CHAPTER - 5

DISCUSSION
Azolla is a fast growing floating aquatic plant which lives in symbiotic association with the filamentous nitrogen fixing cyanobacterium Anabaena azollae. The Azolla-Anabaena association has a tremendous potential for providing photosynthetic production of nitrogenous fertilizer. This symbiotic association has been used as a green manure in rice fields in Vietnam and China for centuries. Currently, China has more than 1.34 million hectares of rice fields under the Azolla cover. Although it has a long history in agriculture, it was not until the late 1950's that techniques for growing Azolla as nitrogen fertilizer for rice were extensively studied in different parts of rice-growing areas. Actually, Food and Agriculture Organization, Rome, took the lead in order to attract the attention of the scientists world over on the practical benefits that can be derived from Azolla which was based mainly on the experience of the farmers of China and Vietnam. Since the mid. 1970's, the International Rice Research Institute, Manila, in cooperation with other national and international institutes has taken a pivotal role in research and training of this promising renewable resource. Now the utility of Azolla has been explored in many directions. But there are some problems too that limit its wide utilization in rice cultivation. Primarily, the survival of Azolla inoculum beyond the temperature of 14 – 35°C and under intense solar insolation poses a great problem. Moreover, heavy infestation of pests during Kharif season and requirement of large quantity of nutrients particularly phosphorus are of serious concern to the management of Azolla.
technology. The sporocarps takes 25 – 30 days to develop into mature plant, the
growth of which is very slow at first. Moreover, during dry season, water involving
energy is a costly resource for production of Azolla inoculum. So the ecological
success of Azolla depends upon its capacity to cope with its physical environment and
with associated species. Considering the high potential of Azolla in various ways as
well as the problems associated with its multiplication, certain technological break­
throughs are needed in order to make it ecologically successful in areas where it is
desperately needed. Although selection of Azolla species or strains and improvement
of cultural practices are particularly important in overcoming constraints and
increasing its nitrogen fixing activity, genetic enhancement of the Azolla–Anabaena
system by sexual hybridization and exchange of algal partners between different
species increase the efficiency of the system. In some areas, hybrids show better
performance than the parents. It is expected that some of the unfavourable
characteristics of various Azolla species, high P requirements, low nutrient content
and low digestibility to animals, susceptibility to high temperature, and sensitivity to
insect attack may be improved by hybridization or by changing the symbiotic
cyanobacteria or even by introducing foreign DNA like insect resistance genes to
either the fern or the symbiont (Watanabe and Liu, 1992a).

Despite the well known constraints for the successful multiplication of Azolla,
it has been possible to introduce the plant in certain areas of Philippines, Thailand,
Indonesia and India where ecological amplitude of essential requirements fairly exists
or have been manipulated. In India sporadic attempts have been made in certain areas
to extend Azolla in farmers field based on the development of appropriate technology.
The primary need for large-scale application of *Azolla* in farmers’ field is to produce bulk quantity of inoculum at a low cost in a simple way. Production site of *Azolla* inoculum should be near the rice field and these sites should contain high concentration of nutrients most likely conducive to the rapid growth of *Azolla*. Hence suitable sites for inoculum production that do not impinge on the productivity of agricultural lands are stagnant waterbodies like ponds and ditches as well as slow flowing waterbodies that receive good quantity of nutrients through run-off. Millions of tonnes of top soil and nutrients are displaced every year by erosion and other processes. Much of these nutrients run off into waterbodies. Soil erosion is dubbed as a ‘double disaster’; a vital resource disappears from where it is desperately needed only to be dumped in areas where it is unwanted causing environmental degradation in land and water. If the nutrients are reclaimed from the water and replaced on the crop-field, the degradation of both the soil and water could be reduced considerably and the farmers would be benefited ecologically and economically (Kushari *et al.* 1992; Kushari, 1994). There are plenty hypertrophic waterbodies in different parts of India, particularly in eastern India. According to Swaminathan (1991), in eastern India, new approaches are needed for breeding and feeding the rice plant. Instead of recommending a uniform package of practices, it would be useful to provide the farmers with a basket of choices. Plant breeding procedures should produce cultivars that fit the environment. This will call for location specific varietal and management choices. A mere repetition of the same approach will not help eastern India. During the south-west monsoon season, fertilizer losses are heavy. Hence, the new crop feeding strategy should include green manure crops and biofertilizers. So in order to
accelerate the rice production, utilization of Azolla in dry season rice has been found promising with the development of new technology and more understanding of the mechanism of Azolla-Anabaena symbiosis associated with the rice plant.

