its own meristem(Nitayangkua, et al., 1980). The cap initial gives rise to two layers through first an anticlinal and then periclinal divisions and then repeated anticlinal divisions gives rise to a large number of cells(Konar and Kapoor, 1972). The second layer of the root sheath then disorganizes(Rao, 1935; Konar and Kapoor, 1972) thus enclosing the root in a single layered sheath and a two layered cap(Konar and Kapoor, 1972).

The development of the root tissues are the result of the three internal derivatives of the apical cell which undergoes a series of divisions that result in four tissue layers. The outer most layer becomes the epidermis which will divide again later to give rise to the root hairs. The next layer inward will give rise to the outer cortex while the third layer will divide resulting in its outer layer giving rise to the inner cortex and its inner layer, the endodermis. The fourth layer also divides to form two layers, the outer most giving rise to the pericycle of which two cells opposite each other and separated by xylem will divide later to form the phloem. The inner layer of the fourth tissue layer will result in the vascular cylinder composed of two tracheids and four parenchymatous cells. Even at maturity the apical cell retains its identity and at the base of the root, remnants of the sheath and cap are present(Konar and Kapoor, 1972). When the root has matured and reached its maximum length it is shed through the formation of an abscission layer at its base(Rao, 1935; Konar and Kapoor, 1972).

The epidermal cells divide tangentially to give rise to an outer larger cell which will develop into the root hair and a smaller inner cell which will divide to form four intercalary cells(Konar and Kapoor, 1972). As the hair cells develop they push the cap away from the epidermal cells, and finally the cap is sloughed off when the root is

mature(Shen, 1960; Konar and Kapoor, 1972). At maturity the root hairs are approximately one cm., unicellular, and vaculated except at the tip where the nucleus is surrounded by dense cytoplasm(Konar and Kapoor, 1972).

1.4.2 STEM

The stem apex of Azolla is slender and finger-like(Gifford and Polito, 1981) and curves up and away from the surface of the water(Rao, 1935; Konar and Kapoor, 1972; Peters, et al., 1978; Gifford and Polito, 1981). The meristematic region of the stem is protected by the overlapping of the forming leaves(Peters, et al., 1978). The apical cell of the stem is wedge-shaped with two cutting faces, which form immediate derivatives to the right and left(Sud, 1934; Rao, 1935; Konar and Kapoor, 1972; Gifford and Polito, 1981). These two cells divide anticlinally to form a quadrant and then an octant(Rao, 1935; Konar and Kapoor, 1972; Gifford and Polito, 1981), after which one cell on the dorsal side and one on the ventral side of this octant cut off two small cells in the center which are termed "the central cells"(Konar and Kapoor, 1972). All the cells except the central cells now divide periclinally to form two layers, the outer of which will function as the epidermis after an anticlinal division. The inner layer will give rise to two more layers, the outer of which will form the cortex. From this point on, the development of the dorsal side will

continue while the ventral side will lag behind until after the stele has differentiated on the dorsal surface. The layer that is adjacent to the central cell on the dorsal side that was formed by the previous division will now undergo successive periclinal divisions to give rise to the endodermis, xylem, pericycle, phloem and parenchyma tissue. The ventral region undergoes several vertical and transverse divisions, of which the outer cells of these divisions give rise to the xylem and the inner cells, the pith. The phloem, pericycle and endodermis are produced from the periclinal divisions of the cells located away from the central cells. In the lateral regions of the stem the phloem fails to differentiate and the xylem is in contact with the pericycle(Konar and Kapoor, 1972).

The result is a stem composed of a single-layer of epidermis , a few layers of cortical cells that are mostly parenchymatous(Konar and Kapoor, 1972), an endodermis that lacks a Casparian strip(Rao, 1935; Konar and Kapoor, 1972), a layer of pericycle, and a phloem composed of sieve cells separated from the xylem by parenchymatous cells(Konar and Kapoor, 1972).

1.4.3 BRANCH

The structure of the branch is similar to the stem(Rao, 1935; Konar and Kapoor, 1972) except that the branch stele is of a smaller diameter(Konar and Kapoor, 1972). The

branch arises from a wedge-shaped apical cell that formed from two oblique divisions in a superficial cell of the stem(Konar and Kapoor, 1972). At the base of the branch there is an abscission layer composed of smaller cells. It is at this point that the plants fragment to form new plants(Rao, 1935; Konar and Kapoor, 1972).

1.4.4 LEAF

The leaves are arranged into two rows on the stem and the leaf arises from the dorsal surface of the stem(Sud, 1934; Rao, 1935; Shen, 1960; Konar and Kapoor, 1972). It is interesting to note here that there was some disagreement among the earlier literature as to the presence of an apical leaf cell. Sud(1934) reported that Campbell(1918) did not find any definite apical leaf cell in A. filiculoides while Sud reported that in A. pinnata there was a definite two-sided apical leaf cell. Rao(1935) on the other hand states that there is no apical leaf cell in A. pinnata and instead refers to a mother cell of a leaf. Konar and Kapoor(1972) state that in the absence of an apical cell the growth of the leaf takes place by the activity of the marginal cells, and they further state that Sud(1934) must have mistaken one of the marginal cells for an apical cell which Rao(1935) also noted.

The leaf initial differentiates on the dorsal stem surface and is recognizable by its large nucleus and dense

cytoplasm(Konar and Kapoor, 1972). The cell then divides anticlinally into two cells which will give rise to the two lobes of the leaf. The upper larger cell will develop into the dorsal lobe while the lower smaller cell will become the ventral lobe(Rao, 1935; Konar and Kapoor, 1972). The early divisions in both lobes are similar until the dorsal lobe divides to form a lobe several cells thick. The upper lobe initial divides anticlinally to form two unequal cells, the smaller of which divides anticlinally again. The resulting cell which lies adjacent to the ventral lobe initial does not divide further, while the other cell and the larger cell from the first division undergo periclinal divisions to cut off small cells towards the center. The outer cells (daughter cells) form a row of tangentially elongated cells by anticlinal divisions. These elongated cells now undergo a single periclinal division, and the outer daughter cells divide by an anticlinal and then a periclinal division and the newly formed outer cells repeat these two divisions. A depression now separates the two leaf lobes due to the fact that a few cells in between the lobes have failed to divide.

Alternating anti- and periclinal divisions occur in the upper lobe followed by irregular divisions which then result in the formation of the flat, oval dorsal lobe. This one-cell thick lobe now divides periclinally to form two cell layers the outer of which divides periclinally again to give rise to the epidermis and the middle layer. The dorsal

lobe now bends inward as a result of the greater number of anticlinal divisions in the outer epidermal cells. The cells of the middle layer now divide in the subapical region, forming a broad flat zone, while those of the basal region do not.

A depression now begins to form at the base of the dorsal lobe towards the inside as the cells of the terminal region enlarge. Below the depression the middle layer undergoes only anticlinal divisions and the epidermal cells around the depression grow inward to form the cavity leaving a small opening. The cells that surround the opening close it partially thus entrapping Anabaena colonies which are found in association with the shoot apex at all times(Konar and Kapoor, 1972). The inner epidermal cells lining the cavity develop a number of simple and branched hairs with which the Anabaena become entangled(Rao, 1935; Konar and Kapoor, 1972; Peters, et al., 1978; Calvert and Peters, 1981).

The mature dorsal lobe outer epidermis has stomata and single cell papillae(Sud, 1934; Rao, 1935; Shen,1960; Konar and Kapoor, 1972). The stomata arise from a small triangular cell that enlarges and divides by a transverse wall resulting in two guard cells of equal size. These cells then gradually separate from each other to form a small pore that is streched in the direction of the long axis of the leaf due to the elongation of the leaf. At the

same time the walls separating the two guard cells breaks down and the cell contents fuse thus forming a single annular guard cell with a central pore(Konar and Kapoor, 1972).

The epidermal cells of the mature dorsal lobe contain numerous chloroplasts and the epidermis is followed by the middle layer of chlorenchymatous cells that enlarge and separate forming intercellular spaces. This middle layer functions as a palisade tissue.

The ventral lobe initial divides similar to the dorsal lobe to produce a one cell thick lobe except at the base(Rao, 1935; Konar and Kapoor, 1972). This thick basal portion then forms intercellular spaces which make it spongy. Numerous chloroplasts and some granular substances are present in these cells(Rao, 1935; Konar and Kapoor, 1972) as well as a few stomata on the ventral surface of the basal lobe(Konar and Kapoor, 1972).

The stem vascular bundle branches out laterally to produce a foliar trace which bifurcates before entering the leaf(Konar and Kapoor, 1972; Peters, et al., 1978). One of the branches enters the ventral lobe and terminates in the spongy tissue, the other branch enters the dorsal lobe where it encircles the cavity(Konar and Kapoor, 1972; Peters, et al., 1978) and extends approximately half the length of the lobe(Konar and Kapoor, 1972). Peters, et al.(1978) states that "This leaf trace is separated from the cavity by at least one cell layer."

1.4.5 CYANOBIONT PACKETS

Peters, et al.(1978) employed enzymatic digestion to isolate packets of the cyanobiont and found a limiting envelope which conforms to the size and contour limits of the cavity that surrounds the cyanobiont. They noted that outlines of the epidermal cells lining the cavity were at times visible on this packet. Also in some instances a pore was observed in an epidermal cell but the origin of the pore is unknown and no more than one per cavity has been observed when they are present. It has been noted that the epidermal cells bordering the large circular cavity opening do create a pore that in some cases closes and seals the cavity(Konar and Kapoor, 1972; Peters, et al., 1978). In an earlier study Peters(1976) noticed that the hair cells were retained within the packets. This was confirmed by later experiments in which the packets were turned inside out by osmotic shock treatment and this showed that the basal cells of the epidermal hairs were contiguous with the outer surface of the envelope. In addition to Anabaena the packets contain a very viscous material or mucilage which exudes when the packet is ruptured(Peters, et al., 1978).

1.4.6 HAIR CELLS

Hair cells have been observed by Konar and Kapoor(1972), Becking(1974), Duckett, et al.(1975), Peters, et al.(1978) and Calvert and Peters(1981). These cavity hairs consist of

two or more cells, a basal cell in contact with the epidermal cell from which it is derived and a terminal cell, while other hair cells may have one or more stalk cells between the basal and terminal cells(Konar and Kapoor, 1972; Peters, et al., 1978). In mature cavities the hair cells are densely cytoplasmic with a prominant nucleus and outer walls with wall ingrowths(Duckett, et al., 1975; Peters, et al., 1978). The basal cells are separated from the foliar trace by at least one row of highly vacuolated cells(Duckett, et al., 1975; Peters, et al., 1978). The cytoplasm of the hair cells contain numerous mitochondria and ribosomes, vacuoles rarely occupy more than 1/4 of the cell volume and various rudimentary plastids (i.e., proplastids and amyloplasts) are present as well as golgi bodies(Duckett, et al., 1975).

The presence of branched hair cells was reported by Peters, et al.(1978) but the number and types of hairs were not reported until Calvert and Peters(1981) conducted a morphological study . In this study they noted two distinct types of hair cells, simple and branched, and that there were about 20 simple hairs and only two branched hairs present in a mature dorsal cavity. The simple hairs are randomly distributed over the cavity surface except for the lower distal quadrant. This region is bounded by a double layer of adaxial epidermis while the remaining wall tissue is mesophyll tissue subtending the epidermal cell layer.

The location of the branched hair cells, unlike the simple, are the same in all leaves. The larger hair composed of seven to 10 cells called the primary branched hair (PBH) is always located in the lower proximal quadrant of the cavity where the foliar trace bifucates from the stem stele. The other smaller branched hair composed of four to five cells is the secondary branched hair (SBH) and is formed midway along the central cavity axis near the dorsal lobe foliar trace.

The population of the simple hairs increases with the development of the leaf, while the PBH is present in the forming cavities of leaves one to four and the SBH develops by leaf 10. The first leaf to have a fully enclosed cavity, leaf five has approximately 10 simple hairs, leaf nine has 15 simple hairs and by leaf 19 there are 20 simple hairs. This data from serial reconstruction showed an increase in simple hairs from 10 in leaf five up to 22 in leaf 12 and then a decrease to 19 in leaf 15(Calvert and Peters, 1981).

The presence of the hair cells may be the result of growth on combined nitrogen in the medium(Duckett, et al., 1975). However Calvert and Peters(1981) have compared A. mexicana Presl., A. filiculoides Lam. and A. pinnata R. Br. grown on nitrate and found the hair distribution to be similar to that observed when grown on nitrogen free media. The A. pinnata did present a problem to these researchers in that none of the cells stained with Safranin O and few hair

cells were discernable with differential interference optics so they were unable to compare the A. pinnata with the other species. There is also some question as to the presence of branched or multicellular hair cells in A. pinnata. Konar and Kapoor(1972) state that A. pinnata has "multicellular branched or unbranched" hair cells in the cavity. They report the more common cavity hair is a two celled hair that enlarges but seldom divides. This would seem to be the simple hairs of Calvert and Peters(1981). In contrast Neumuller and Bergman(1981) report no branched or multicellular hairs and that the hair cells are two celled with a round basal cell connected to the cavity wall.

In light of the findings that the two hair types have different distributional patterns as well as developmental differences, Calvert and Peters(1981) suggested different functions for the hair types. They noted that prior to cavity closure the increase in simple hairs parallels increasing heterocyst frequency and nitrogenase activity. The fact that neither they nor Duckett et al.(1975) noted any cytological specialization in the leaf cells or leaf trace near the cavity or hairs, and because of the hairs random pattern, they suggested that if the hairs are involved in any metabolite transfer it is most likely only an exchange of solutes between mesophyll and the cavity. The branched hairs on the other hand exhibit transfer cell ultrastructure(Duckett, et al., 1975; Kremer, 1978; Peters,

et al., 1978; Calvert and Peters, 1981) and Calvert and Peters(1981) observed that the foliar trace in the area of the branched hair also shows transfer ultrastructure. They suggested then that the branched hairs could be mediating the movement of nitrogen compounds out of the cavity to the rest of the fern as well as being a source of nitrogen for the non-heterocystous Anabaena associated with the PBH in the developing dorsal leaf cavities.

1.5 NUTRITION OF THE INTACT ASSOCIATION

As mentioned previously the macro- and micro-nutrients required for the growth of Azolla are the same ones that higher green plants require. Of these the macronutrients phosphorus (P), potassium (K), calcium and magnesium are the most important for growth of the intact fern(Ashton and Walmsley, 1976; Becking, 1979). The elements iron, cobalt, and molybdenum are essential to the nitrogen fixation process in the cyanobiont and of these iron is the most critical due to its dependence on pH for availability to the fern(Ashton and Walmsley, 1976; Becking, 1979).

The elements which have received the most attention in nutrient studies are phosphorus and nitrogen. When nitrogen is added to the medium of the intact association the results are generally comparable to growth on nitrogen free medium. Singh(1977) added nitrogen in the form of ammonium sulfate to his medium and reported that it did not increase the

growth of the fern as measured by fresh weight. Holst and Yopp(1979) also added nitrogen in the form of ammonia and they reported that the growth of the fern was considerably less than for those grown on nitrogen free medium. They also noted that the pH had dropped from 6.5 to 3.9 in three days, this is a problem with the use of ammonia and urea and should be corrected by the use of buffers. Peters, et al.(1981) reported good growth with buffered ammonium at pH 6.0 using 10mM MES buffer when compared to a control grown on nitrogen free medium.

When nitrate was added to the medium Holst and Yopp(1979) reported that growth was comparable to that on a nitrogen free medium, and the same can be said for Peters et al.(1981) results. When Peters, et al.(1981) added 25mM NO_3^- to the medium the doubling time was slightly increased over the control. When they added urea to the medium it had no significant effect on the doubling time. In all cases when some form of nitrogen was added to the medium the nitrogenase activity was decreased, while growth was not significantly affected. The addition of up to 2.5mM ammonium and 25mM nitrate increased the chlorophyll content while urea had no effect on chlorophyll. Tomlinson(1979) reported an increase in growth of A. caroliniana on nitrate and noted that Peters, Evans and Toia(1976) and Holst(1977) reported slight increases in growth of A. caroliniana and A. mexicana respectively. Subudhi and Singh(1979) reported

that growth was significantly decreased on ammonium chloride as compared to the control and that although growth was less in calcium nitrate it was not depressed as severely as that in the ammonium chloride.

