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India is basically an agricultural country and more than 75% people are in the rural sector depending mainly on agriculture. Paddy is one of the major cereal crops for Indians covering approximately 40 million hectares area. On global scale it covers more than 150 million hectares with an average production of 3.5 tonnes per hectare. India is the largest rice growing country of the world producing 84.7 million tonnes of rice grain. The population in India is steadily growing posing a serious threat to the national food-security. In order to meet the need of the growing millions, attention must be focused on improving the productivity and stability of rice production on an ecologically sustainable basis (Swaminathan, 1991).

Nitrogen is the key element for realizing the potential yield from modern high yielding rice-varieties. It is well-established that 19-21kg. N is removed from soil to produce 1 tonne of brown rice (Murayama, 1979). The total nitrogen requirement for a potential yield of 15 tonnes per hectare is estimated at 300kg per hectare (Setter et al., 1994). Considering that soil supplies about 50-80kg nitrogen per hectare and the efficiency of fertilizer N is about 50%, 440-500kg of fertilizer nitrogen per hectare will be required in order to achieve this goal. So chemical nitrogen fertilizer application is still an indispensable phenomenon of modern agricultural practices. But in order to promote sustainable rice production, there should be a good management of practices so that the cost of production as well as environmental degradation should be kept in mind without lowering the production. This calls for a new judicious crop-feeding strategy of utilizing organic manures and biofertilizers along with the appropriate doses of chemical fertilizers.

The production of organic manures does not keep pace with the increasing demand of the crop production. Therefore, various types of living organisms are used as biofertilizers. These include symbiotic systems like Rhizobium, Azolla, etc. and free living systems like Azotobacter, Cyanobacteria, etc. The symbiotic system is much superior to the free living system with respect to nitrogen fixation.
The practice of green manuring where feasible is the principal supplementary means of adding organic matter to the soil. This includes a fast growing nitrogen-fixing crop and incorporation of the biomass into the soil so as to be decomposed equally rapidly in order to make the nutrients including nitrogen in available form. In this context *Azolla-Anabaena* symbiotic system has been regarded as a very promising component in rice cultivation. *Azolla*, the popular water-fern fixes atmospheric nitrogen with the help of symbiotic cyanobacteria *Anabaena azollae* present in its specialized leaf-cavities. All the six species of *Azolla* are widely distributed under the diverse agro-ecological conditions (Lumpkin and Plucknett, 1982). *Azolla-Anabaena* system has many uses. It can be utilized as a biofertilizer on rice and many other crops, an animal feed, a human food, a medicine, and a water purifier. It may also be used for the production of hydrogen fuel, the production of biogas, the control of mosquitoes, and the reduction of ammonia volatilization that accompanies the application of chemical nitrogen. Its application also improves soil fertility and has a residual effect on the yield of other crops succeeding rice (Ventura and Watanabe, 1993). Moreover, the fern acts as a scavenger of phosphorus and potassium from underlying water several times of their metabolic demand (Liu, 1987; Kushari and Watanabe, 1991). But there are some problems too that limit its wider utilization in rice cultivation.

Despite the traditional use of this plant as biofertilizer in China and Vietnam for over a century, it has been extended only in certain areas of Philippines, Thailand, Indonesia and India etc. where ecological amplitudes of essential requirements fairly exist or have been manipulated. In India, technology for large scale production and use of *Azolla* in rice farming has been developed (Singh, 1989; Kannaiyan, 1994; Kushari, 1987). Sporadic attempts have been made in certain areas in extending *Azolla* in farmers’ field based on the development of appropriate technology but have met with little success. Recently National Cooperative Development Corporation, Govt. of India is trying to extend the *Azolla* Biofertilizer Technology through Cooperative Societies (Kushari and Kushari, 2000). 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. The production site of *Azolla* inoculum should be near the
rice-field and these sites should contain high concentration of nutrients most likely to suit the rapid vegetative growth of *Azolla*. Hence suitable sites for inoculum production that do not impinge on the productivity of agricultural lands are stagnant water bodies like ponds and ditches as well as slow-flowing water bodies that receive good quantity of nutrients through runoff. Here again the production of vegetative inoculum suffers from certain limitations. It is very difficult to transport over a long distance and is easily decomposable. Moreover it can not tolerate extreme cold or extreme heat. So the alternative way of vegetative inoculum production is to produce bulk quantity of spores of desirable species.