Azolla propagates sexually and asexually. Asexual method of fragmentation is the common procedure for maintenance. But in favourable conditions sexual method through sporulation or sporocarp formation is followed, although this method is not practiced in practical application. The environmental factors responsible for triggering sporocarp formation are not well understood, it is only known that temperature, light quality and quantity, photoperiod and plant density are somehow involved with the triggering of sporulation. Thus it is not possible intentionally to induce sporocarp formation in order to produce large number of spores for storage and seeding. Secondly, germinating megaspores and sporelings grow very slowly. As a result it takes 25 – 30 days to become a mature plant. Thirdly, rice crops do not permit the growth of Azolla after 40-45 days of transplantation because of dense overhead canopy shade. As a result, in order to achieve effective application of Azolla sporocarp for more than one crop within the time schedule of 40-45 days of rice crop, the growth of the sporelings must be significantly enhanced. Moreover, sporocarps may be used effectively for over-summering and over-wintering because of both high temperature and low temperature which are detrimental for the growth of vegetative propagates. So the entire work has been divided broadly into two sections.

1. Study of sporulation capacity of six species of Azolla at different seasons of the year in order to identify the species with better sporulation capacity.
2. Study of germination and viability of spore of most potential species. Evaluation of the growth of sporelings (Plantlets) in different seasons of the year. Manipulation of the growth rate of slow growing sporelings by treatment with Phytonol, IBA, GA₃ and NAA in relation to important environmental factors.

5.1 Sporulation capacity of different species.

The sporulation process in *Azolla* is very complex and the causative agent(s) is not clearly understood. As mentioned earlier, several factors or agents like temperature, photoperiod, light intensity and plant density are believed to play important role (Ashton 1974). The observation in both the ecological conditions of Burdwan and Bolpur on six *Azolla* species including one hybrid in different seasons clearly supports the earlier observations. The climatic changes across the whole year through different seasons particularly with regard to temperature, light intensity, photoperiod and relative humidity significantly regulate the sporulation process of a particular species. As a result, different species exposed to the differential climatic conditions behaved differently in the process of sporulation. Six species collected from different geographical regions of the globe, although cultured in the same environmental conditions of Burdwan and Bolpur behaved differently in the process of sporulation. This differential behaviour of the six species warrants certain internal regulatory mechanism, precisely unknown currently, that influenced the induction of the sporocarps.
Out of the six *Azolla* species, *A.pinnata, A.microphylla, A.filiculoides* and one Hybrid sporulated throughout the year while *A.mexicana and A.caroliniana* produced sporocarps only in February-March. The observations in this ecological condition, further indicates that sporulation capacity of Hybrid *Azolla* was prolific throughout the year exhibiting a lower value in June-July but higher value in February-March, while the other species except *A.microphylla* and *A.pinnata* showed poor response to the changes of the climatic conditions. Of the different seasons, April-May was not at all favourable for the sporulation. The mean maximum and minimum temperatures were much lower and photoperiod was shorter in February-March than that in April-May. This characteristic inhibition of sporulation during this period of summer (April-May) evidently strengthens the significant influence of very high temperature ranging between 35.4 to 37.0°C, long photoperiod of more than eight hours, strong sunlight covering 530.08μmol.m⁻².s⁻¹ to 568.70μmol.m⁻².s⁻¹ and low relative humidity of 44% to 85.8% (Meteorological data as referred in Table MD-1and MD-2). In contrast to the unfavourable conditions of April-May, February-March was very conducive for the sporulation of different *Azolla* species particularly for Hybrid, *A.microphylla* and *A.pinnata* (native species) when the temperature ranged between 14.06°C to 34.2°C, light intensity having range between 421.08μmol.m⁻².s⁻¹ to 480μmol.m⁻².s⁻¹, photoperiod of less than eight hours, and relative humidity ranging between 36.4% to 86.75%. The low temperature of 14.06°C to 20.8°C and the wide temperature difference between day and night temperatures have a crucial role in this sporulation process. Watanabe et al. (1981) observed higher sporulation in low temperature in *A.mexicana*. Herd et al. (1989) observed that lowering of day and
night temperatures from 26°C/18°C to 26°C/13°C or 21°C/13°C enhanced sporulation in three strains each of *A.pinnata* and *A.mexicana*. However, it has either no effect or decrease sporulation in three strains of *A.microphylla* and one strain of *A.mexicana*. Thus our observation confirms the earlier observations suggesting that the environmental conditions, particularly of a suitable range of temperatures, is more crucial for the induction of sporocarps and this characteristic is not only species specific but also strain specific. The moderate sporulation capacity of the six species represented by the sporulation index and sporocarp output during August-September and October-November further substantiates some important derivations regarding this complicated mechanism of sporulation. Despite the high temperature and light intensity during June through September which are likely to cause inhibition of sporulation, the profuse sporulation particularly in Hybrid, *A.microphylla* and *A.pinnata* during this season warrants some other crucial environmental factor(s). Photoperiod of more or less eight hours’ duration and high relative humidity of 72.8% to 93% are thought to be deciding factors in this process. So the interplay of various combinations of different environmental factors might trigger some endogenous stimulants for the induction of sporocarps and these species characteristics might have a significant role in this process. The sporulation index in *A.microphylla* in the Philippines was also maximum during November-January when the average temperatures were relatively low and photoperiod was shorter (Palaywal and Paderon, 1986). Kannaiyan (1988) recorded maximum sporocarp output in *A.mexicana* at 25°C/15°C and there was a complete inhibition of sporulation at 38°C/25°C. Conflicting reports are also available in connection with the influence of different
environmental variables. Becking (1979) observed sporocarps during the late summer in Netherlands. Moore (1969) reported that sporocarps were abundantly found in cold weather of Central China. Konar and Kapoor (1974) found sporocarp in winter months. Singh (1979) found sporocarps of *A. mexicana* throughout the year but profusely during winter months. Shen (1960) observed sporocarps during winter in Taiwan. Ashton (1974) reported the formation of sporocarps in summer in temperate region.