Phosphorus, unlike nitrogen, has been found to be necessary for the growth and multiplication of Azolla(Ashton and Walmsley, 1976; Singh, 1977; Watanabe, et al., 1977; Becking, 1979; Subidhi and Singh, 1979; Watanabe, et al., 1980; Subudhi and Watanabe, 1981). Phosphorus deficient plants are described as having reddish brown discoloration, small frond size, longer roots, browning of the roots and fragile fronds(Singh, 1977; Watanabe, et al., 1977; Kulasooriya, et al., 1980; Subdhi and Watanabe, 1981). Subudhi and Singh(1979) have also noted that phosphorus deficiency results in the accumulation of carbohydrates and reducing sugars as well as disturbing the nitrogen metabolism of the fern. Watanabe, et al.(1977) reported a 22% reduction in fresh weight and a 16% reduction in total nitrogen content of a phosphorus deficient fern as compared to a control.

The minimum phosphorus requirement for A. pinnata was calculated using two methods by Subudhi and Watanabe(1981). They noted that most previous experiments had used the batch method in which the supply of the nutrient depended on concentration, volume and frequency of renewal of the batch medium. In order to better determine the minimal phosphorus

requirement they felt that a continuous flow method was better because it reproduced the gradual but constant release of phosphorus from the solid state to the liquid state found in nature, thus the concentration remained more stable. Using the batch method they noted that the amount of fresh matter produced increased with increasing levels of phosphorus up to five ppm phosphorous and that levels above this had no significant effect. In the batch method they concluded that 0.3% phosphorus content was the maximum for increasing A. pinnata growth. In an earlier paper Watanabe, et al.(1980) reported the threshold for phosphorus deffeciency was between 0.1 and 0.3% super phosphate(P_2O_5) in dry matter. The data from these continuous flow experiments showed that above 0.06 ppm(2uM) phosphorus, no increase in fresh weight occurred, while 0.03 ppm(1uM) phoshporous resulted in a 22% decrease in the fresh weight when compared to the 0.06 ppm level.

Their third experiment compared the growth of several species on 0.03 ppm phosphorus in a continuous flow system. This experiment showed wide differences in the species, especially between the A. pinnata species, which grew fairly well, and the new world species A. mexicanna, A. caroliniana and A. filiculoides which could not grow at this level of phosphorus(Subudhi and Watanabe, 1981).

Watanabe, et al.(1977) also used calcium, potassium and magnesium deficient media and reported a reduction in fresh

weights of 9, 32 and 82% respectively. They noted that the potassium deficient treatment reduced the number of cyanobacteria and those left were less healthy, and that calcium deficient medium resulted in browner, smaller fronds that were cyanobacteria free. The magnesium deficient cultures did not differ in appearence from the controls.

1.6 PHYSIOLOGY OF THE INTACT ASSOCIATION

1.6.1 NITROGEN FIXATION

The ability of the Azolla fern to fix atmospheric nitrogen was known for almost sixty years, but the site of nitrogen fixation was not determined to be the cyanobiont Anabaena azollae Stras. until Peters and Mayne(1974b) isolated the symbionts. Hill(1977) reported that the ability to fix nitrogen in the fern parallels the developmental pattern of the fern. He noted that the fixation rate at the apex was negligible, but began to increase with leaf age up to leaf 10 and remained fairly constant to leaf 20, after which the rate decreased as these leaves senesced and died. Kaplan and Peters(1981) also reported similar results in a leaf by leaf assay study.

The comparison of nitrogen fixation data as measured by the acetylene reduction assay(ARA) is somewhat difficult since different researchers have used different parameters to relate acetylene reduction to, and also the incubation and growth conditions are variable(Peters, et al., 1978).

The best way to compare these data are to use the ratio of acetylene reduced to the amount of nitrogen fixed by an equivalent sample. This conversion factor is determined by comparing the ARA rate to total nitrogen increase during the incubation period(Watanabe, et al., 1977). Watanabe, et al.(1977) reported ratios of acetylene:nitrogen of 3.4, 1.6, and 2.4 for samples of 14, 19 and 22 days old respectively. Peters, et al.(1977) in Peters, et al.(1980a) reported ratios of 2.77 and 3.38 for 30 and 60 minute incubation periods respectively. From this Peters, et al.(1980a) noted that these values are well within the range of values reported for other nitrogen-fixing organisms and also that they agree with Watanabe, et al.(1977).

A number of researchers have reported that various factors (light, temperature, nutrients and nitrogen) affect the acetylene reduction rate of Azolla(Becking, 1974; Peters and Mayne, 1974b; Peters, et al., 1978; Holst and Yopp, 1979; Kularoosiya, et al., 1980; Peters, et al., 1980a; Peters, et al., 1980b; Tung and Shen, 1981). Peters, et al.(1980b) showed that there was an increase in acetylene reduction from 15C up to the optimum growth temperature for each of the five species they observed. A 16 hour light photoperiod resulted in a slightly higher ARA rate over the 14 hour light photoperiod treatment, while the ARA activity was saurated at a light intensity of 100-200 umoles/sq. M/sec. Becking(1974) reported an ARA rate of 7 to 10.6

nmoles ethylene/mg sample protein/min. under laboratory conditions and a light intensity of 14-27 klux(175-337.5 uM/sq M/sec). Becking(1974) stated that he observed no light intensity effect, this could be accounted for by Peters, et al.(1980b) results which placed the light saturation point for the ARA at 8-16 klux(100-200 uM/sq M/sec.) and thus Becking was above the light saturation point.

Kulasooriya, et al.(1980) conducted several experiments using A. pinnata to study the effects of various light, temperature and phosphorus levels on nitrogenase activity and growth. Their results showed that maximum nitrogenase activity was reached at their medium light intensity of 30 klux(375 uM/sq M/sec) while high light intensity (45-75 klux) caused a decrease in nitrogenase activity. Increasing the phosphorus level in the medium showed an increase in nitrogenase activity for all light intensities, while phosphorus starvation resulted in a decrease in nitrogenase activity with increasing light intensity.

Studies with labeled nitrogen using the isolated cyanobiont have shown that it releases approximately half of the nitrogen it fixes into the incubation medium as ammonium with only small amounts of organic nitrogen(Peters, et al., 1980a). Both the fern and cyanobiont have the ability to metabolize ammonia (Peters, et al., 1981). While both the fern and cyanobiont exhibit glutamine synthetase (GS),

glutamate dehydrogenase (GDH), and glutamate synthase (GOGAT) activities the fern accounts for 90% of the GS and 80% of the GDH activities of the symbiosis. Peters, et al.(1981) noted that Stewart(1977)and Haselkorn(1978)suggest decreased GS activity in the cyanobiont could be the cause for the release of ammonia and that the fern is utilizing this ammonia. Based on nitrogen fixation and nitrogen content profiles of the stem, Kaplan and Peters(1981) concluded that the older leaves were fixing the nitrogen and transporting it to the stem tip. Their pulse-chase experiments confirmed these suppositions but they do not know the exact mechanisms nor the form of nitrogen being transported. So it is possible that the ammonia released by the cyanobiont is being transported to the stem tip or the ferns GS is metabolizing it to glutamine and then transporting it, or even both are occurring simultaneously (Kaplan and Peters, 1981). Kaplan and Peters(1981) also speculated from the results of Calvert and Peters(1981) study of the cavity hairs, that the simple hairs may be involved in fixed carbon transport while the branched hairs are involved in fixed nitrogen transport.

1.6.2 PHOTOSYNTHESIS

The partners in the Azolla symbiosis contain light harvesting pigments which are complementary to each other. The fern contains chlorophylls a and b as well as

carotenoids, while the cyanobiont contains chlorophyll a, phycobilins and carotenoids(Peters, et al., 1981). The cyanobiont has been estimated to contribute 15-20% of the associations total chlorophyll content(Peters, et al., 1978). The association as well as the isolated partners exhibit Calvin cycle intermediates of photosynthetic carbon dioxide fixation with phosphoglyceric acid as the initial product of carbon dioxide fixation followed by hexose phosphates and finally sucrose(Peters, et al., 1973).

Photosynthesis is also the source of electrons and ATP for the nitrogenase enzyme. The role of photosystem II is to produce the reducing power for photosynthesis and studies with DCMU, a photosystem II inhibitor, have shown that carbon dioxide fixation can be completely inhibited while the acetylene reduction rate shows less than a 30% inhibition, provided that endogenous reserves of reductant have not been depleted. From this Peters, et al.(1981) concluded that photosystem I was the primary source for ATP in light-driven nitrogenase-catalyzed reduction. The structures which capture the energy for these reactions, the chloroplasts of the fern, are reported to have a chlorophyll a/b ratio of 2.78(Lumpkin and Plucknett, 1980).

Tinh and Faludi-Daniel(1981) observed the effects of temperature on the photosynthetic activity of A. pinnata and reported that a temperature of 10C resulted in a lowered carbon dioxide fixation rate as well as a lower carbon

dioxide incorporation rate. The effect of 35C resulted in an increase in labeled carbon fixation and they felt that the adverse effect of this temperature on the growth of the fern was due to the high temperature affecting protein synthesis.

1.6.3 GROWTH RATES

Azolla growth measured in terms of biomass doubling time has been reported in the range of two to five days depending on the conditions (Peters, et al., 1980b; Tung and Shen, 1981). The major factor affecting growth on floodwater was phosphorus and its deficiency resulted in decreased growth and acetylene reduction. Tung and Shen(1981) reported that the first week after transfer to phosphorus deficient medium the ferns showed no symptoms but during the next two weeks the growth rate declined rapidly and by the fourth week they had ceased to grow. From this and previous information it is clear that phosphorus is a very necessary nutrient for the proper growth and activity of the intact association.

1.7 MORPHOLOGY AND PHYSIOLOGY OF CYANOBACTERIAL FREE FERNS

The natural occurrence of cyanobacteria free (CBF) ferns in nature is rare but has been observed by some researchers(Huneke, 1933; Shen, 1960). Attempts to produce CBF ferns has met with varying results. Huneke(1935) attempted six different treatments; alcohol, calcium

hypochlorite, starvation of immature seed plants, frost, starvation of mature plants, and nitrogen free medium. Of these she reported success with calcium hypochlorite, starvation of mature plants, nitrogen free medium, and frost treatments. Lumpkin and Plucknett(1980) reported that Schaede(1947) used dilute medium and low light intensity. Antibiotics have been used with success by Nickell(1958), Peters and Mayne(1974a), Ashton and Walmsley(1976) and Becking(1978). Becking(1978) also used calcium hypochlorite as a surface sterilizing agent. Duckett, et al.(1975) and Pai Ke-chih, et al.(1978) have also reported success with surface sterilization techniques. Hill(1975) used low light followed by high light intensity to produce CBF ferns. Flint(1942) tried UV irradiation, he was unsuccessful because the symbionts showed no difference in susceptibility. Watanabe, et al.(1977) reported that a calcium-deficient medium resulted in CBF ferns in two weeks.

In recent years the production of CBF ferns has been solely for the purpose of comparative morphological or physiological studies, and the information usually cited in the literature is minimal. The morphological information on CBF ferns indicates that the CBF ferns are very similar to the intact association in most respects. Duckett, et al.(1975) and Calvert and Peters(1981) have reported the presence of the cavity hairs in CBF ferns and Ashton and

Walmsley(1976) have reported that the cavities are also similar. Calvert and Peters(1981) found no effect on the cavity hair populations of CBF ferns grown on combined nitrogen which was consistent with the findings of Duckett, et al.(1975). Duckett, et al.(1975) did notice that in mature hair cells of CBF A. pinnata the cell membrane did not exhibit as marked a separation from the cell wall as the intact associations hair cell showed. Peters, et al.(1978) agreed with this observation and also reported that they were unable to isolate empty algal packets from CBF ferns. Duckett, et al.(1975) also reported the absence of the evidence of mucilage in the leaf cavity of CBF A. pinnata.

Physiological studies of CBF ferns has been limited to the determination of photosynthetic pigments, and the role of the fern chloroplast photosystems in nitrogen fixation(Peters, et al., 1981). To my knowledge there have not been any reports of growth studies using CBF ferns. The only information reported has been that the CBF fern appears less green and/or more compact(Peters and Mayne, 1974a; Ashton and Walmsley, 1976; Becking, 1978). There is also only one report of successful reinfection from Pai Ke-chih, et al.(1978) in China but the methods are very sketchy.

1.8 OBJECTIVES

From the literature it is obvious that the Azolla Anabaena azollae symbiosis has been studied in depth, especially the species A. pinnata and A. caroliniana, whereas, the literature lacks information on CBF ferns. The successful reassociation of the separated symbionts depends on the ability to maintain CBF cultures as well as provide sufficient amounts of material with which to work.

The goals of my research were to study the nitrogen, phosphorus and potassium requirements of CBF ferns as well as to quantify any morphological differences between CBF and intact ferns of the species A. pinnata and A. filiculoides. To accomplish these goals the following objectives were established:

1. Produce CBF A. pinnata and A. filiculoides using either surface sterilization or antibiotic treatments.
2. Compare the sizes of leaf seven as well as the distances between leaves six, seven and eight and the distance between the branches nearest leaves seven and nine.
3. Investigate the nitrogen, phosphorus, and potassium requirements of CBF ferns.

II

MATERIALS AND METHODS

The water ferns A. pinnata R. Brown (from D.W. Rains via G.A. Peters; from the Malaysian population at the International Rice Research Institute in the Philippines) and A. filiculoides Lam. (from D.W. Rains via G.A. Peters from the Kauai, Hawaii population) were used in this study. These cultures were maintained under aseptic culture conditions and were free of epiphytes.

2.1 CULTURE METHODS

The ferns were maintained on two basic types of media. The intact association was cultured on a nitrogen-free medium (I) composed of the macronutrients described by Watanabe, et al.(1977) and the micronutrients of Allen(1968) with the substitution of $CoCl2 \cdot 6H2O$ for $Co(NO3)2 \cdot 6H2O$ (see Table 1). The cyanobacterial free ferns were cultured on the basic I medium with the addition of 1.5ml/L of 1M KNO3 and 1.0ml/L of 1M $Ca(NO3)2 \cdot 4H2O$, this is I+ medium. Both media were adjusted to pH 5.6 with 1N KOH and dispensed into flasks which then were sterilized in an autoclave for 25 minutes at 1.05kg/sq cm pressure and 120C prior to use. The cultures were maintained in 500ml Erlenmeyr flasks containing 200ml

Table 1. Standard I Medium.

MACRONUTRIENTS	STOCK CONCENTRATION MOLARITY	WORKING SOLUTION ml/L
K_2SO_4	0.5	1.0
$CaCl_2 \cdot 2H_2O$	1.0	1.0
$MgSO_4 \cdot 7H_2O$	1.0	1.5
$NaH_2PO_4 \cdot H_2O$	1.0	0.75
$FeC_6H_5O_7 \cdot 5H_2O$	0.035	1.0
MICRONUTRIENTS	mM	2.0
H_3BO_3	46.0	
$MnCl_2 \cdot 4H_2O$	9.14	
$ZnSO_4 \cdot 7H_2O$	0.77	
$NaMoO_4 \cdot 2H_2O$	1.6	
$CuSO_4 \cdot 5H_2O$	0.31	
$CoCl_2 \cdot 6H_2O$	0.16	

of medium and stoppered with cotton dental plugs wrapped in cheese cloth. The cultures were transferred every 12-15 days or when 2/3 of the surface of the medium was covered with growth. All transfers were done in a hood using standard aseptic techniques. The cultures were maintained in a growth chamber under a 16/8 light/dark photoperiod using Westinghouse Cool-White 40 Watt fluorescent bulbs at an intensity of 68.75 umole/sq m/sec. The temperature was held constant at 23+2C. This will be considered standard Azolla growth conditions.

2.2 PRODUCTION OF CYANOBACTERIAL FREE FERNS

To produce CBF ferns three methods were used with the two species of ferns. The first method, adapted from Duckett, et al.(1975), was surface sterilization with Clorox. Utilizing fresh stocks of A. pinnata and A. filiculoides, the apical meristem along with two-three leaves were excised, rinsed in a solution of 10% Clorox + 0.01% Tween 20 in glass distilled water for approximately one minute, followed by two rinses with sterile glass distilled water. The apices were then transferred to I+ medium and placed under standard Azolla growth conditions. Three replicates of each fern species were set up with five meristems per replicate.

The second technique used to produce CBF ferns was the sequential antibiotic exposure method of Peters and

Mayne(1974a). The three treatment regimes that they reported success with were utilized (see Table 2). The antibiotics(Sigma Chemical Co.) were added to sterilized 125ml flasks containing 50ml of I medium. The cultures were grown on antibiotic containing medium for one week, with a change to fresh antibiotic I medium after the first three days. The cultures were then transferred to antibiotic free I medium for a week, and then transferred to the next antibiotic treatment I medium in the sequence. Three replicates of each treatment were run simultaneously under standard Azolla growth conditions.