Sporocarp technology helps in the maintenance of *Azolla* germplasm particularly under unfavourable conditions. Sporocarps can resist adverse conditions and remain viable for a longer period because of the sporangial covering around them. Their storage, transportation and distribution are easier and economical. But the use of sporocarps has also certain limitations. All the species do not produce sporocarps round the year. The most serious limitation of sporocarp technology is that the spores take about 25 – 30 days to develop mature plant. So in order to remove this bottle-neck and to develop the appropriate sporocarp technology; detailed investigations are needed on the ecological factors influencing profuse sporulation of the desirable *Azolla* species as well as rapid growth of the sporelings (plantlets) so that sporocarp technology can be used directly in the field or as a means of producing vegetative inoculum depending on the various factors involved in agricultural practices.

Based on the above problems of sporocarp technology, the objective of this work was aimed at two directions: -

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 spores of most potential species. Evaluation of the growth of sporelings in different seasons of the year. Manipulation of the growth rate of slow growing sporelings by Phytonol, IBA, GA₃ and NAA in relation to important environmental factors.
1. Sporulation of different species of *Azolla*

Sporulation of different species of *Azolla* was studied during different seasons of the year and showed wide fluctuation. This characteristic is represented by Sporulation Index, Sporocarp Output and ratio of Mega- and Microsporocarp. During June-July, Sporulation Index was highest in Hybrid followed by *A.microphylla*, *A.pinnata* and *A.filiculoides*. The Sporocarp Output and the ratio of Mega- and Microsporocarp production of the above species showed more or less same trend where Hybrid was followed by *A.microphylla*, *A.pinnata* and *A.filiculoides*. There was no sporulation in *A.caroliniana* and *A.mexicana* during this season. With the onset of rainy season (August-September) the sporulation of different species showed better result compared with the previous season. During October-November, sporulation of different species gradually increased and the Hybrid took the lead in producing more sporocarps as well as in increasing the ratio of Mega- and Microsporocarps. It was followed by *A.microphylla*, *A.pinnata* and *A.filiculoides*. But *A.caroliniana* and *A.mexicana* did not produce any sporocarp. The same trend of sporulation among the different species but having higher values was noted during December-January. The maximum sporulation value was obtained during February-March in both controlled as well as field conditions. Hybrid showed 100% Sporulation Index in February-March when the sporocarp Output (9.48) and the ratio of Mega- and Microsporocarp (6.5:10) were also maximum. This was followed by *A.microphylla*, *A.pinnata* and *A.filiculoides*. However in field conditions of both Burdwan and Bolpur, only Hybrid and *A.pinnata* showed sporulation. Evidently, Hybrid showed better performance than the *A.pinnata* in ecological condition of Burdwan. The two species, however differed in some respects in a different ecological condition of Bolpur where *A.pinnata* showed higher performance than the Hybrid.
Proportion of mega- and microsporocarp bearing plants of different species

Azolla species differed also widely with respect to the sporocarp bearing plants. Most of the species did not bear megasporocarps singly but they bore either microsporocarps or both the sporocarps. During June-July out of 10 plants selected at random, there were four microsporocarp bearing plants and six plants bearing both types of sporocarps in Hybrid *Azolla*. *A.microphylla* contained six and four corresponding plants. *A.filiculoides* contained seven microsporocarp bearing plants leaving only three megasporocarp bearing plants while *A.pinnata* having six and four plants respectively *A.caroliniana* and *A.mexicana* did not produce any kind of sporocarps during July though January. During August-September Hybrid *Azolla* produced 3 micro- and 7 bisporocarp bearing plants. *A.microphylla* produced 4 and 6 micro- and bisporocarp bearing plants respectively. *A.filiculoides* showed reverse production i.e., 6 micro- and 4 biosporocarp bearing plants. *A.pinnata* showed 5 micro- and 5 biosporocarp bearing plants. In October-November the tendency of developing more bi-sporocarp bearing plants was found in Hybrid and *A.microphylla* while equal number of two types of sporocarp bearing plants was found in *A.filiculoides* and *A.pinnata*. In December-January Hybrid produced all the bi-sporocarp bearing plants while *A.microphylla* produced more microsporocarp bearing plants. However, *A.filiculoides* produced equal number of both types of plants but *A.pinnata* produced one mega-, four micro- and 5 bi-sporocarp bearing plants. During February-March again Hybrid produced all the bi-sporocarp plants. *A.microphylla* produced lesser number of bi-sporocarp plants but *A.filiculoides* having higher number of bi-sporocarp plants. *A.pinnata* produced 2 mega-, 3 micro- and 5 bi-sporocarp plants. In contrast, during this season, only *A.caroliniana* and *A.mexicana* produced both types of sporocarp bearing plants.
2. Development of sporelings