Concomitant with the increase in sporulation index and sporocarp output along with the changes in the climatic conditions right from April-May to February-March, another important characteristic event of different *Azolla* species is the variation in the ratio of mega- and microsporocarp. This ratio is very important for the development of the number of sporelings. The different *Azolla* species particularly Hybrid, *A. microphylla*, *A. filiculoides* and *A. pinnata* behaved differently in the production of megasporocarps. This differential production was very conspicuous in different seasons having different climatic conditions. The higher megasporocarp formation of Hybrid and *A. microphylla* during June through September was probably much influenced by the higher temperature during June-July followed by August-September while the higher megasporocarp formation of *A. pinnata* during the colder months of November through February could be the result of the predominating influence of low temperature. Traore *et al.* (1995) determined the sporulation period of various strains of *Azolla*. Seven out of nine *Azolla* strains observed actually sporulated with maximum rates at the time of years when temperature were low (22-27°C, i.e., from January to February. In ecological condition of Burdwan, Hybrid
showed highest number of both micro- and megasporocarps. This unique property of the Hybrid necessarily deserves special attributes for which the germplasm can be exploited commercially and needs further detailed analysis of the behaviour of the sporocarp germination and development of sporelings or plantlets. One notable exception is the scanty information of sporocarps formation particularly megasporocarps of *A. filiculoides* during colder months in contrast to the prolific vegetative growth during this period. Such attributes of differential formations of either microsporocarp or megasporocarp still remain unanswered and seem to be a characteristic of species specificity. Calvert *et al.* (1983) had noticed higher number of megasporocarps in the cultures of *Azolla mexicana* in comparison to microsporocarps. Nayak and Singh (1986) observed male and female sporocarps per plant were 48 to 100 in *A. mexicana*, 11 to 18 in *A. filiculoides*, where as among *A. pinnata* isolates (India, Vietnam and Thailand), Thailand isolates showed highest number of 12 to 25. They also observed that male/female ratio during winter months remained highest in all species and isolates than in summer months.

Proportion of megasporocarp and microsporocarp bearing plants in a particular season is also characteristic feature of a species as noted in the result of the Table 5. Mostly the species did not have the ability to bear megasporocarp solely, either they were bisporocarpic i.e., the same plant bears both types of sporocarps or monosporocarpic having only microsporocarp. This characteristics widely varied from species to species as well as with seasons and seemed to be related with the growth behaviour in a particular season. Hybrid and *A. filiculoides* particularly during the colder months of the year (October through February) having indications of a high
frequency of sporocarp bearing plants showed a shift towards higher bisporocarpic characteristics. *A. caroliniana* and *A. mexicana* exceptionally produced both types of sporocarp bearing plants only during February-March. On the contrary, *A. microphylla* which is more prolific during the period of warm weather produced more microsporocarp bearing plants during this period while *A. pinnata* which is a native subtropical species and adapted to fluctuating climatic conditions, produced a more or less uniform proportion of both types of sporocarp bearing plants throughout the year. These evidences logically indicate the assumption that low temperature especially along with other environmental factors may trigger endogenous stimulus of a species to shift the mono ----- microsporocarpic characteristics to the bisporocarpic habit and it is inherently a species --- specific character. Based on these characteristics, the species could produce larger number of sporelings as found in the case of Hybrid *Azolla* in both controlled and field cultures of two ecologically distinct places of Burdwan and Bolpur.