A third method used to produce CBF ferns was that of Pai Ke-chih, et al.(1978). This method involved the rinsing of the fern in cold (16C) tap water, removal of several branches with shoot tips and placement of these branches in a gauze bag. The branches were then rinsed in cold tap water for 30 minutes, after which they were submerged in a solution of 2% Clorox + 0.01% Triton X-100 for four minutes and then rinsed with 200ml of sterile once glass distilled water. The branches were then placed on sterile Whatman paper moistened with sterile distilled water in a sterile Petri dish and sterile needles were used to excise the shoot apices which were then placed on I+ medium under standard Azolla growth conditions. Nine replicates of each species were used each containing five apices per replicate.

Table 2. Antibiotic Treatment Sequences. From Peters and Mayne, 1974a.

TREATMENT SEQUENCE A
1- 2ug/ml Aureomycin
2- 50ug/ml Na penicillin G
3- 10ug/ml Streptomycin sulfate
4- 10ug/ml Bacitracin + 12.5ug/ml Polymyxin B sulfate
TREATMENT SEQUENCE B
1- 25ug/ml Polymyxin B sulfate
2- 10ug/ml Tetracycline
3- 20ug/ml Bacitracin
4- 20ug/ml Streptomycin sulfate + 100ug/ml Na penicillin G
TREATMENT SEQUENCE C
1- 10ug/ml Tetracycline + 25ug/ml Polymyxin B sulfate + 20ug/ml Bacitracin
2- 100ug/ml Na penicillin G + 20ug/ml Streptomycin sulfate + 4ug/ml Aureomycin
3- 10ug/ml Tetracycline-HCl + 25ug/ml Polymyxin B sulfate + 20ug/ml Bacitracin
4- 100ug/ml Na penicillin G + 20ug/ml Streptomycin sulfate + 4ug/ml Aureomycin

For all three techniques the ferns were checked for the presence of cyanobiont as described below. The antibiotic treated ferns were checked after one week on antibiotic free I medium following the last antibiotic treatment. The Clorox treated ferns were checked for absence of cyanobiont after four-six weeks of growth. Those ferns which were found to be CBF were transferred to fresh I+ medium and maintained under standard Azolla growth conditions.

2.3 TEST FOR CBF FERNS

In order to demonstrate that the treated ferns were in fact CBF the following three methods were employed. To be declared CBF the ferns had to pass all three tests for the absence of the cyanobiont. The first check was to remove several of the number five to seven leaves from a sample and place them on a microscope slide. The leaves were then sliced with a sterile needle into several pieces and gently squashed under a coverslip in a drop of water, and observed with a light microscope for the presence of any cyanobacterial filaments.

The second method was to take those ferns which showed no visible signs of cyanobacterial filaments and subject them to the acetylene reduction assay (ARA). A sample of one gram fresh wet weight of fern was placed into a serum bottle with 1.0ml of once glass distilled water and sealed with a serum stopper. The atmosphere inside the bottle was

flushed for three minutes with Argon, the length of time necessary to flush the bottle three times. Then 11% of the volume was removed and replaced with 1% carbon dioxide and 10% acetylene (Peters and Mayne, 1974b). The flasks were the incubated under standard Azolla growth conditions and assays were made at 30 minutes and approximately 16 hours incubation times. The following control flasks were set up and also sampled; blank flask, blank flask + 1.0ml of distilled water, blank flask + 1.0ml of water + 1% carbon dioxide + 10% acetylene, intact fern association + 1.0ml distilled water, intact fern association + 1.0ml distilled water + 1% carbon dioxide + 10% acetylene, and CBF fern + 1.0ml distilled water. A 0.5ml sample of the incubation gas phase was removed from the bottles and injected into a Varian/Areograph Model 600-D gas chromatograph with a hydrogen flame ionization detector. The column was a Porpac N 80/100 mesh, 1/8 inch diameter by two meters. The column and injector were maintained at 60C by the Varian/Areograph Model 328 Programmer. Nitrogen was used as the carrier gas at a flow rate of 25ml/minute, the hydrogen flow rate was 25ml/minute and the air flow rate was approximately 250-300 ml/minute. The pen deflections generated by the hydrogen ionization detector were recorded on a Varian Model 9176 chart recorder (see Table 3). These deflections were compared to a standard curve generated from ethylene standards (see Figure 2.)

Table 3. Ethylene Standards Data.

ETHYLENE STANDARD CONCENTRATION ug/ml	PEN DEFLECTION UNITS
0.01	4.5
0.025	11.75
0.05	23.00
0.10	44.00

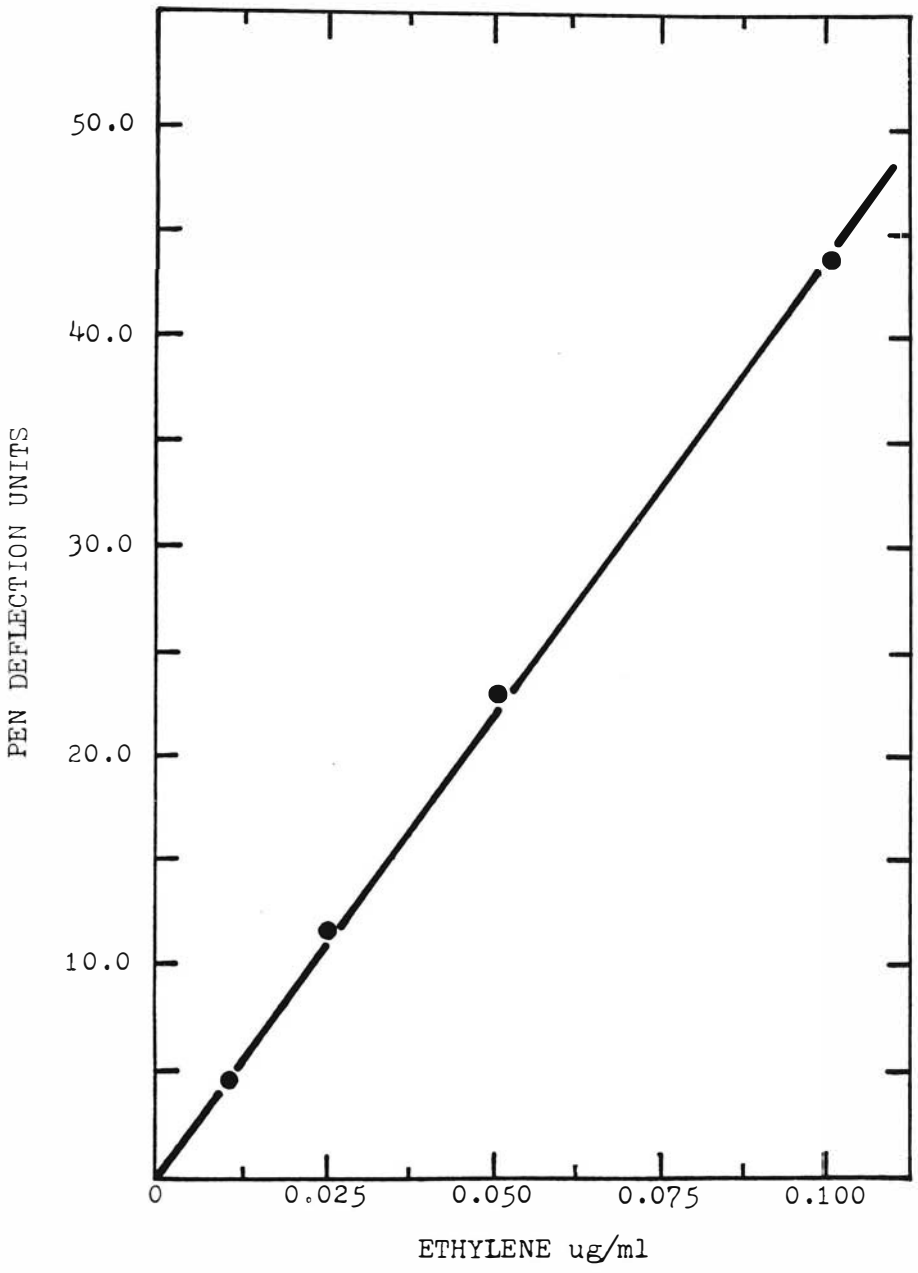

Figure 2. Ethylene standard curve.

The presence of cyanobiont in the fern would result in the reduction of acetylene to ethylene by the cyanobiont's nitrogenase enzyme system which would then show up as an ethylene peak on the recorder output. The lack of nitrogen fixation as measured by the absence of ethylene in the ARA was considered evidence of CBF ferns.

The third method used to establish a fern as CBF involved the ability of the fern to grow on I medium. A 0.25 gram sample of an intact fern and the CBF fern to be tested were both placed on I medium in triplicate and allowed to grow for four weeks under standard Azolla growth conditions. Since the I medium lacks a nitrogen source, the ferns must provide their own nitrogen source to grow. Intact associations, having the cyanobiont to fix atmospheric nitrogen, would grow on I medium. CBF ferns lacking the cyanobiont to fix nitrogen would not grow because of the lack of a nitrogen source. After four weeks the ferns were weighed and observed for growth.

2.4 CBF FERN NUTRIENT STUDIES

Nutrient studies were conducted on CBF ferns to determine the effects of N, P and K on the ferns growth, as measured in terms of change in wet weight and total chlorophyll content. The media used in these studies was the basic I+ medium with the levels of N and P adjusted to be 1/2, 1 and 2 times the concentration of these nutrients in I+, which

resulted in concentrations of 0.875 mM, 1.75 mM and 3.5 mM nitrogen and 2.17 mM, 4.35 mM and 8.70 mM phosphorus. Levels of 1/2, 1 and 3 times the concentrations of K in I+ resulted in concentrations of 0.25 mM, 0.50 mM and 1.50 mM potassium. To prepare these media 9 liters of twice glass distilled water was used. To this was added 14.4 ml of 1M $MgSO4 \cdot 7H2O$ stock, 9.0 ml of 0.035M $FeC6H5O7 \cdot 5H2O$ stock and 18.0 ml of micronutrient stock. Then 900 ml of this new stock solution was removed and the appropriate N and P solutions added and the volume brought up to 1 liter. Then 300 ml were dispensed into 3 beakers to which the appropriate amount of K was added to give 300 ml solutions of each of 1/2K 1K and 3K. This was repeated for each of the 9 different N and P combinations. In the cases where the N values were 1/2 the standard I+ values 1 ml of 1M $CaCl2 \cdot 2H2O$ stock was added to maintain the calcium level in these solutions. Also only 1M $Ca(NO3)2 \cdot 4H2O$ was used as the nitrogen source so as not to add any potassium in the form of $KNO3$ to the media. The pH of each treatment was adjusted to 5.6 using 0.25N NaOH. The treatments were run in triplicate and all the flasks were autoclaved at 1.05 kg/sq cm pressure and 120C prior to inoculation with 0.20 to 0.25 grams wet weight Azolla which had been blotted dry with three layers of Kim-wipes to remove the excess medium. The flasks were then arranged at random in a growth chamber under the standard Azolla growth conditions for five weeks.

At this time the ferns were removed from the flasks, blotted on three Kim-wipes and then weighed. Chlorophyll was extracted for two hours in the dark using 10.0 ml of 80% acetone(Ross, 1974). The acetone solution was then removed, placed in a cuvette and the absorbance read at 645 and 663 nm on a Turner 350 spectrophotometer and these values used to calculate the total chlorophyll content per gram fresh weight of fern.

The SAS statisticalpackage was used to establish a random number sequence in order to assign the flasks in a random manner in the growth chamber. The SAS package was also used to analyze the data using the general linear model (GLM) procedure to construct the analysis of variance (ANOVA) tables, with a confidence interval of 90% and to test the linear orthogonal contrasts.

2.5 TISSUE PREPARATION AND MORPHOLOGY STUDIES

The tissues utilized for light microscopy (LM) studies were prepared using the procedures of Duckett, et al.(1975) with modifications as suggested by Dr. Carolyn Conway, Dr. Steven Quattropani and myself. The seventh leaf from the apex was removed, placed in a gauze bag and fixed in 3% gluteraldehyde in 0.05M Na cacodylate buffer for 2.5 to 3 hours at a temperature of 25C. The fixative was then removed and the leaves placed in 0.05M Na cacodylate buffer overnight at room temperature. The next morning the buffer

was removed and the tissue was post-fixed in 1% osmium tetroxide in 0.05M Na cacodylate buffer for two hours at 4C. Following this the tissue was dehydrated in a graded acetone series for 15-20 minutes in each of 50, 70, 90, 100 and 100% acetone. The leaves were then removed from the gauze bag and placed in a 1:1 mixture of Effapoxy resin (Ernest Fullum Co.) and acetone overnight at a temperature of 25C, from which they were placed into fresh 100% embedding resin overnight at 4C. Finally the fronds were placed in fresh 100% embedding resin in plastic molds and placed in a drying oven at 65C for 48 hours to cure the resin.

Upon removal from the oven the specimens were removed from the mold and mounted on aluminum the sectioning studs with epoxy cement. The specimens were then trimmed and thick sections (0.5um) were cut using an LKB Ultrotome model 4801A and glass knives. The sections were then placed on a slide in a drop of 20% ethanol and dried on a hot plate after which the slide was stained with 1% Toluidine Blue in borax for approximately 15 seconds.

Preliminary morphological data on the CBF ferns was obtained by measuring the distance along the stem axis between leaves six and seven, and seven and eight. In addition the length and width of leaf seven was recorded as well as the distance between the branches from near leaf nine to near leaf seven. All measurements were made using a Nikon dissecting scope fitted with an occular micrometer.

Fifty plants of both intact and CBF ferns of the two species were measured for leaf and branch distances. Twenty five plants of both the intact and CBF fern of the two species were measured for leaf seven size.

III

RESULTS

3.1 CBF FERN PRODUCTION

The production of CBF ferns was accomplished through the use of two different treatments,: surface sterilization with clorox and incorporation of antibiotics into the fern medium. The clorox treatments resulted in the production of A. filiculoides CBF(AF-CBF) ferns within 60 days after sterilization while no A. pinnata CBF(AP-CBF) ferns were produced using either the standard clorox method or that of Pai Ke-chih, et al.(1978). The AF-CBF ferns produced by this treatment were a lighter green color than the intact association.

The sequential antibiotic treatments listed in Table 2 resulted in the successful production of AP-CBF ferns with all three different treatment sequences, while AF-CBF ferns were produced in all three replicates of the B and C sequences but in only two of the A sequence replicates. At the end of the eight week treatment the AP-CBF ferns were pale yellow with brown center fronds. The AF-CBF ferns of sequence A were green with some brown center fronds, sequence B was light green with some brown center fronds and sequence C ferns were a pale green-yellow with brown center

fronds. After three months under standard Azolla growth conditions the antibiotic treatments resulted in AP-CBF ferns from the A and B sequences, but all the C sequence treated ferns had died. The AF-CBF ferns of treatment sequence A had reverted back to the intact association and sequences B and C remained CBF. Thus the attempts to produce CBF ferns resulted in only AF-CBF ferns from the clorox treatments and both AF-CBF and AP-CBF ferns from the sequencial antibiotic treatments. Due to contamination by green algae the AF-CBF, B and C sequences were discarded, thus no AF-CBF antibiotic treated ferns were available for this study. Also the AP-CBF antibiotic B treated ferns were very slow to recover and grew at a rate which would not provide sufficient experimental material.

3.2 TEST FOR CBF FERNS

As described in the Materials and Methods, three methods were used to determine if the ferns were CBF. The first method used was the whole leaf squash method. The AF-CBF ferns were observed to be CBF after 60 days and again at 150 days after treatment. The ferns have remained visually free as observed by periodic checks. The AP-CBF cultures have also been observed to be free 90 days after the end of the treatment regime and have remained free since then.

The second method used was the acetylene reduction assay(ARA), the results of which are presented in Table 4.

Table 4. Acetylene Reduction Assay Results for 4 Varieties of Azolla Under Standard Azolla Growth Conditions.

SAMPLE	ng C_2H_4 produced/ml/g fresh wt/30 min.
Blank	0.0
Blank + H_2O	0.0
Blank + H_2O + C_2H_2	0.0
AP + H_2O	0.0
AP + H_2O + C_2H_2	31.0
AP-CBF + H_2O	0.0
AP-CBF + H_2O + C_2H_2	0.0
AF + H_2O	0.0
AF + H_2O + C_2H_2	38.0
AF-CBF + H_2O	0.0
AF-CBF + H_2O + C_2H_2	0.0

Only the intact ferns had nitrogenase activity Figure 3 shows the actual chart recorder graph of the ARA for both AF and AF-CBF cultures in the presence of acetylene. This figure is presented to show the sensitivity of the assay for nitrogenase activity as well as the difference between an intact fern and a CBF fern.