Germination behaviour of spores of different *Azolla* species

The germination behaviour of the spores (fertilized megasporocarp) of four species viz. Hybrid, *A.microphylla*, *A.pinnata* and *A.filiculoides* during the consecutive three months since February after one month of harvest in December showed that Hybrid spores germinated earlier followed by *A.microphylla*, *A.pinnata* and *A.filiculoides*. Longer time was required for germination during February but lesser time was required during March and April. Number of germinated sporelings gradually increased and after one month of harvest (February), maximum number of sporelings (38) were developed in Hybrid after 35 days of sowing. This was followed by *A.microphylla*, *A.pinnata* and *A.filiculoides*. The same trend was noted with increasing number of sporelings in all the species during March-April. Germination of Hybrid spores gradually increased upto the 7th month after harvest when after sowing 109 sporelings were recorded after 17 days and 145 and 164 sporelings were recorded after 22 and 27 days respectively. The minimum time required for germination during this period was 12 days only. After 18 months after harvest, germination of spores was very low and after 23 months practically germination of spores ceased.

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

The germination of Hybrid spores varied widely with the changes of temperature. The effect of temperature had direct relevance with the seasonal variation of temperature particularly during summer. The incubation of the spores with varying degrees of temperatures starting from 30°C influenced the rate of germination and progressively the germination increased upto the temperature of 60°C. The rate of germination of spores was much influenced by the direct exposure of the spores in high temperature and as a result varied with the depth of
soil. The optimum range of temperature for spore germination was 55-60°C but remained viable at much higher temperature (70°C) beyond the optimum range of temperature of 25-30°C for the growth of the *Azolla* plant. However lower temperature was more effective in lower depth of soil and in higher depth of soil higher temperature was more suitable.

**Identification of size range of sporelings (Plantlets)**

The sporelings were grouped critically according to certain characteristics. According to the size of the sporelings (0-0.5mm, 0.5-1.0mm, 1.0-2.0mm and 2.0-3.0mm) the growth of the sporelings was characteristically different and 2.0-3.0mm sporelings showed maximum growth behaviour and fertilized megasporocarp attained 2.0-3.0mm within 18-21 days. The Hybrid sporelings showed effective growth response to IRRI’s medium. Among the various sizes of the sporelings, 2.0-3.0mm size showed maximum growth response in August-September followed by February-March, June-July, October-November, December-January and April-May in IRRI’s medium.

**Effect of growth regulators on the growth of sporelings**

*Phytonol* (n-triactanol) is a nontoxic bioregulator of growth of plants and is suitably tested in plant system for the benefit and growth of tea bushes and other plants. The effect of phytonol was studied in both quantity controlled and concentration controlled conditions. 5ml to 50ml volumes of 0.05ppm phytonol were used in this investigation to regulate the growth of *Azolla* sporelings having various maturities like 9-12days (0-0.5mm), 12-15 days (0.5-1mm) 15-18days (1.0-2.0mm) and 18-21days (2.0-3.0mm) in four seasons of April-June, July-October, December-January and February-March. The smallest sporeling of 0-0.5mm size showed maximum stimulating growth in a volume of 25ml phytonol, while the growth of 0.5-1.0mm sporelings showed better response than the previous one in both concentration controlled condition as well as treatment with higher quantities (40ml) of phytonol.
(quantity controlled condition). With increasing size of the sporelings, their growth also increased which was evidently more marked in sporelings larger than 1.0mm due to application of 40ml phytonol and maximum response was noted in 2.0-3.0mm sporelings. So the percentage increase data clearly reveal the steady increase of the growth of sporelings with the increment of sporeling size starting from 0-0.5mm and culminating in 2.0-3.0mm sporelings. Seasonal influence further stimulated the promotive effect of phytonol and the growth of the sporelings was maximum in July-October followed by February-March, April-June and December-January. In extreme season of December-January the sporelings of varying sizes required higher amount of the hormone compared with the requirement of lower amount in favourable seasons. As a result during December-January, 40ml phytonol was effective in 0-0.5mm sporelings but 25ml in other seasons while 50ml phytonol was more effective in 2.0-3.0mm sporelings in December-January in comparison to the requirement of 40ml phytonol in favourable season of July-October. The percentage increase of shoot growth (no. of leaves and size of leaves) due to the treatment of phytonol was significantly higher than that of root growth (no. of roots and size of roots) particularly in larger sporelings. The trend was maintained in both the favourable season (July-October) and unfavourable season (December-January). Phytonol had an overall promotive effect on the heterocyst frequency of the symbiont, *Anabaena azollae* that is related also to the growth of the sporelings irrespective of the size of the sporelings and seasonal variation.