5. 2a Germination behaviour of megaspore/megasporocarp of different *Azolla* species.

The variation of sporulation in different *Azolla* species has been well established as recorded in our previous discussion and is dependent on many factors. Sporulation particularly the megasporocarp and microsporocarp ratio influences the success of a species to a considerable extent. So germination behaviour of megasporocarps of different species was studied in controlled condition as well as in the field condition and the results as noted in Table no. 6 showed interesting
information. Usually the spores of many Pteridoptytes remain dormant for one year or more (Rashid 1976). Although the fertilization process of Azolla is accomplished by a complicated mechanism but occurs rapidly under favourable conditions. Once the megaspore is fertilized it starts diploid sporophytic generation within a very short time as found in our observation. Most of the species which produced sporocarps (eg. Hybrid Azolla, Azolla microphylla, A. filiculoides and A. pinnata), the fertilized megaspore / megasporocarp produces sporelings after a dormancy of one month only. But the germination behaviour varied sharply from species to species as well as with the changes of seasonal factors. Moreover, the germination percentage of the megasporocarp gradually increased with the ageing of the megasporocarps due to storage under natural conditions. This behaviour might be due to the specific characteristic of different species. The gradual increase in the number of sporelings due to the ageing of the sporocarps of four species might be due to the interactive influence of both the endogenous and environmental factors. Hillel et al. (1986) found a positive relationship in spore germination of an ostrich fern (Matteuccia struthiopteris) with the peak activity of endopeptidase after 12 to 24 hours’ imbibition of spores under light. Beri and Bir (1995) also suggested a significant role of total proteins stored in some fern spores on the spore germination, the survival of species and ultimately their conservation.

Despite the variation of germination percentage among the four species with the increase of storage period, the germination after one month of harvest increased gradually with the increase of incubation period in the medium. This characteristic might be due to the fact that there might have some stimulants exuded from the
germinating spore that induces further germination of fertilized megaspore. In order to elucidate this process further, the germination behaviour of the Hybrid spores was tested critically as it was found to be most successful plant type both for sporulation as well as spore germination. The continuation of the experiment on viability and germination behaviour of the fertilized megaspore for 25 months after storage under natural conditions in polythene bags led to the very significant conclusion that germination of the Hybrid megasporocarp may be of immense practical importance for the maintenance of germplasm and development of sporelings for commercial exploitation. The result as indicated in Table no. 8 clearly indicates that the germination was maximum after seven months of harvest (July), thereafter it declined gradually to a considerably low number at eleventh month (November) but after the eighteenth month (June) the germination was drastically reduced. The variation in germination of the megasporocarp of the Hybrid *Azolla* over the long eighteen months clearly indicates that seasonal factors predominantly higher temperature and moderate light intensity were crucial for the germination of the megasporocarp and development of sporelings. Singh *et al.* (1990) observed that germination did not occur in dark-incubated megasporocarps under either culture room on greenhouse conditions. Qing Yuan *et al.* (1987) also observed similar findings. This is in conformity with our observations as higher germination was noted during May through September under the condition of partial shade having higher temperature (33.408°C) moderate irradiance of 541.76μmol.m⁻².s⁻¹. Bewley and Black (1982) observed increased germination under greenhouse condition when compared with the culture room conditions due to higher temperature and irradiance. The viability of the
megasporocarp gradually decreased after 7 months of harvest to a considerably low level at eleventh month indicating the weak regulatory processes involved in germination coupled with the low temperature prevailing during November-December (14.86°C and 413.2 μmol.m⁻².s⁻¹). This is further confirmed by the very low germination after seventeen months of harvest despite the favourable temperature and light intensity prevailing in June-July indicating the loss of viability of the spores.