A representative photograph of the sectioned dorsal lobe cavity of each species of Azolla studied is shown in Figure 4. The absence of the cyanobiont in the CBF ferns was obvious as there were no cyanobacterial filaments around the hair cell or the outer edge of these cavities. The presence of hair cells in both the intact and CBF ferns was shown as well as the attachment of the hair cell to the epidermal cells lining the cavity.

The third test for CBF ferns was Nitrogen starvation on I medium. The results, presented in Figure 4 and Table 5, showed that the intact association was able to grow in the absence of a combined nitrogen source while the CBF ferns were not. The CBF ferns were completely brown and dead while the intact ferns were uniformly green with good frond and root growth. These results showed that there were no cyanobionts present in the CBF ferns since the CBF ferns : 1) were visually free of cyanobionts, 2) were unable to reduce acetylene, (i.e., fix atmospheric nitrogen), and 3) died when grown on a nitrogen free medium.

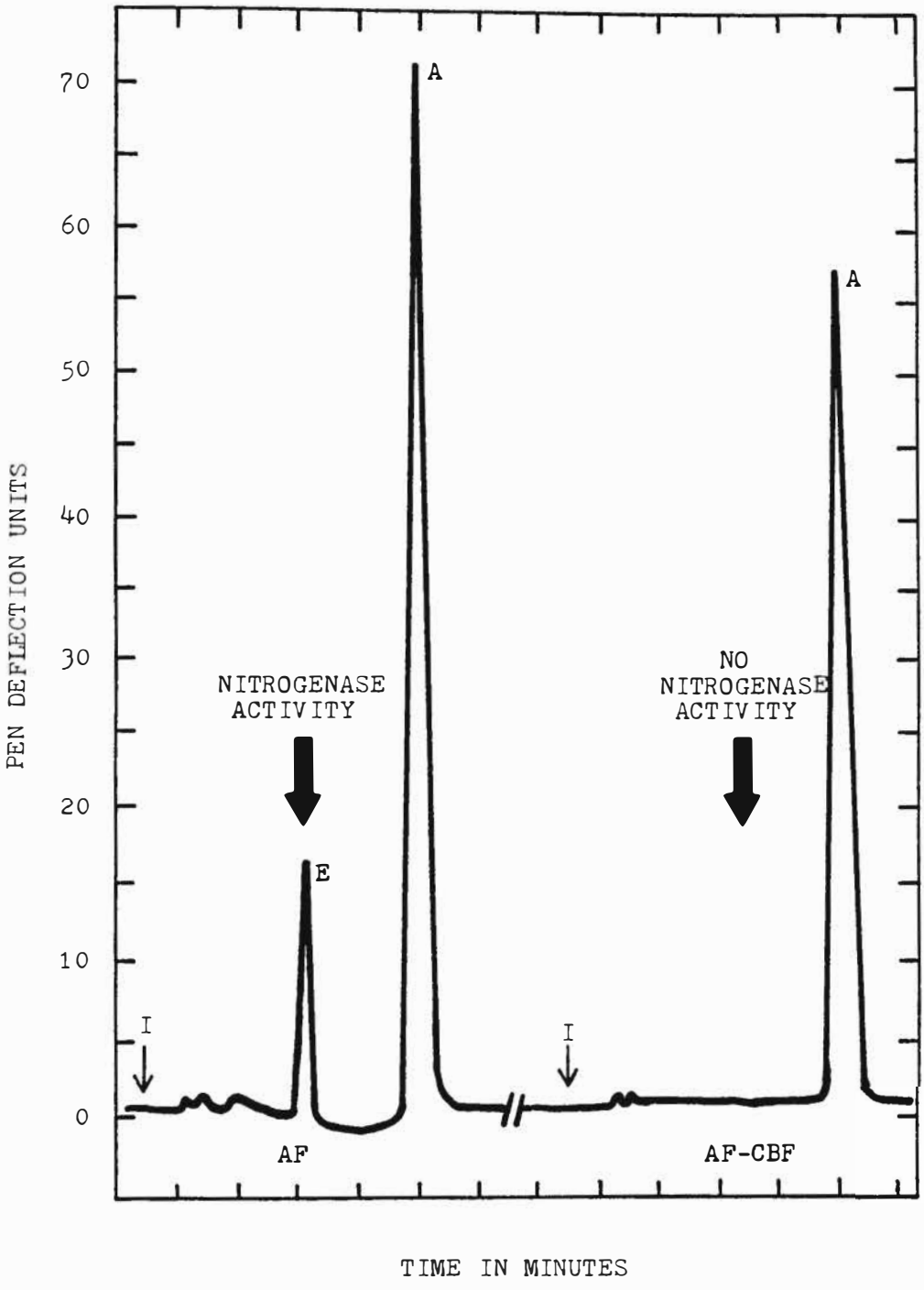

Figure 3. Acetylene reduction assay showing injection time (I), ethylene peak (E), and acetylene peak (A). Acetylene peak is 1/640 actual size.

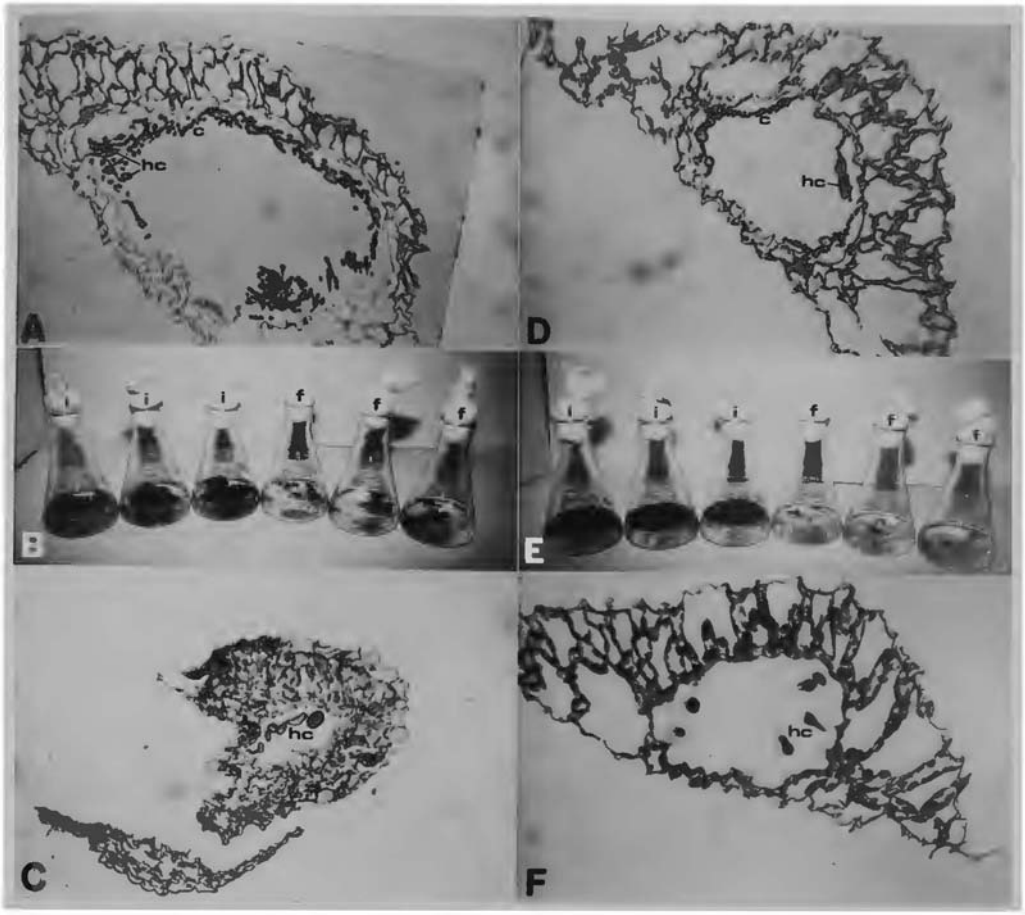

FIGURE 4. A, AZOLLA FILICULOIDES DORSAL LOBE CAVITY OF LEAF 7, FROM APEX, SHOWING HAIR CELL (hc) AND CYANOBACTERIAL FILAMENTS (c). B, GROWTH OF AZOLLA FILICULOIDES INTACT ASSOCIATION (i) AND AZOLLA FILICULOIDES-CBF (f) ON I MEDIUM. C, AZOLLA FILICULOIDES-CBF DORSAL LOBE CAVITY OF LEAF 7 SHOWING HAIR CELL (hc); NOTE ABSENCE OF CYANOBACTERIAL FILAMENTS. D, AZOLLA PINNATA DORSAL LOBE CAVITY OF LEAF 7 SHOWING HAIR CELL (hc) AND CYANOBACTERIAL FILAMENTS (c). E, GROWTH OF AZOLLA PINNATA INTACT ASSOCIATION (i) AND AZOLLA PINNATA-CBF (f) ON I MEDIUM. F, AZOLLA PINNATA-CBF DORSAL LOBE CAVITY OF LEAF 7 SHOWING HAIR CELL (hc); NOTE ABSENCE OF CYANOBACTERIAL FILAMENTS.

Table 5. Growth of 4 Azolla Varieties on I Medium for 4 Weeks. Means are result of 3 replicates.

SPECIES		WT. INITIAL grams	WT. FINAL grams	WT. CHANGE grams
AP	$\bar{X}$	0.250	2.933	2.683
	S.D.	0.000	0.185	0.185
AP-CBF	$\bar{X}$	0.220	0.366	0.146
	S.D.	0.010	0.011	0.015
AF	$\bar{X}$	0.246	2.873	2.626
	S.D.	0.005	0.066	0.068
AF-CBF	$\bar{X}$	0.250	0.640	0.393
	S.D.	0.000	0.017	0.020

3.3 MORPHOLOGY RESULTS

Table 6 presents the data for the distances between leaves six and seven and seven and eight as well as the distance between the two branches located basipetal to leaf seven. The means and standard deviations were used to test the null hypothesis that the means of the leaf and branch distances in the intact fern were equal to those of the CBF fern of the same species, verses the alternative hypothesis that the mean of the CBF fern was less than the mean of the intact fern at the 10% significance level. A t-test was used to test this hypothesis and the table value at the 10% significance level was determined to be 1.684. Any t-value calculated from the data that was greater than this table value would indicate that the means of the intact and CBF fern distances were significantly different. The calculated t-values are given in the table below the means for each species and it is clear that in all cases the hypothesis that the means are equal was rejected. Thus the data clearly shows that the leaf spacing in the CBF ferns is closer than in the intact association. It should also be noted that in the AP species the ratio of the leaf six to seven to leaf seven to eight is virtually the same while in the AF species this ratio is different.

The branch spacing in the CBF ferns was significantly shorter than that in the intact ferns for both species. It

Table 6. Leaf and Branch Spacing for 4 Azolla Varieties. Means are based on 50 randomly sampled plants, except for AP branch where n=49. The critical t-value at 10% significance level is t=1.684. A calculated t-value which exceeds this is considered significant.

SPECIES		LEAF 6-7 SPACING mm	LEAF 7-8 SPACING mm	BRANCH SPACING mm
AP	$\bar{X}$	0.788	0.822	0.886
	S.D.	0.082	0.081	0.110
AP-CBF	$\bar{X}$	0.690	0.734	0.814
	S.D.	0.111	0.093	0.119
Calculated t-value for difference in means		4.995	5.003	3.162
AF	$\bar{X}$	0.352	0.462	1.070
	S.D.	0.073	0.067	0.182
AF-CBF	$\bar{X}$	0.290	0.325	0.926
	S.D.	0.051	0.063	0.204
Calculated t-value for difference in means		4.884	10.475	3.714

was also interesting to note that the branch distance means of the AP and AP-CBF ferns are very close to the leaf distance means while in the AF and AF-CBF ferns the branch distance means were two to three times greater than the leaf distance means. This pattern as well as that noted in the leaf spacing ratios may be explained by the different growth patterns exhibited by these two species. The AP ferns grow in a very regular triangular pattern while the AF ferns do not show any regular shape and tend to grow in tangles.

The leaf size data is the mean of 25 random samples of leaf seven for each species and is presented in Table 7. The length of the CBF leaf was significantly less than that of the intact fern as determined from the calculated t-values for these comparisons in both AP and AF. Leaf seven of AP is longer than that of AF but the difference between the AP intact and CBF ferns was greater than that between the AF intact and CBF. The mean width of leaf seven in the intact and CBF ferns were not significantly different from each other. From this data it can be concluded that the CBF fern leaf seven was shorter than the intact leaf seven but that the width of leaf seven did not differ significantly from the intact to the CBF fern.

Thus the CBF ferns were different from the intact ferns in that the CBF ferns were more compact as seen by the shorter branch distances and closer leaf spacing of the CBF ferns. Also the length of leaf seven in the CBF ferns was

Table 7. Leaf 7 Size In 4 Azolla Varieties. Means are based on 25 random samples, and the critical t-value at the 10% significance level is t=1.684.

SPECIES		LENGTH mm	WIDTH mm
AP	$\bar{X}$	1.192	0.682
	S.D.	0.078	0.045
AP-CBF	$\bar{X}$	0.990	0.692
	S.D.	0.086	0.051
Calculated t-value for difference in means		8.634	-0.0729
AF	$\bar{X}$	0.842	0.514
	S.D.	0.057	0.072
AF-CBF	$\bar{X}$	0.806	0.538
	S.D.	0.052	0.083
Calculated t-value for difference in means		2.315	-1.084

shorter than in the intact fern. These differences result in a fern which was more compact than the intact fern. intact ferns are similar in that they both follow the same general growth pattern as seen by the similarity of the leaf and branch distances between the intact and CBF ferns. This information indicated that there was a difference between the intact and CBF ferns in their morpological appearence and indirectly supports the hypothesis that the removal of the cyanobiont had something to do with causing these differences.

3.4 NUTRIENT STUDY RESULTS

3.4.1 AP-CBF

At the conclusion of this experiment a 3K effect on coloration of the ferns was observed at all levels of N and P. The 3K ferns were more uniformly green than either the 1/2K or 1K treatments. All the treatments showed some brown center fronds but the 1/2N 1/2P 3K treatment resulted in fewer brown fronds per plant. Some black fronds were observed at N P K levels of 1 1 1, 1 2 1/2, 1 2 1, 2 1/2 1/2, 2 1/2 1, 2 1 1, 2 2 1/2, and 2 2 1. The morphology of the leaves and roots was not visibly affected in any of the treatments.

The analysis of variance(ANOVA) for the data is presented in Table 8 for the effect of N P and K on weight of the AP-CBF ferns. At the 10% level of significance the

interaction of N and P(N*P) was significant in determining the weight of the fern, K alone is also significant. Since the N*P effect was significant it is difficult to dissociate the effects of N and P. So to interpret the data the sums of squares for N*P and P were pooled to give six degrees of freedom which were then broken down into the six meaningful linear contrasts(Table 9) allowing discussion of the data in terms of the effect of N at different levels of P.

The graph of the mean weights plotted against N at the different levels of P is given in Figure 5. At 1/2N the 1/2P and 1P levels resulted in significantly better increases in weight, but 1/2P and 1P were not significantly different from each other. At 1N, 2P resulted in a significant increase in weight. At 2N, both 1P and 2P resulted in a significant increase over 1/2P but 1P and 2P were not significant from each other. From this analysis I concluded that at levels of 1N and 2N, 2P gave better growth of AP-CBF while at 1/2N, 2P resulted in a decrease in growth. Therefore, from these results it appears that the best growth of AP-CBF occurred at 1N 2P.

As mentioned previously K alone had a significant effect on the increase in the ferns weight. Figure 6 shows the effect of the three levels of K on the increase in weight. Weight increased linearly with increasing levels of K. A pair of contrasts were constructed to test the significance of these values(see Table 10). The values were

Table 8. ANOVA Table for Effect of Nutrients on Weight of AP-CBF Fern.
An effect is considered significant if the PR>F value is less than the 10% significance level (0.10).

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE
MODEL	26	10.04970617	0.38652716	3.16	0.0002	0.603106
ERROR	54	6.61353333	0.12247284		STD DEV	WT MEAN
CORRECTED TOTAL	80	16.66323951			0.34996120	1.91086420

SOURCE	DF	TYPE I SS	F VALUE	PR > F
N	2	2.95971358	12.08	0.0001
P	2	0.96575802	3.94	0.0252
N*P	4	2.07096790	4.23	0.0048
K	2	2.80093580	11.43	0.0001
N*K	4	0.28292346	0.58	0.6801
P*K	4	0.42405679	0.87	0.4906
N*P*K	8	0.54535062	0.56	0.8083

Table 9. Contrasts for N*P Interaction Effect on Weight of AP-CBF Fern. A contrast is considered significant if the PR > F value is less than the 10% significance level (0.10).

CONTRAST	DF	SS	F VALUE	PR > F
1/2P + 1P vs 2P at 1/2N	1	0.36836296	3.01	0.0886
1/2P vs 1P at 1/2N	1	0.00222222	0.02	0.8933
1/2P + 1P vs 2P at 1N	1	1.52006667	12.41	0.0009
1/2P vs 1P at 1N	1	0.03380000	0.28	0.6015
1P + 2P vs 1/2P at 2N	1	0.95733519	7.82	0.0072
1P vs 2P at 2N	1	0.15493889	1.27	0.2657

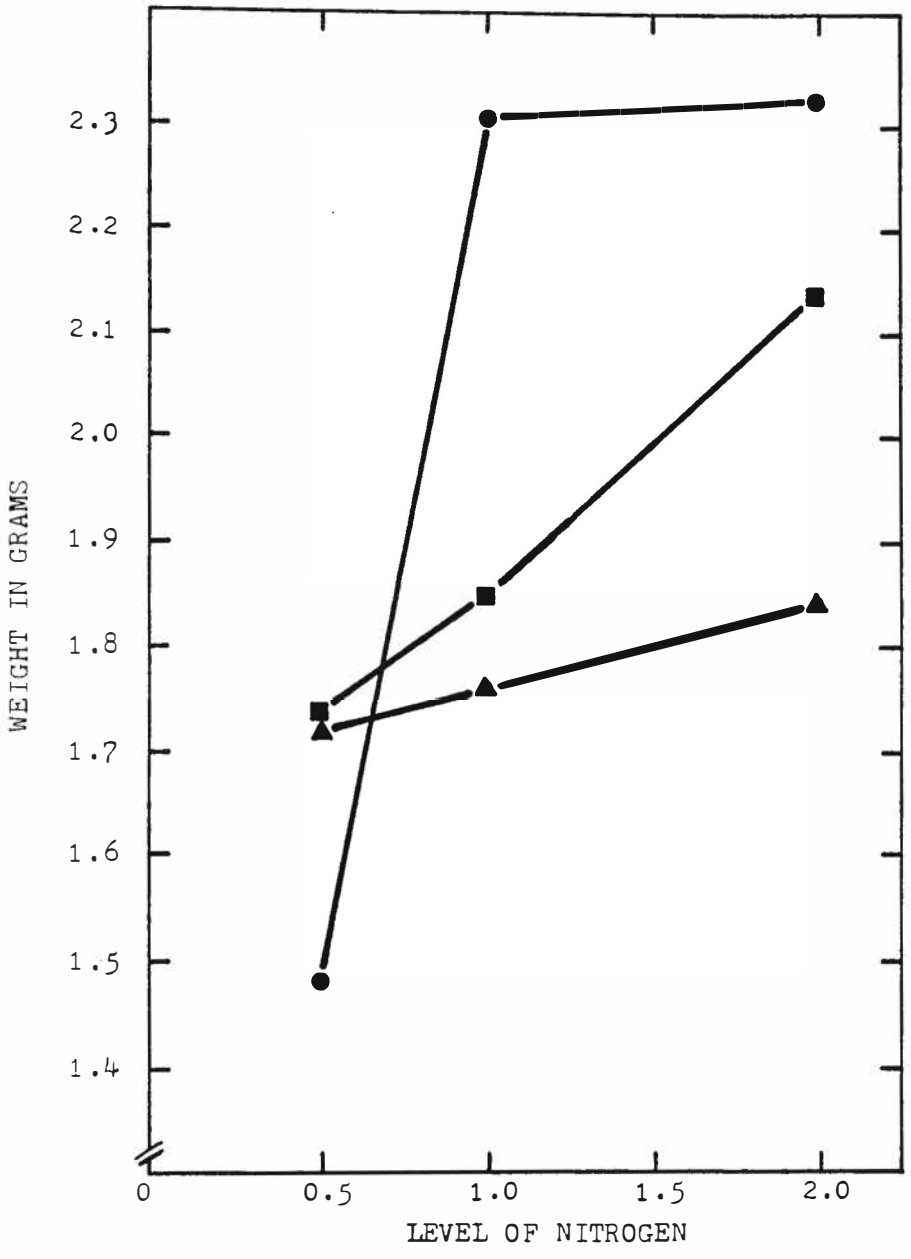

Figure 5. Effect of N and P on AP-CBF fresh weight. 1/2P (▲—▲), 1P (■—■), 2P (●—●).

significantly different at all levels of K and thus increased levels of K resulted in an increase in AP-CBF fern weight.

The ANOVA table for the effects of N P and K on the total chlorophyll content of the AP-CBF ferns is presented in Table 11. Each nutrient alone had a significant effect on total chlorophyll content. The effect of N level on chlorophyll content is in Figure 7. Total chlorophyll increased from 1/2N to 1N. This increase in chlorophyll was significant but the 2N level did not produce any further significant change in the total chlorophyll content(see the contrasts in Table 12). Figure 8 shows that increasing the level of P from 1/2 to 1 resulted in a significant decrease in chlorophyll while increasing P from 1 to 2 was not significant. Figure 9 shows that only the increase of K from 1 to 3 was significant and resulted in a decrease of the ferns total chlorophyll.

These nutrient experiments suggested the best medium for increasing AP-CBF ferns weight was I+ medium with levels of 1N, 2P, 3K. These levels gave the greatest increase in weight and there were no visible signs of stress on the ferns. To achieve the highest total chlorophyll content the best mediun would be 1N 1/2P 1/2 or 1K. Although Figure 7 and Table 12 show no significant difference in total chlorophyll content the 2N treatment did result in some black fronds while the 1N treatment did not, so this is why

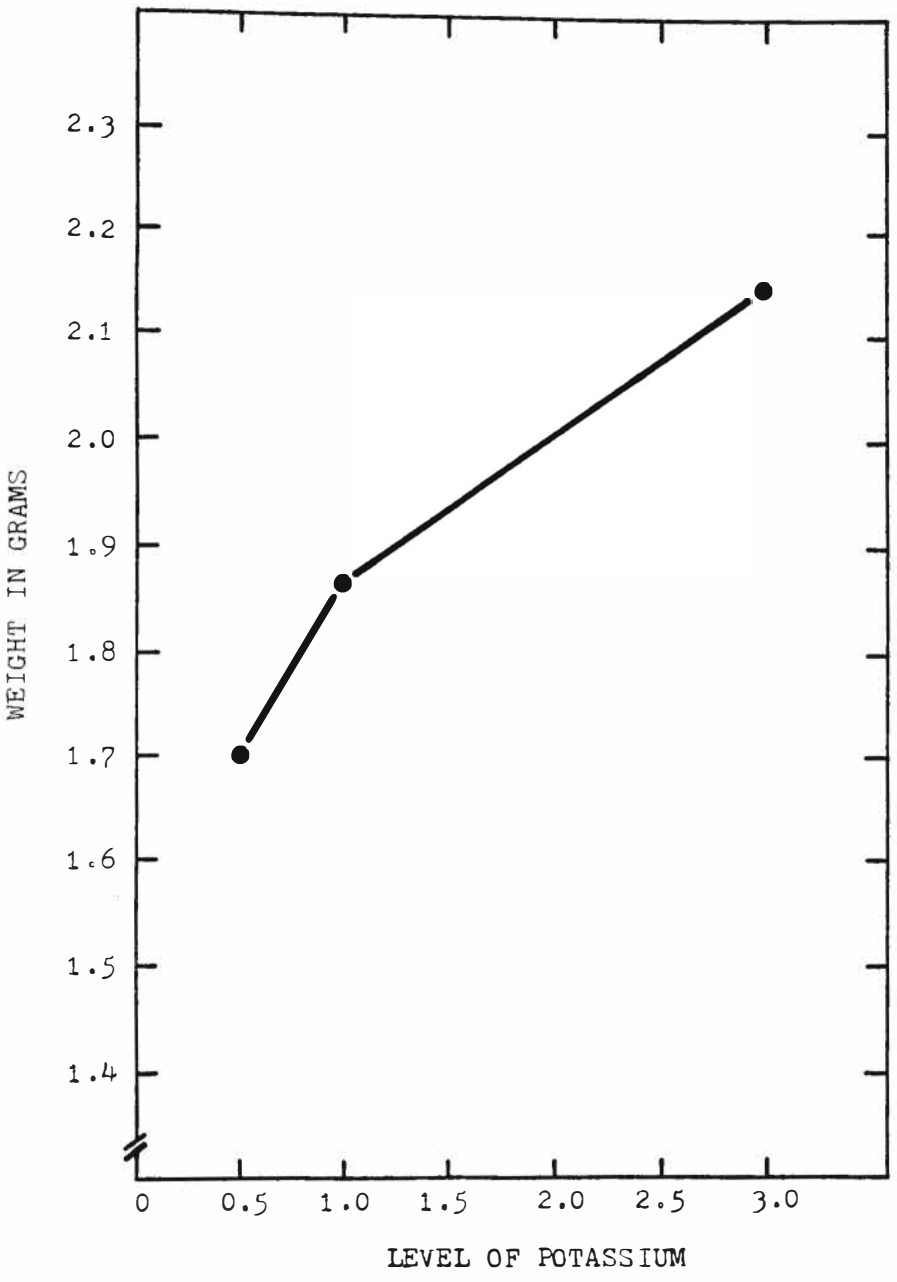

Figure 6. Effect of K on AP-CBF fresh weight.

Table 10. Contrasts for Effect of K on Weight of AP-CBF Fern.

CONTRAST	DF	SS	F VALUE	PR > F
1/2K + 1K vs 3K	1	2.41755617	19.74	0.0001
1/2K vs 1K	1	0.38337963	3.13	0.0825

Table 11. ANOVA Table for Effect of Nutrients on Total Chlorophyll of AP-CBF Fern.

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE
MODEL	26	0.02287125	0.00087966	5.21	0.0001	0.715105
ERROR	54	0.00911181	0.00016874		STD DEV	CHL MEAN
CORRECTED TOTAL	80	0.03198305			0.01298989	0.09410401

SOURCE	DF	TYPE I SS	F VALUE	PR > F
N	2	0.00328547	9.74	0.0002
P	2	0.01367885	40.53	0.0001
N*P	4	0.00127011	1.88	0.1269
K	2	0.00125393	3.72	0.0308
N*K	4	0.00122202	1.81	0.1402
P*K	4	0.00100098	1.48	0.2202
N*P*K	8	0.00115989	0.86	0.5561

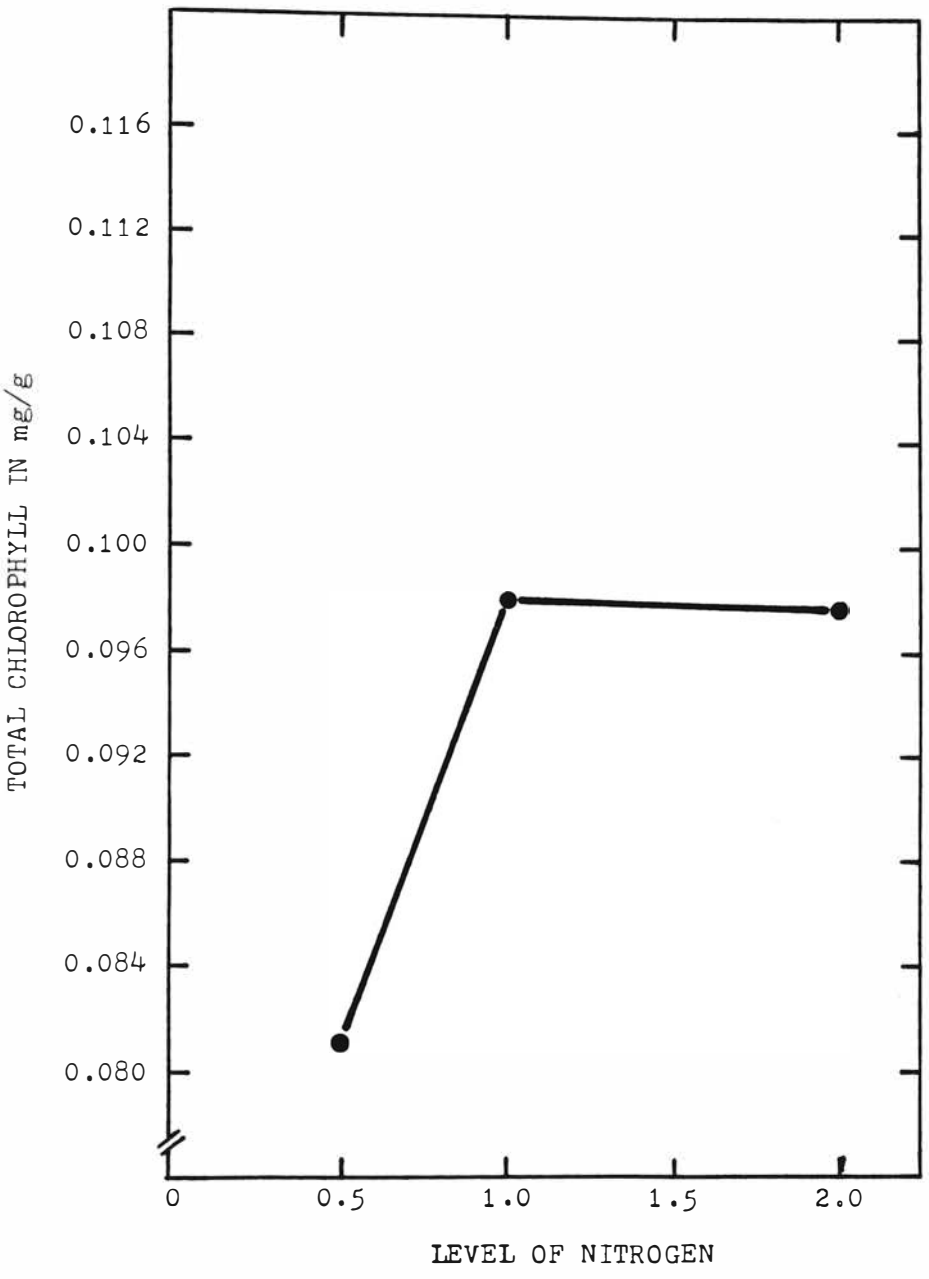

Figure 7. Effect of N on total chlorophyll in AP-CBF.

Table 12. Contrasts for Individual Effects of N P and K on Total Chlorophyll of AP-CBF Fern.

CONTRAST	DF	SS	F VALUE	PR > F
1N + 2N vs. 1/2N	1	0.00328108	19.44	0.0001
1N vs. 2N	1	0.00000439	0.03	0.8725
1P + 2P vs. 1/2P	1	0.01323936	78.46	0.0001
1P vs. 2P	1	0.00043949	2.60	0.1124
1/2K + 1K vs. 3K	1	0.00125390	7.43	0.0086
1/2K vs 1K	1	0.00000004	0.00	0.9882

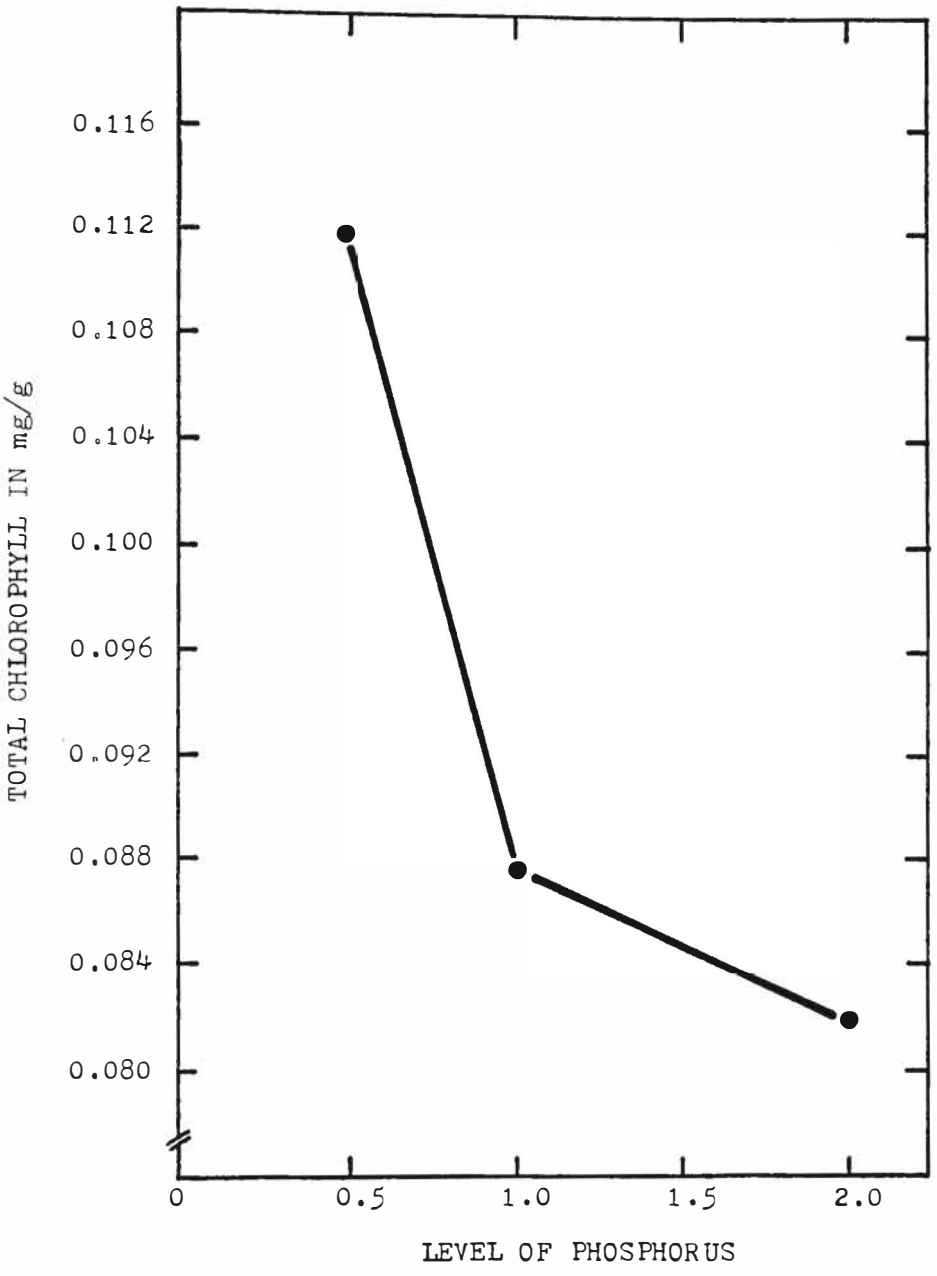

Figure 8. Effect of P on total chlorophyll in AP-CBF.