Effect of temperature on viability of spores and germination of fertilized megasporocarps:

Spores and seeds of plants show differential response to temperature and the germination of spores and seeds as a result is quite variable depending on the changes of temperature. Generally the spores and seeds are prone to desiccation and loss of viability at very high temperature particularly during the summer when the normal temperature in subtropical countries like India exceeds 40°C. But plants adapt various processes to overcome these unfavourable conditions by way of oversummering. Peter et al. (1988) found that like Dryopteris Osmunda i.e., photo-induced fern spores, photo-induced seeds of Oenothera Lettuce and other species can recover from thermal inhibition with the aid of a red light pulse, suggests possible similarities in the mechanism of light stimulated recovery from high temperature inhibition. Another mechanism of oversummering the high temperature is the placement of spores under varying depths of soil due to siltation of soil particles as a result of soil erosion. The result as shown in the Table no. 8 indicates that the incubation of the
spores with varying degrees of temperature starting from 30°C influenced the rate of germination and progressively increased up to the temperature of 60°C, thereafter it declined with a very low degree of response to 80°C and virtually there was no germination at 100°C. This result clearly indicated that the high temperature of above 60°C is detrimental to the survival of the spores. As a result, the subsequent fertilization and development of embryo and sporelings are drastically reduced (e.g. at 0.0cm depth of soil, emergence of sporelings was 29 only). Contrastingly this inhibitory effect of high temperature might be reduced considerably due to the soothing effect of the soil particles particularly at a depth of 1.0cm soil (emergence of sporelings was 46) when the development of the sporelings even above 60°C was considerably high. The stimulating effect of moderately high temperature of 55°C – 60°C due to the exposure of the spores on the soil surface as represented by higher number of sporelings (57) in comparison to the lower number of sporelings (35) under the 1.0cm soil depth in a particular season might be due to the fact that higher depth of soil may hinder the attainment of required stimulating temperature for the optimum germination and shooting of the megasporocarps.

5.2b Development of sporelings

Some practical problems for using *Azolla* sporocarps to raise sporelings (plantlet) in the rice field are very important, as there are many factors responsible for release of microspore and megaspore, fertilization of the megaspore, germination of fertilized megaspore and development of sporelings. These events should be meticulously analysed and proper care should be taken at the seedling bed, seeding,
mulching, fertilizing and floating young sporelings. The sporelings under favourable environmental conditions emerged into 2-3 leaved sporelings. But subsequently the formation of leaves and the development of the sporelings are very slow and take about 35 – 40 days to develop a mature plant. As the rice canopy becomes very dense after 40 – 45 days it is very difficult to harvest more than one crop of Azolla raised from the sporelings. So in order to overcome this critical problem of raising more than one crop by the use of sporocarps, techniques must be developed to promote the growth of sporelings. So the analysis of the growth and development of the sporelings of varying maturities (size ranges 0-0.5, 0.5-1.0, 1.0-2.0 and 2.0-3.0mm) clearly indicates that size of the sporelings is an important characteristic in the rapid development of the sporelings into mature plant. Thus the growth rate of larger sporelings 2.0-3.0mm size) are more vigorous compared with the smaller sporelings (0-0.5mm). Thus the 2.0-3.0mm sporelings produced 8.9 leaf number, 1.70mm leaf size, 1.78mm root size, 1.2 root number and 12% heterocyst frequency of the symbiont and the time required to develop this size of the sporeling was 18-21 days. The growth in dry weight (mg) although variable seasonally was significantly higher in these larger sporelings as represented by 0.25mg dry weight compared with 0.2mg dry weight 0-0.5mm sporelings during April-May while in February-March 2.0-3.0mm sporelings produced 0.63mg dry weight compared with 0.35mg in 0-0.5mm sporelings. All other growth parameters followed the same trend. This observation clearly indicates the suitability of the size of the sporelings in using sporelings and sporocarps in practical application in the rice field. The promotive growth behaviour of the larger sporelings in developing mature plant within a comparatively short time.
might be due to different endogenous as well as exogenous factors associated with the utilization of the environmental resources and rapid metabolism resulting in the addition of new tissues and their differentiation.