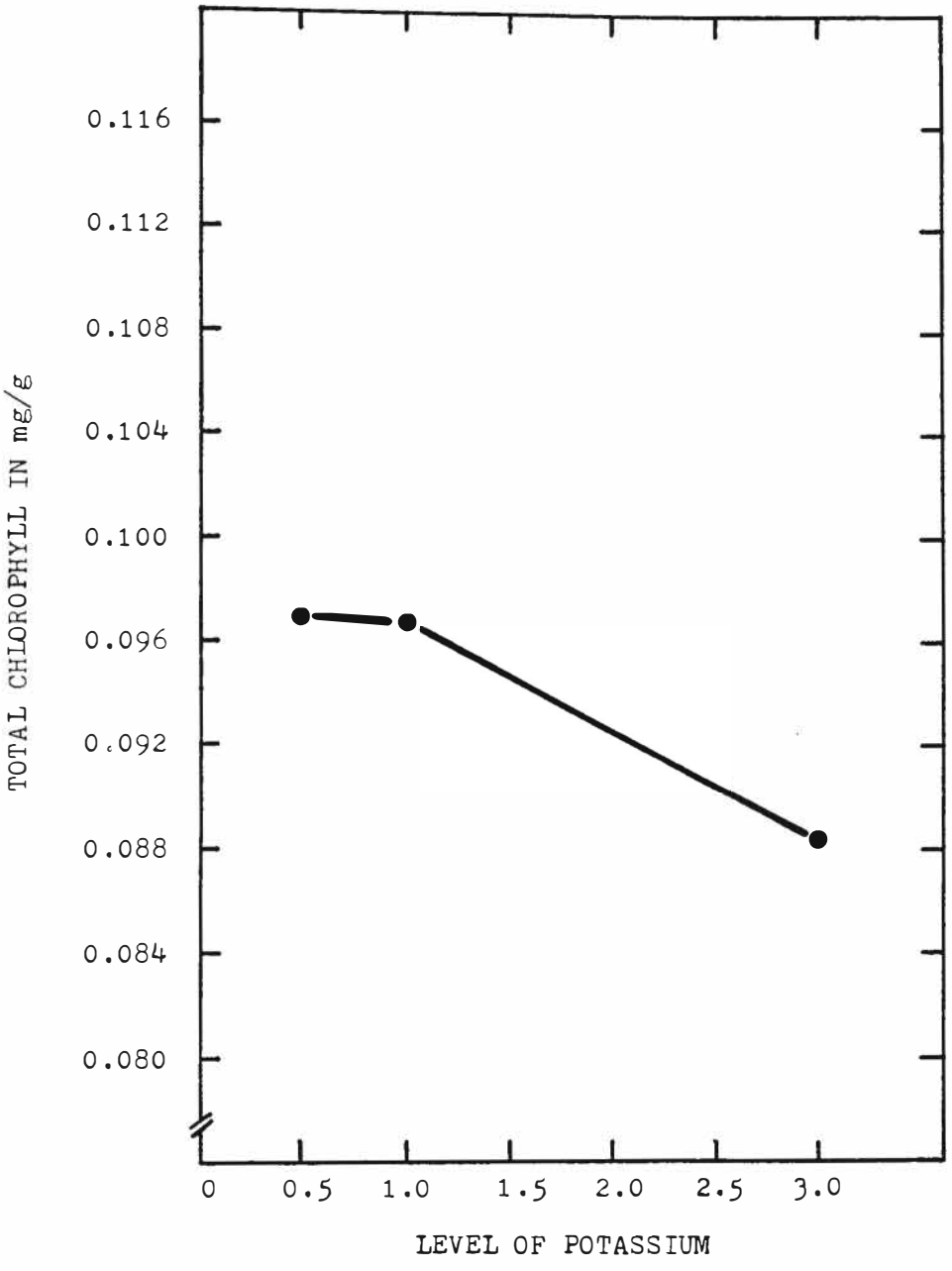

Figure 9. Effect of K on total chlorophyll in AP-CBF.

1N was chosen. It is also interesting to note that in the 3K treatments the ferns were observed to be more uniformly green than in either the 1/2K or 1K treatments but that the 3K ferns showed a significantly lower total chlorophyll content than either 1/2 or 1K treated ferns.

The above results indicate that the best growth medium for the AP-CBF ferns was the 1N, 2P, 3K I+ medium because it resulted in the greatest increase in fern tissue with the least amount of stress as observed through visual cues. The reason that the medium found to be optimal for total chlorophyll content was not suggested as the best was because it resulted in less than optimal growth of the fern.

3.4.2 AF-CBF

The AF-CBF ferns showed a greater increase in weight during the experiment than the AP-CBF ferns, but this was most likely due to the greater number of roots in AF-CBF. The AF-CBF ferns at 1/2N 1/2K and 1/2N 1K were more fragile than the 1/2N 3K ferns, they easily fragmented when disturbed or upon removal from the flasks for weighing. It was also noted that the 1N 1/2P 1/2K, treated ferns were also fragile and the roots fell off very easily and a larger number of loose roots were present in the culture flasks.

Table 13 contains the ANOVA data for the dependent variable weight, for AF-CBF ferns. The three factor interaction N*P*K was significant at the 10% significance

level. Since this interaction effect requires one to speak about all three effects at once the sums of squares for P, N*P, P*K, and N*P*K were pooled to give 18 degrees of freedom and then 18 linear orthogonal contrasts were created, they are presented in Table 14. In order to discuss the data in the same manner as the AP-CBF data, the data was first grouped according to the level of K, this resulted in three graphs each corresponding to the appropriate level of K with weight vs N as the axises of these graphs. The effect of N and P along with 1/2K is given in Figure 10. At 1/2N the 1/2P treatment resulted in a significant increase in weight as compared to 1P or 2P which were not significantly different from each other. However at 1N, the 2P level gave a significant increase in weight over either 1/2P or 1P both of which were not significantly different from each other. At 2N all three levels of P were significantly different from each other with 2P resulting in the highest weight gain, 1P the next and 1/2P the least. From these data it was clear that at 1/2K the best growth occurred at the 1N 2P and 2N 2P levels.

The effect of N and P at 1K on weight is presented in Figure 11. At 1/2N both 1/2P and 2P resulted in significantly better growth than 1P, while 1/2P and 2P were not significantly different from each other. At both 1N and 2N, 2P resulted in a significant increase in weight over both 1/2P and 1P which were not significantly different from

Table 13. ANOVA Table for Effect of Nutrients on Weight of AF-CBF Fern.

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE
MODEL	26	31.74618765	1.22100722	17.84	0.0001	0.895743
ERROR	54	3.69500000	0.06842593		STD DEV	WT MEAN
CORRECTED TOTAL	80	35.44118765			0.26158350	3.39567901

SOURCE	DF	TYPE I SS	F VALUE	PR > F
N	2	4.07640247	29.79	0.0001
P	2	4.60868395	33.68	0.0001
N*P	4	3.28083457	11.99	0.0001
K	2	15.71863951	114.86	0.0001
N*K	4	0.51521235	1.88	0.1268
P*K	4	1.26657531	4.63	0.0028
N*P*K	8	2.27983951	4.16	0.0006

Table 14. Contrasts for N*P*K Interaction Effect on Weight of AF-CBF Fern.

CONTRAST	DF	SS	F VALUE	PR > F
1P + 2P vs 1/2P at 1/2N 1/2K	1	0.48347222	7.07	0.0103
1P vs 2P at 1/2N 1/2K	1	0.00601667	0.09	0.7680
1/2P + 1P vs 2P at 1N 1/2K	1	0.64600556	9.44	0.0033
1/2P vs 1P at 1N 1/2K	1	0.03375000	0.49	0.4855
1/2P + 1P vs 2P at 2N 1/2K	1	1.92080000	28.07	0.0001
1/2P vs 1P at 2N 1/2K	1	0.26460000	3.87	0.0544
1/2P + 2P vs 1P at 1/2N 1K	1	0.39308889	5.74	0.0200
1/2P vs 2P at 1/2N 1K	1	0.01126667	0.16	0.6865
1/2P + 1P vs 2P at 1N 1K	1	1.82405000	26.66	0.0001
1/2P vs 1P at 1N 1K	1	0.12615000	1.84	0.1802
1/2P + 1P vs 2P at 2N 1K	1	3.22580000	47.14	0.0001
1/2P vs 1P at 2N 1K	1	0.04166667	0.61	0.4386
1/2P + 2P vs 1P at 1/2N 3K	1	1.25875556	18.40	0.0001
1/2P vs 2P at 1/2N 3K	1	0.08166667	1.19	0.2795
1/2P + 2P vs 1P at 1N 3K	1	0.43555556	6.37	0.0145
1/2P vs 2P at 1N 3K	1	0.03226667	0.47	0.4952
1P + 2P vs 1/2P at 2N 3K	1	0.55475556	8.11	0.0062
1P vs 2P at 2N 3K	1	0.09626667	1.41	0.2408

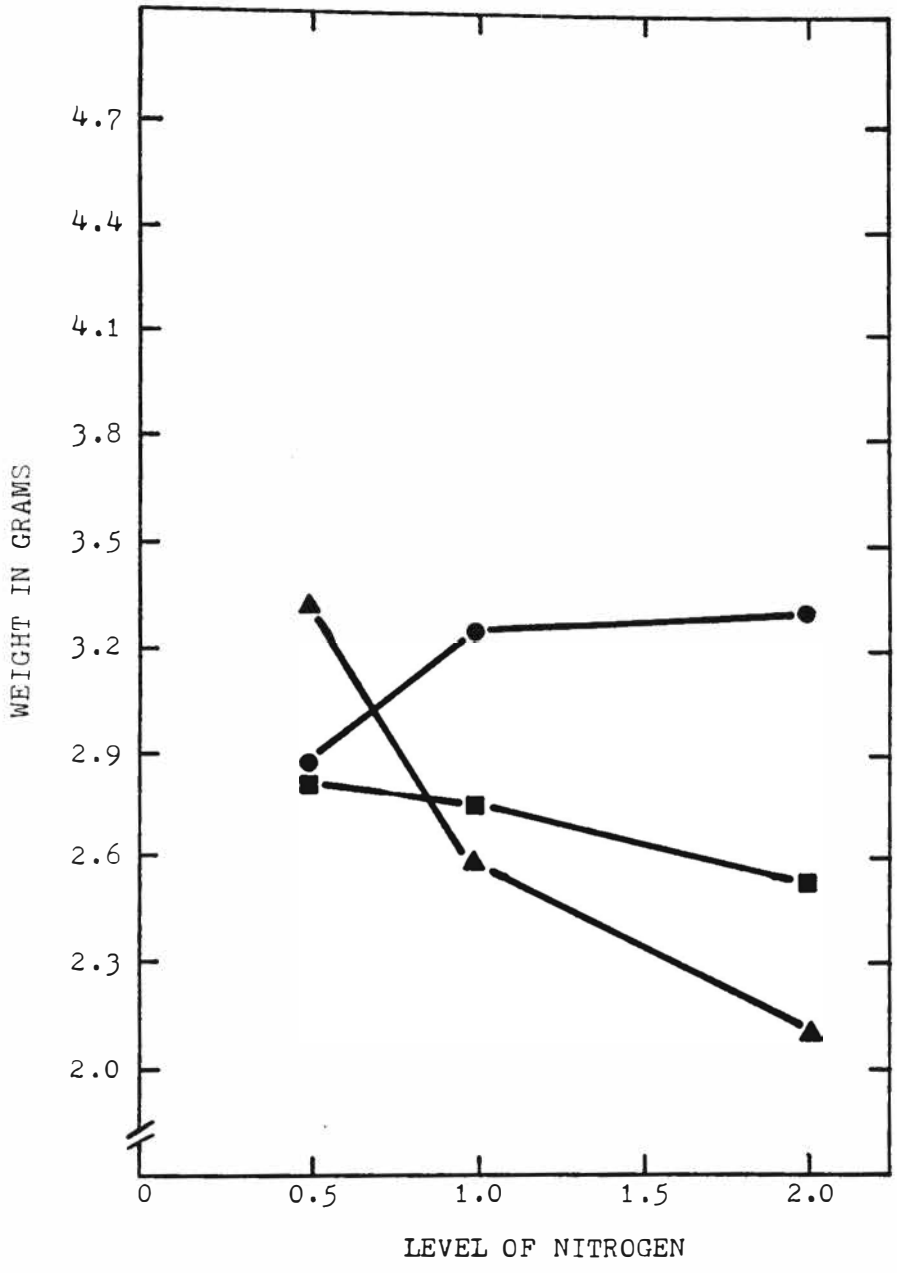

Figure 10. Effect of N and P at 1/2K on AF-CBF fresh weight. 1/2P (▲——▲), 1P (■——■), 2P (●——●).

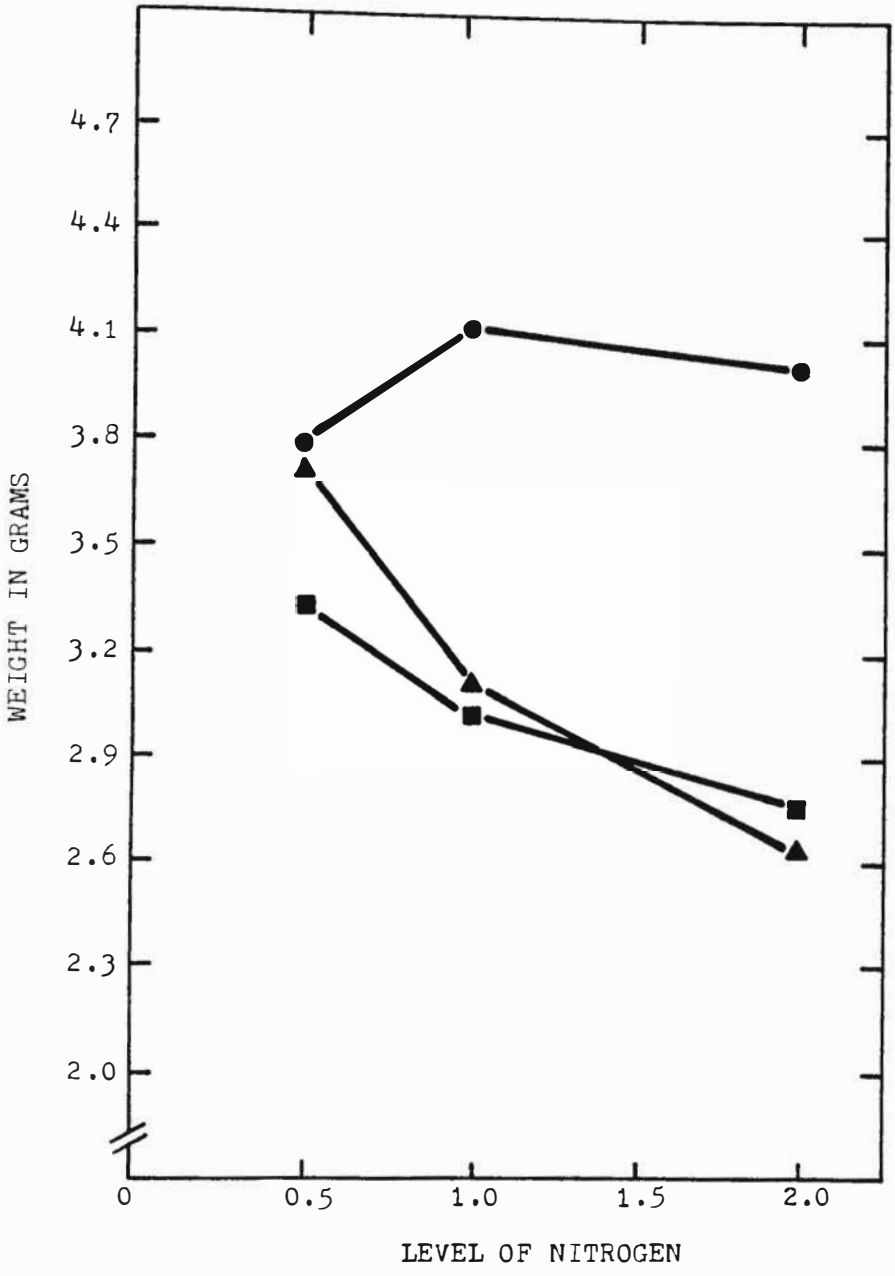

Figure 11. Effect of N and P at 1K on AF-CBF fresh weight. 1/2P (▲——▲), 1P (■——■), 2P (●——●).