In order to elucidate the endogenous factors, hormones play an important role in the development of plants in general and sporelings or seedlings of Azolla in particular. Accordingly, the experimental observations with reference to the application of two growth hormones like phytonol (n-triacontanol) and I.B.A. are very interesting in the sense that phytonol is a potent bioregulator in shoot formation (Anonymous, 1986; Shrivastava et al. 2001) and I.B.A. is a potent growth regulator in the formation of adventitious roots (Kantharaj et al. 1979; Alvarez et al. 1989; Deklerk et al. 1997; Armstrong and Johnson, 2001). So the application of phytonol and I.B.A is primarily aimed at producing larger number of leaves and adventitious roots for increasing photosynthesis and biomass production as well as absorption of nutrients through roots. The promotive effect of phytonol on the formation of new leaves by accelerated photosynthesis and the promotive effect of I.B.A on the increase of adventitious roots of the sporeling were found to be synchronized events to increase the total biomass production. So the standardization of the optimal quantity of the hormones is a first step in this direction. Moreover seasonal factors like temperature, light intensity, humidity etc. profoundly regulated their promotive effect. The differential effect of varying quantities (volumes) of 0.05ppm phytonol and 100ppm I.B.A on both the shoot growth and root growth of Azolla sporelings as well as the heterocyst frequency of the symbiont Anabaena azollae was a very interesting area of this study. The particular effect on the larger sporelings (higher
than 0-0.5mm) substantiates the merit of the observations. This supporting evidence was corroborated by the seasonal influence of not only the favourable seasons but also the unfavourable ones. Larger sporelings were more effective by the higher quantities (Volumes) of phytonol. This indicates that larger quantities were responsible for the induction of cell division as well as tissue formation resulting in higher biomass production. This is exemplified by the 2.0 - 3.0 mm sporelings which required 40ml phytonol i.e., about double the amount of phytonol responsible for induction of cell division in comparison to 25ml phytonol for smaller sporelings (0-0.5mm). The percentage increase of shoot growth proved this promotion as found by the increase of 62.3% in leaf number and 86.7% in leaf size in 2.0 - 3.0mm sporelings over that of 23.8% in leaf number and 23.3% in leaf size in 0-0.5mm sporeling during July to October. This means that largest sporelings are more adaptive to use of higher quantities (volumes) of phytonol for induction of more cell division and assimilation resulting in the rapid biomass production. Srivastava et al. (2001) found that foliar application of triacontanol on Cicer arietinum L. produced the maximum growth and yield components with 25.8% increase in grain yield over no plant growth regulator.

The stimulating effect of phytonol was further enhanced by the seasonal factors. Azolla is particulary very sensitive to the seasonal fluctuations. The ecological amplitude of different environmental factors for the prolific growth of Azolla is generally narrow, so the fluctuation of these factors beyond this optimal range necessarily affected the growth and development of Azolla sporelings (plantlets). The plant prefers a placid water surface, temperature between 20°c and
30°C, light intensity in the range of 25% to 50% of full sunlight, eight hours photoperiod, water pH of 4-7 and rich in all essential plant nutrients particularly phosphorus except nitrogen, solution salt content less than 0.3% and freedom from competitors of insects and diseases (Lumpkin 1987). So the ecological success of the sporelings depends on the adjustment with the narrow amplitude of the different environmental factors. *Azolla* preferentially adapted with the environmental condition of July to October and February-March producing maximum biomass while the extreme environmental conditions of December-January retarded the growth and in April-June it survived very difficultly but managed to escape the adverse condition under the partial shade condition (Pal and Kushari, 1980; Pal, Sinhababu and Kushari, 1982; Kushari 1985, Kushari 1987; Kushari et al. 2000). So the effect of phytonol on the growth and development of sporelings in different seasons more or less followed this general pattern. It has been found that the growth of the sporelings was maximum in July to October followed by February-March, April-June and December-January. The interesting point of observation regarding the interactive influence of season and phytonol on the varying sizes of the sporelings opens up a new avenue for its commercial application. The very slow growth of *Azolla* sporelings in the extreme season of December-January could be partly compensated by the higher quantities of the phytonol hormone compared with the requirement of its lower amount in favourable seasons. The characteristic property of the phytonol might be ascribed to the influence in the endogenous concentration of total cytokinins of the isopentenyl adenine subfamily in the leaf and root tissues influencing the promotion of retarded metabolism and mobilization of nutrients in both root and shoot tissues even in the
unfavourable environmental conditions. Le and Loh (2002) found more than 82% bolting of Arabidopsis thaliana by triacontanol treatment. The promotive effect of phytonol to alleviate the retardation of growth during unfavourable seasons was more or less applicable in both the smaller and the larger sporelings indicating its consistency on the endogenous regulation of cytokinins and tissue proliferation, although more pronounced effect was observed in the larger sporelings of 2.0-3.0mm size. This means that this type of sporelings would be more useful for treatment of phytonol to attain the mature Azolla within a reasonably short period in the unfavourable seasons of December-January, because the production of sporelings during this period is more crucial for their application in the first week of February in rice cultivation. Another interesting point of observation regarding the relative percent increase of the shoot growth (number of leaves and size of the leaves) over the root growth (number of roots and size of roots) due to the treatment of phytonol particularly in larger sporelings in both the favourable seasons (July - October) and unfavourable seasons (December – January) focused a far-reaching information on the suitability of its application in shoot formation, although there are evidences that phytonol induces the formation of cytokinins in both root and shoot tissues. The formation of more leaf tissues due to phytonol clearly indicates its preferential action on the chlorophyll and photosynthesis. Tantos et al. (1999) found a promotive effect of triacontanol on micropropagation of balm, Melissa officinalis. They noticed that there is a differential optimal concentration for the enhanced shoot growth, fresh weight and chlorophyll content. Thus the preferential effect of singular treatment of n-triacontanol (phytonol) on the formation of larger shoot can be justified by the
application of its higher quantity to increase chlorophyll content thereby increasing the photosynthesis. The promotion of the heterocyst frequency of the symbiont Anabaena azollae by the treatment of phytonol again indicates its endogenous formation of cytokinin and indirect regulation of enzymes including the nitrogenase which is responsible for the fixation of nitrogen. As the symbiont, Anabaena azollae is invariably associated with the host plant Azolla since the initial development of fertilized megaspore, it can be presumed that treatment of the sporelings (along with the associated Anabaena azollae) with phytonol is influenced much metabolically so that endogenous nitrogen fixation system is also influenced to increase the heterocyst frequency of the symbiont thereby increasing the molecular nitrogen fixation and metabolism of nitrogenous materials for the synthesis of protoplasm and new tissues.