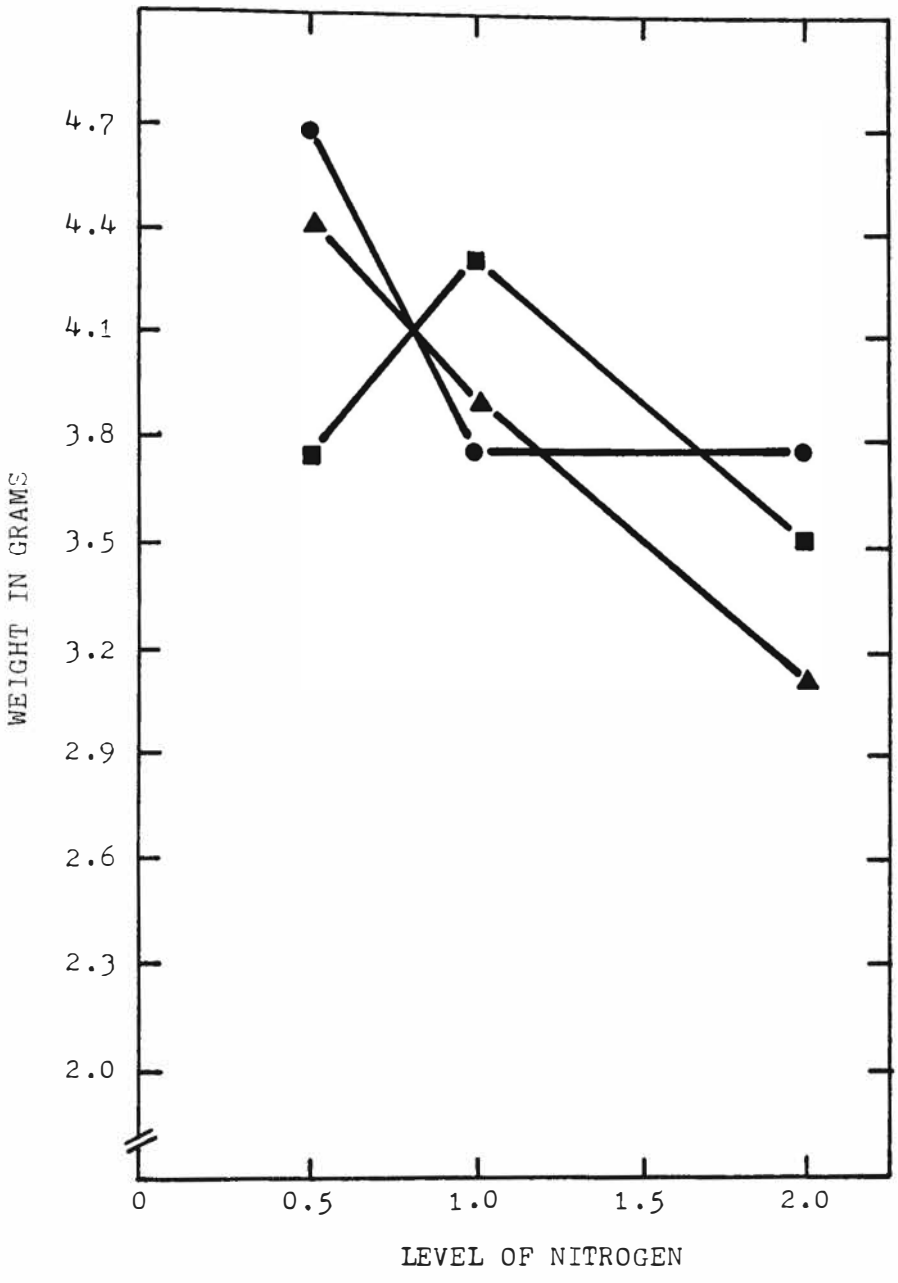

Figure 12. Effect of N and P at 3K on AF-CBF fresh weight. 1/2P (▲——▲), 1P (■——■), 2P (●——●).

each other. Figure 12 gives the effect of N and P at 3K on the weight of AF-CBF. A general decrease in weight with an increase in both N and P was observed. At 1/2N, both 1/2P and 2P were significantly different from 1P but they were not significantly different from each other. At 1N, 1P resulted in a significant increase as compared to 1/2P and 2P which were not significant from each other. At 2N, 1P and 2P were significantly different from 1/2P but not each other.

The total chlorophyll data ANOVA table(see Table 15) also showed a three factor interaction effect. This resulted in the pooling of the sums of squares to give the 18 degrees of freedom needed to make the 18 meaningful linear orthogonal contrasts presented in Table 16. It should be noted that replicate one of treatment 1/2N 1P 1/2K used for the chlorophyll extraction was lost, therefore only two values were available to determine the means for total chlorophyll at this treatment. An analysis of variance can still be performed on this data but, the missing value will create a term which will not cancel out of the other contrasts. Thus each hypothesis will have an extraneous term which will change the meaning of that hypothesis, and adversely affect all the contrasts. To overcome this problem it is standard procedure to estimate the missing values, unfortunately there is no standard equation to calculate a missing value in a completely randomized

Table 15. ANOVA Table for Effect of Nutrients on Total Chlorophyll of AF-CBF Fern.

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE
MODEL	26	0.10658392	0.00409938	56.68	0.0001	0.964653
ERROR	54	0.00390545	0.00007232		STD DEV	CHL MEAN
CORRECTED TOTAL	80	0.11048937			0.00850430	0.09265545

SOURCE	DF	TYPE I SS	F VALUE	PR > F
N	2	0.08689638	600.75	0.0001
P	2	0.00240667	16.64	0.0001
N*P	4	0.00852018	29.45	0.0001
K	2	0.00338860	23.43	0.0001
N*K	4	0.00121531	4.20	0.0049
P*K	4	0.00205335	7.10	0.0001
N*P*K	8	0.00210343	3.64	0.0019

Table 16. Contrasts for N*P*K Interaction Effect on Total Chlorophyll of AF-CBF Fern.

CONTRAST	DF	SS	F VALUE	PR > F
1P + 2P vs 1/2P at 1/2N 1/2K	1	0.00013495	1.87	0.1776
1P vs 2P at 1/2N 1/2K	1	0.00002696	0.37	0.5440
1/2P + 1P vs 2P at 1N 1/2K	1	0.00050475	6.98	0.0108
1/2P vs 1P at 1N 1/2K	1	0.00004737	0.65	0.4219
1/2P + 1P vs 2P at 2N 1/2K	1	0.00430098	59.47	0.0001
1/2P vs 1P at 2N 1/2K	1	0.00040463	5.59	0.0216
1P + 2P vs 1/2P at 1/2N 1K	1	0.00000544	0.08	0.7849
1P vs 2P at 1/2N 1K	1	0.00000299	0.04	0.8396
1/2P + 2P vs 1P at 1N 1K	1	0.00242747	33.56	0.0001
1/2P vs 2P at 1N 1K	1	0.00019795	2.74	0.1039
1/2P + 1P vs 2P at 2N 1K	1	0.00482883	66.77	0.0001
1/2P vs 1P at 2N 1K	1	0.00000153	0.02	0.8847
1/2P + 2P vs 1P at 1/2N 3K	1	0.00012510	1.73	0.1940
1/2P vs 2P at 1/2N 3K	1	0.00000267	0.04	0.8484
1P + 2P vs 1/2P at 1N 3K	1	0.00046534	6.43	0.0141
1P vs 2P at 1N 3K	1	0.00000311	0.04	0.8366
1P + 2P vs 1/2P at 2N 3K	1	0.00155793	21.54	0.0001
1P vs 2P at 2N 3K	1	0.00004564	0.63	0.4304

design(CRD) experiment, so to estimate the missing values the absorbance readings of replicates two and three were plotted on a weight vs absorbance graph, a best fit line was drawn and the estimated value arrived at by using the weight of the missing value to determine the estimated absorbance. By using the estimated absorbance only those two contrasts which include the estimated value were affected and not the remaining 14 contrasts.

Figure 13 presents the effects of N and P at 1/2K on total chlorophyll. A general increase in fern chlorophyll paralleled the N level increase. At 1/2N none of the means were significantly different from each other, and it should be noted that the 1P value at this point is the mean of only 2 observations. At 1N, 2P resulted in a significantly higher chlorophyll content than either 1/2P or 1P which were not significantly different. At 2N we see a leveling off of the 2P effect, all the P levels at 2N were significantly different from each other with 1/2P resulting in the largest chlorophyll content and 2P the least.

Figure 14 shows the effect on total chlorophyll of N and P at 1K. Total chlorophyll increases as the N level increased. At 1/2N none of the P levels were significantly different from each other, while at 1N the 1P level was significantly different from the 1/2 and 2P levels. Again a leveling off of the 2P effect occurred at 2N while the 1/2P and 1P levels continued to increase. The 1/2 and 1P levels

were significantly different from 2P and resulted in a higher total chlorophyll content.

Figure 15 shows the effect on total chlorophyll of N and P at 3K. An increase in total chlorophyll accompanied the increase of N. At 1/2N none of the P levels resulted in significantly different total chlorophyll content, while at 1N the 1P and 2P levels resulted in a significant increase in chlorophyll over the 1/2P level. At the 2N level we do not see the leveling off of the 2P treated ferns, instead all the P levels continued to increase. The 1/2P level though resulted in a significant increase over either the 1P or 2P levels which were not significantly different.

The data from these AF-CBF nutrient studies showed that as the level of K increased the weight increased and that the relationship of N and P remained fairly constant for the 1/2 and 1K levels. From the results the best medium for the growth of AF-CBF ferns as measured in terms of weight increase was 1/2N 2P 3K in I+ medium , even though 1/2P and 2P were not significantly different the 2P level did cause a slightly higher increase in weight. The data for total chlorophyll showed a different trend. The total chlorophyll increased as the N increased at all levels of K. No increases at different levels of K were observed. Therefore, the best medium for total chlorophyll content would be either 2N, 1/2P, 1/2K or 2N, 1/2 or 1P, 1K in I+. These combinations resulted in a total chlorophyll content that differed by only 0.005mg/g.

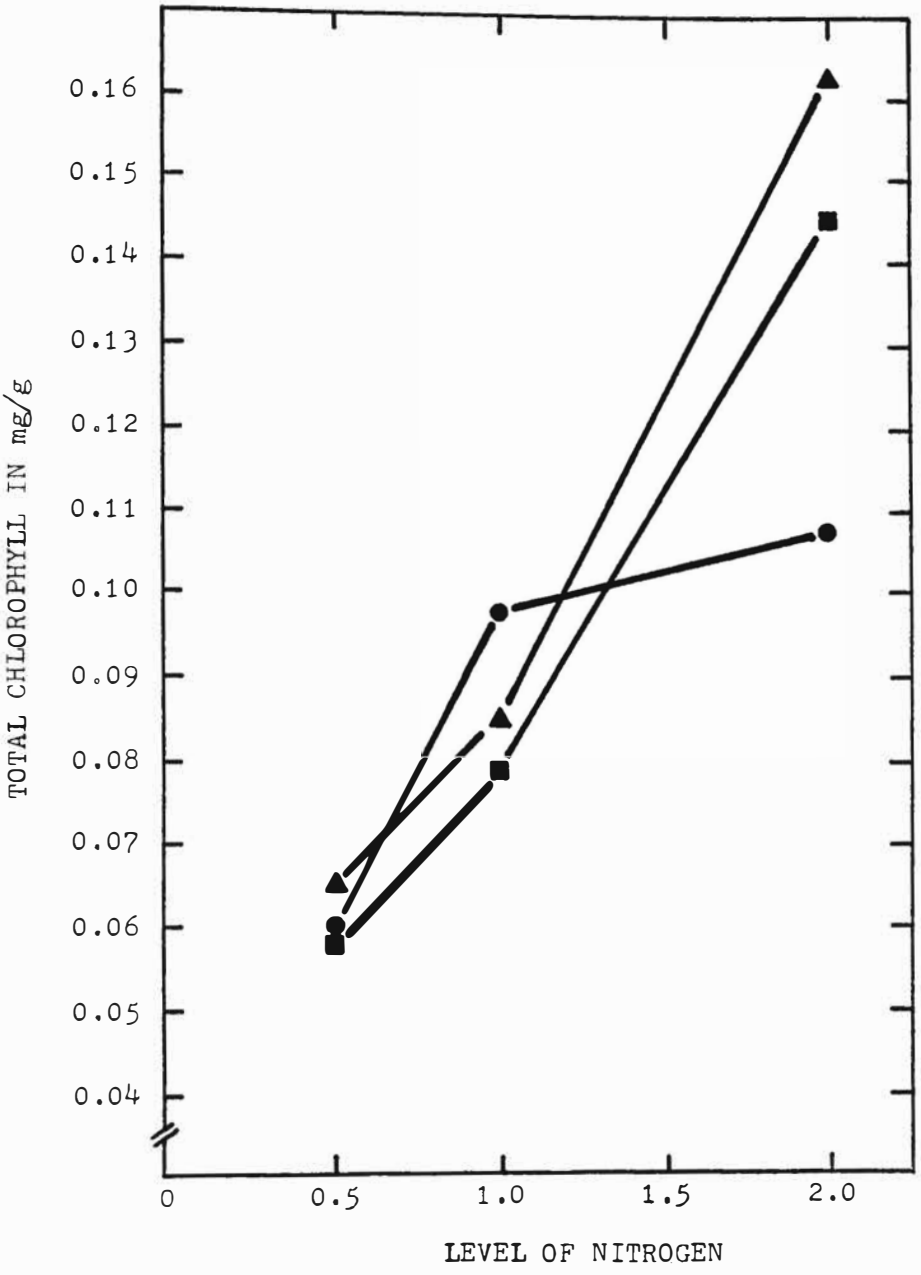

Figure 13. Effect of N and P at 1/2K on total chlorophyll of AF-CBF. 1/2P (▲——▲), 1P (■——■), 2P (●——●).