IBA (Indole Butyric Acid) has a dramatic effect on the initiation of adventitious roots of plants. Azolla sporelings vegetatively propagate but only one root is developed from the rhizomatous stem of young sporelings. The induction of more adventitious roots is to be ensured if the rapid development of sporelings into mature plant is effected. The treatment of different quantities (volumes) of 100ppm IBA following the standard procedure of pretreatment produced remarkable findings but quite variable among the different types of sporelings. Small sporelings required least amount of IBA treatment (10ml) in all the seasons except December-January where larger volume of 20ml IBA was more effective. During favourable season least amount of the hormone is utilized in inducing root formation while the requirement of more amount of IBA in unfavourable season might be due to alleviate the retardation effect of very low temperature prevailing during this period. Thus the important
findings of overcoming the effect of low temperature by pretreatment of higher amount of IBA may be noteworthy for commercial application of growing sporelings during December-January before the onset of dry season rice cultivation in February. Contrasting larger sporelings required larger volume of IBA (30ml IBA). This may be due to the induction of more metabolic activities towards the formation of more roots, as a result more mobilization of nutrients from the medium to the photosynthetic areas. These findings were consistently observed in all the three types of larger sporelings. The pretreatment of the IBA further augmented the stimulating effect of the particular season i.e., the stimulation of IBA was more marked in July to October followed by February-March, April-June and December-January. This means that IBA facilitates the mobilization of nutrients favourably during the favourable growth season. Despite the more stimulating effect of IBA on root growth, shoot growth was also affected positively but with a lesser degree and this was noted in all the four types of sporelings of different maturities. This observation might be explained on the concept that IBA is primarily a promoter of initiation and development of roots, as a result the improved root formation may increase the shoot formation indirectly by mobilization of different types of growth promoting substances. Hartman and Kester (1972) has discussed elaborately on the role of IBA in the root induction and formation. Kushari (1981) found stimulation on the number of roots and length of root of Azolla plants due to foliar application of IBA. It was explained that the higher production of biomass due to application of IBA might be due to the higher capacity of absorption by increasing the number and length of roots as found by Kushari (1986). The dramatic role of IBA during adventitious root
formation in the hypocotyl of *Phaseolus vulgaris* has been explained by Kantharaj *et al.* (1979) due to enhanced rate of total protein synthesis. Recently Armstrong and Johnson (2002) found relatively higher effectiveness of IBA over NAA in rooting of tissue culture of *Ceratopetalum gummiferum*. The effectiveness of growth promoters like phytonol and IBA could be enhanced by the appropriate concentration and quantity (volume) of phytonol and IBA. The combined effect of these two growth promoters due to basal pretreatment was more marked by the seasonal influence i.e., February-March was marked with the mixture of 30ml IBA and 30ml Phytonol on 0-0.5mm sporelings while the growth of other larger sporelings showed best promotion of growth in combined mixture of 30ml IBA and 40ml phytonol. The growth of all types of sporelings having more or less similar trends was evidently higher in July-October, followed by February-March, April-June and December-January. Thus two concluding points can be emerged that would address in the control of differential effectiveness of the growth promoters on the size (maturity) of the sporelings and the influence of the climatic variables in a particular season. Secondly differential action of phytonol preferentially on shoot growth and the effect of IBA preferentially on root growth harmonize the overall growth of shoot and root due to the appropriate combinations of phytonol and IBA and this effect was evidenced significantly on the larger (2.0 - 3.0mm) sporelings. This effectiveness of combined applications was further corroborated by the combined foliar application in a large scale in the field and more or less same trend of growth promotion clearly advocated the preferential action of shoot and root promotion by the phytonol and IBA respectively. This evidence was carefully testified further in large scale for commercial application of
the combined application of phytonol and IBA on 2.0-3.0mm sporelings at a low cost to harvest the mature Azolla plants in a considerably shorter duration of 16 days during the most favourable seasons of July to October by foliar treatments.