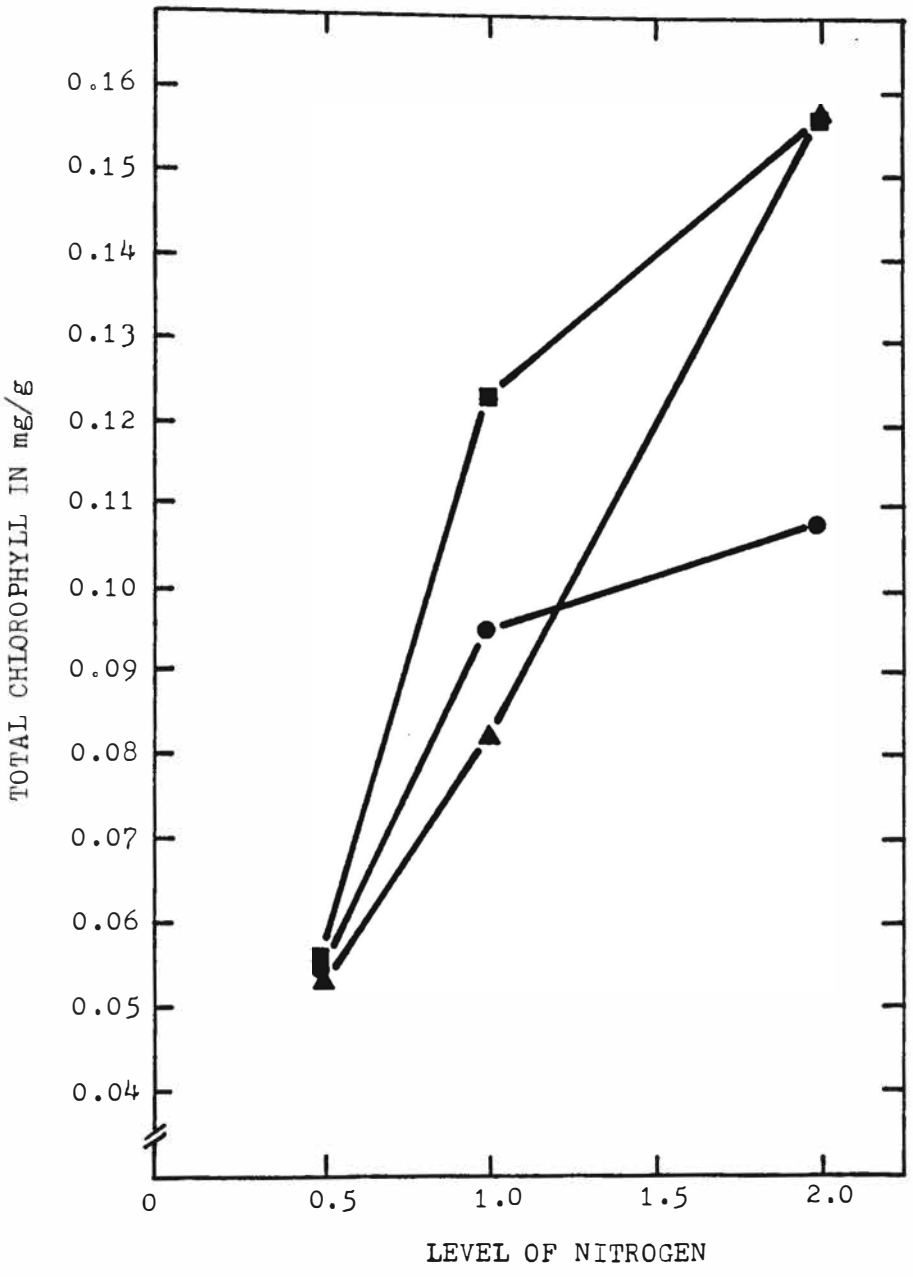

Figure 14. Effect of N and P at 1K on total chlorophyll of AF-CBF. 1/2P (▲——▲), 1P (■——■), 2P (●——●).

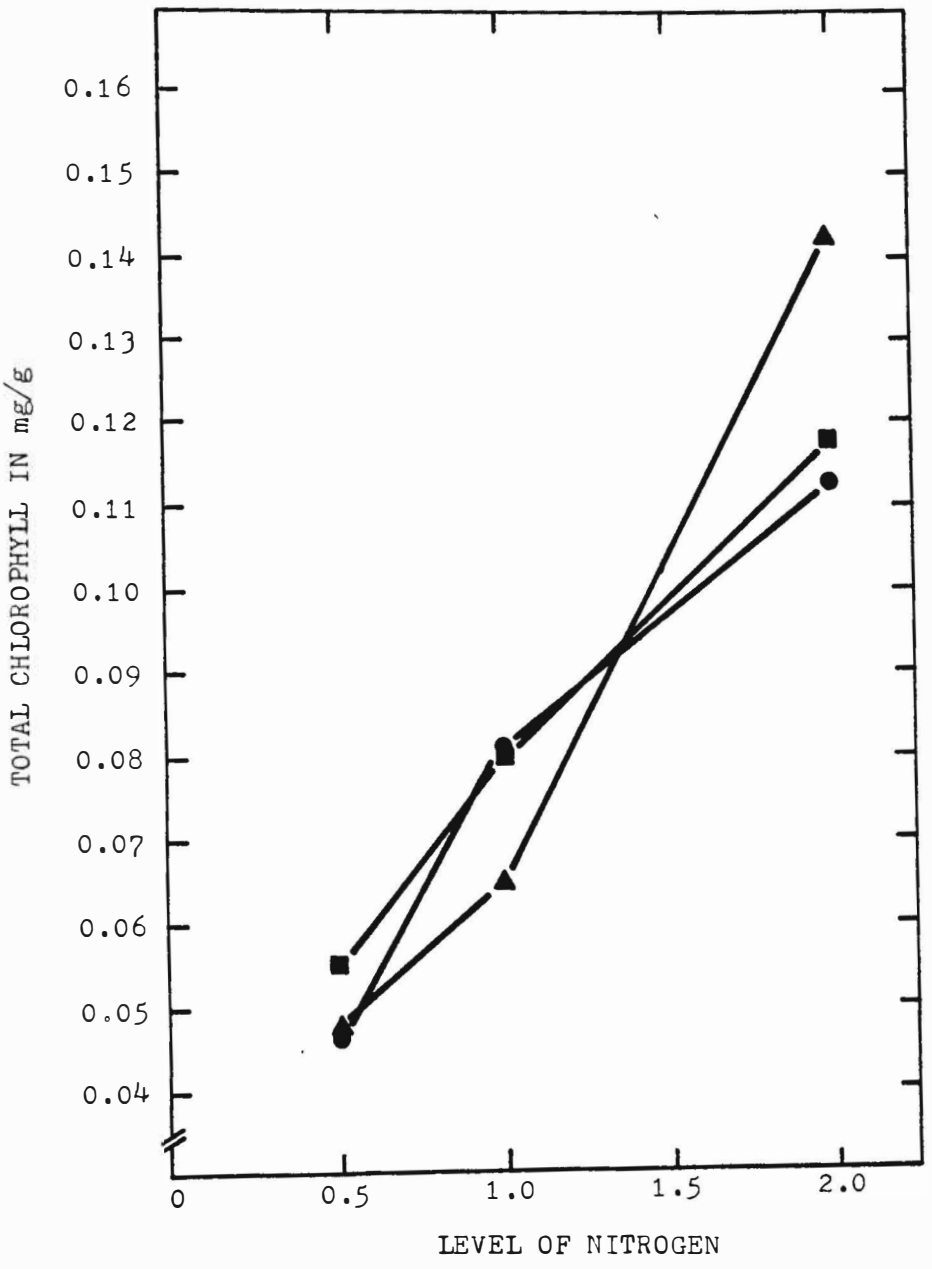

Figure 15. Effect of N and P at 3K on total chlorophyll of AF-CBF. 1/2P (▲——▲), 1P (■——■), 2P (●——●).

IV

DISCUSSION

4.1 CBF PRODUCTION

The limited success of the clorox surface sterilization technique indicated that there may be a difference in the organization of the stem apex in AF and AP. The literature contains only one report(Pai Ke-chih, et al., 1978) of the successful use of surface sterilization of an AP species A. imbricata Nakai. All other reports of surface sterilization have used AF(Huneke, 1935; Ashton and Walmsley, 1976; Becking, 1978) or AC and AF(Duckett, Toth and Soni, 1975) which are all members of the subgenera Euazolla. From these reports it is not clear whether there is a difference in the apex organization. A comparison of the AP apex(Konar and Kapoor, 1972) to the AF apex(Becking, 1978) shows a very similar arrangement of the leaves. Thus further research with the use of clorox as a surface sterilizing agent is needed to determine if the AP species does exhibit a resistance to this method that is due to a morphological difference.

The antibiotic sequence treatments used were those found successful by Peters and Mayne(1974a) which were based on the sequential treatment used by Nickell(1958), both

researchers utilized A. caroliniana for their experiments. The difference in the success of the sequences used in my research again showed some difference between the AP and AF species. The AF species though exhibited resistance to the antibiotic treatments since the less rigorous A sequence failed to produce AF-CBF ferns but the more rigorous B and C sequences did. The AP ferns on the other hand were freed by all three treatment sequences but the C sequence treated ferns died soon after the conclusion of the treatments and the B sequence ferns were very slow to recover from the treatments. This would indicate that the B and C sequences were possibly more severe than necessary.

The results from the use of these two methods on the two species of Azolla indicated some difference in the ferns. The difference in the clorox surface sterilization experiment indicated some difference in the morphology of the AP stem apex, perhaps in the overlap of the leaves protecting the apex or else in its curvature away from the water which prevented the entry of the clorox into the apex. The results of the antibiotic treatments suggest some difference in uptake of the antibiotics by the ferns. It appears that the AP ferns very readily take up antibiotics while the AF ferns do not, as exhibited by the need for higher doses and use of several antibiotics in unison to produce a CBF fern. The roots are the probable site of uptake of the antibiotics and so some difference may exist in the roots which may lead to a difference in uptake.

Therefore more research is needed to determine why these differences exist. These further experiments should concentrate on the apex and compare the overlap of the outer leaves, the curve of the apex away from the water and possibly composition of the cuticle layer of each species. It is also suggested that uptake and mineral transport studies be initiated to determine what if any differences exist between the species.

4.2 MORPHOLOGY

The finding that the leaf and branch spacing is closer in the CBF ferns was not surprising since a compactness of the CBF ferns had been observed in our lab as well as by others(Peters and Mayne, 1974a; Ashton and Walmsley, 1976; and Pai Ke-chih, et al., 1978), but no quantitative data had ever been presented. The fact that the length of leaf seven was shorter in the CBF ferns but not the width was interesting. The absence of visible changes in the compactness or size of the CBF ferns either during or after the nutrient studies indicated that the changes in morphology were due to the absence of the cyanobiont and not a loss of nitrogen. Since the addition of phosphorus or potassium had no effect on the CBF ferns' morphology these nutrients could also be ruled out as the reason for the changes in morphology of the CBF ferns. Possibly some substance secreted by the cyanobiont or else the physical

presence of the cyanobiont itself was the reason for the morphological changes. Nickell (1961) conducted a hormone study on A. caroliniana using indoleacetic acid, 2,4-D, and gibberellin but he did not look at their effects on leaf or branch spacing. Dusek and Bonde(1965) used gibberillic acid and indoleacetic acid on A. mexicana. No hormone related increase in stem internode length was noted. This information indicates that these hormones do not affect the growth pattern of the intact association, whether this can be extended to the CBF ferns needs to be studied. It is also possible that the cyanobiont produces some morphogen that is not a hormone which affects the fern. To test this a study using isolated cyanobiont pure cultures would be needed from which the possible morphogen could be isolated and added to the fern medium.

4.3 NUTRIENT STUDIES

4.3.1 AP-CBF

The nutrient studies were undertaken to determine if the slow growth of the CBF ferns were due to the concentrations of N, P and K in the nutrient medium presently being used in our lab. The results of these experiments showed that indeed a nutrient medium of 1N, 2P, 3K in I+ medium gave better growth as measured in terms of fresh weight than the presently used 1N, 1P, 1K in I+ medium used for the AP-CBF ferns. The interaction effect observed in the fresh weight

results, between nitrogen and phosphorus is not surprising. The removal of the cyanobiont from the fern resulted in the loss of the ferns nitrogen supply, which must be added to the medium to support the growth of the CBF ferns. Several researchers(Subudhi and Singh, 1979; Kulasooryia, et al., 1980; Watanabe, et al., 1980; and Subudhi and Watanabe, 1981) have shown that phosphorus is a major limiting factor in the growth and nitrogen fixation in intact Azolla. Subudhi and Singh(1979) have also reported that a phosphorus deficiency results in a disturbance in the ferns nitrogen metabolism as well as the accumulation of plant carbohydrates. There was no reason to believe this was not also true for the AP-CBF ferns which would explain the interaction of nitrogen and phosphorus in determining the ferns weight. The role that phosphorus plays in the nitrogen metabolism in the fern can be seen in Figure 5 where the 2P level at 1/2N resulted in the poorest growth while at 1N this same 2P level results in the greatest increase in growth.

The effect of potassium on the weight of the AP-CBF fern indicated that the maximum level of K may not have been reached in this experiment. The ferns grown at 1/2 and 1K levels did show some signs(i.e., necrotic fronds) of K deficiency while at the 3K treatments the ferns did not exhibit this deficiency symptom. Potassium plays a role in stomate movement, enzyme activation and also ion transport

in higher plants(Salisbury and Ross, 1978) and it probably plays the same role in the fern. So the results indicated that an even higher level of K may have increased the AP-CBF ferns weight even more.

The data for the effects of N, P and K on total chlorophyll showed that each effect was independent of the other. The increase in chlorophyll from 1/2N to 1N indicated that the optimum N level occurred between 1 and 2N for chlorophyll. Subudhi and Singh (1979) reported for AP intact that as nitrogen is added to the medium the chlorophyll content decreases. These results support the suggestion that the optimum N level for chlorophyll has been reached. Subudhi and Singh(1979) also reported that above their optimum phosphorus dose of 1.2mM further increases in P resulted in a decreased chlorophyll content. This agrees with the results in Figure 8 which shows a decrease in chlorophyll from 1/2P to 2P. The decrease in total chlorophyll that resulted from increasing the potassium level is not clearly understood. A possible explanation might be that the increased K levels were facilitating the transport of nitrogen and thus possibly chlorophyll is being broken down for its nitrogen or else N is being used up to handle the excess potassium and not incorporated into chlorophyll. Further studies are needed to determine the effect of K on the fern and these studies should also involve a wider range of nutrient levels.

All of these studies on AP-CBF should be expanded to determine if the effects seen on fern weight in the N P interaction would be repeated at higher levels or whether a maximum level was reached above 2N or 2P.

4.3.2 AF-CBF

The finding of a three factor interaction for the AF-CBF ferns served to reinforce the premise that the two fern species are different in their metabolic processes. This premise is additionally supported by the findings of Subudhi and Watanabe(1981) that AF intact ferns exhibited poorer growth in a phosphorus limiting medium than AP ferns. They also reported that AF ferns exhibited little or no phosphorus uptake but that the phosphorus content of AF ferns were higher than AP. They suggest that the AF species may "require a high level of tissue phosphorus to maintain normal metabolic activity." This can be seen in the 1/2K and 1K weight graphs where the 2P level resulted in the highest weight increase. AT 3K the highest weight increase occurred at 2P, 1/2N indicating that the optimum N level may have been exceeded.

This phosphorus effect was not seen in the total chlorophyll graphs where 1/2P and 1P levels resulted in the highest chlorophyll contents at 2N levels in all three K levels. In contrast to the effects on weight, K increases did not cause an increase in chlorophyll. Instead the

chlorophyll graphs overlap fairly closely perhaps indicating that the optimum P concentration for chlorophyll had been reached. The fact that the three factors interact is reasonable since K is involved in the transport of both NO_3^- and phosphates and phosphorus is involved in nitrogen metabolism. This interrelationship between the nutrients in fact almost seems more reasonable than the lack of the interaction of K with N and P in the AP-CBF ferns.

In conclusion it is clear that the absence of the cyanobiont placed a stress on the ferns that could be partially removed by addition of nutrients to the medium. It was also obvious that the removal of the cyanobiont resulted in morphological changes in the ferns which were not due to the loss of the nutrients supplied by the cyanobiont. Instead it was indicated that the cyanobiont may produce some morphogen which is responsible for the changes observed in leaf and branch spacing as well as in leaf length between intact and CBF varieties. It is also interesting to note that the loss of the cyanobiont affected the integrity of the AF-CBF fern as observed when preparing this tissue for plastic embedding. The AF-CBF fern tissue when removed from the gauze bag after the last dehydration in acetone, prior to placement in the epoxy, plasmalyzed very easily. This was not the case with the AF intact ferns or the AP or AP-CBF ferns. What caused this was not known but it served to indicate that there are differences between

the ferns and even between intact and CBF ferns of the same species.

From these experiments it was clear that the growth of AP-CBF and AF-CBF ferns could be improved by changing the medium they are presently being cultured on to better suit the needs of each species. The AP-CBF fern medium should be adjusted to contain 1N, 2P, 3K in I+ to produce the best growth under the standard Azolla growth conditions outlined previously. The AF-CBF ferns should be maintained on I+ medium containing 1/2N, 2P and 3K under standard Azolla growth conditions. Further research into the nutrient requirements of the CBF ferns is needed especially a more comprehensive study of the concentrations of nutrients at the optimum levels indicated in this preliminary study. Further research with these CBF ferns should concentrate on the following areas.

1. Effects, after long term growth on the suggested optimal media, on CBF fern cavity.
2. Production of cyanobacterial/bacterial free (CBF/BF) ferns.
3. Initiatiion of Azolla tissue cultures using CBF or CBF/BF ferns.
4. Production of Azolla protoplasts from CBF of CBF/BF ferns.
5. The use of CBF and CBF/BF ferns for reinfection and genetic studies.

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