Based on the concept that mixtures of different root promoting and shoot promoting substances are more effective than either component alone (Leopold, 1975), further study was conducted on the use of GA₃ to promote essentially shoot formation and naphthalene acetic acid for promotion of roots. The effect of the two hormones individually as well as in combination with Phytonol and IBA was found to be very encouraging after foliar treatment at a particular concentration and quantity, because application of growth hormones at high concentration can inhibit development of any organ. Among the different concentrations of Gibberellin ranging from 10ppm to 100ppm, only 40ppm was most effective and considerably decrease the time required for the development of mature plant. The dramatic effect of Gibberellin has been found in many cases and it enhanced the growth of 2.0-3.0mm sporeling into a mature plant within 24 days in comparison to 37 days required for developing sporelings in controlled conditions even in the unfavourable period of April-June. Yuan et al. (2001) experimented on stimulation effect of gibberellic acid on leaf photosynthesis. They applied for a short-term (one hour) treatment of gibberellin of concentration 9µM on surfaces of broad bean leaves through painting and they also applied 90µM Gibberellin on surface of soyabean leaves. They found that more than 20% increment of net photosynthetic rate compared with control. These results suggest that increment of net photosynthetic rate by short-term treatment is mainly due to the increase in content and activity of RuBP case, and that
gibberellin (GA₃) stimulates the synthesis of RuBP case at the transcription level of leaf-cell protoplast.

Naphthalene acetic acid (NAA) like Indole Butyric Acid (IBA) is also a potent promoter of formation and development of roots. Out of the several concentrations (0.1ppm to 1.0ppm) tested, 0.4ppm NAA was most effective by promoting root development particularly over shoot development. As a result, it hastened the maturity of 2.0 – 3.0mm sporelings to 32 days in comparison to 37 days in control. Probably the same mechanism of hormonal action like IBA might be displayed by increasing the number of roots and length of roots by which mobilization of more amount of nutrients through roots was possible. D’Souza et al. (2001) described a protocol for invitro propagation of Bixa orellana. Plants were regenerated from shoot-apex explants and nodal explants. Shoots regenerated from nodal explants needed 2.7 μM NAA for rooting and shoots regenerated from shoot apex explants also needed a definite amount of NAA for rooting in combination with Murashige and Skoog’s basal medium. In order to exploit these hormones (Phytonol, IBA, GA₃ and NAA) tested above, the combined application was found to be more effective than solitary application. The combination of NAA+Phytonol+GA₃ was most conducive in promoting the growth of sporelings and this was followed by IBA+Phytonol+GA₃. As a result only 20 days of four successive combined treatments at an interval of 5 days was required to obtain mature plants from 2.0 – 3.0mm sporelings. But the shortest time of 19 days only was required to raise the 2.0-3.0mm sporelings into mature plants after four successive treatments of 5ml phytonol (0.05ppm), 5ml IBA (100ppm), 5ml GA₃ (40ppm) and 5ml NAA (0.4ppm) at an interval of 5 days when
the total volume was applied in equal split volumes. Kissimon et al. (1999) studied the effect of natural growth regulator triacontanol in combination with glucose and sucrose of different concentrations under the unfavourable conditions on two woody plants. It was found that triacontanol altered the inhibitory effect of stress condition by changing the carbohydrate concentration and enhanced the stimulating effect of the optimal carbohydrate concentrations, which indicated its specific importance under such stress conditions.

Hoque et al. (2001) studied the efficiency of invitro germination and shoot proliferation of chilling treated water chestnut (Trapa japonica Flerov) embryonal explants. Embryonal explants from water chestnut seeds germinated with high efficiency following a 40 day control in (Murashige and Skoog), medium supplemented with 2.7μM N-6-benzyL adenine (BA), 0.5μM 1-napthalene acetic acid with different duration. The control and chill-treated (different durations) embryonal explants were cultured into media which contained half-strength MS medium in combinations of cytokinins [BA, thidiazuron, (TDZ)], auxin (NAA) and GA3. It was found that shoot proliferation was found better in liquid half-strength MS medium containing 1.1μM BA, 0.5μM NAA, 1.1μM IBA and 0.5μM GA3 in comparison to control or chill-treated explants.