**IBA** (**Indole butyric acid**) has a dramatic effect on the initiation of adventitious roots. *Azolla* sporelings vegetatively propagate but only one root is developed from the rhizomatous stem of growing sporelings. So different quantities (1-60ml) of 100ppm IBA was applied to different types of sporelings having various maturities. 10ml of 100ppm IBA was most effective in 0-0.5mm sporelings in all the seasons except December-January where 20ml was most effective. The response of 0.5-1.0mm, 1.0-2.0mm and 2.0-3.0mm sporelings to the supply of different quantities of IBA indicated that treatment of 30ml IBA was most pronounced irrespective of any season. The growth of different types of sporelings was usually high during July-October followed by February-March, April-June and December-January. Despite the
more stimulating effect of IBA on growth of root, shoot growth was also affected positively but with a lesser degree and this was noted in all the four growth stages of sporelings. The percentage increase data clearly reveal the steady increase of the growth of sporelings with the increment of sporeling size starting from 0-0.5mm and culminating in 2.0-3.0mm sporelings.

The comparative effect of various combinations of 100ppm IBA and 0.05ppm phytonol was thoroughly studied and showed encouraging information regarding the suitable mixture of the promotive chemicals on a particular size of sporelings. The combined effect of those two growth promoters during February-March was mostly 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 in combined mixture of 30ml IBA and 40ml phytonol with a steady increase from the combination of 10ml IBA and 30ml phytonol. In pursuance of the study of the different combinations of phytonol and IBA during the optimum season of February-March, the encouraging result prompted to search for the effective combinations of these two growth regulators during other seasons of the year for commercial utilization at a particular season. The growth of all types of sporelings having more or less similar trend was evidently higher in July-October, followed by February-March, April-June and December-January. In order to derive maximum benefit of growth promoters foliar application of phytonol and IBA at the most suitable concentrations and at the most suitable quantities was analyzed on 2.0-3.0mm sporelings. The treatment with 30ml IBA and 40ml phytonol mixture was most suitable in this regard during different seasons of the year followed by the treatment of phytonol, IBA and control. In the crop field the effect of foliar spray was more encouraging as compared with the effect in net house. During the most favourable season of July through October season combined foliar application of IBA and phytonol required only 16 days to raise the 2.0-3.0mm sporelings into mature plant in comparison to 18, 19 and 33 days required by the sporelings in the respective treatments of phytonol, IBA and control. Maximum increment of growth was found in July-October season followed by February-March, April-June and lastly December-January. Percentage increase of growth parameters over control followed the same seasonal trend.
The role of gibberellins in the regulation of growth is well known \( \text{GA}_3 \) (Gibberellic acid) of different concentrations from 10ppm to 100ppm was used. Among them 40ppm concentration was most effective and showed most pronounced growth promoting effect over control. 24 days of successive treatments at an interval of 6 days was enough to get mature \( \text{Azolla} \) plants from 2.0-3.0mm sized sporelings in the unfavourable period of April-June.

\( \text{NAA} \) (Naphthalene acetic acid) is a synthetic auxin which is found to be equally important in causing formative effects on plants especially it has effects on adventitious root formation. Different concentrations ranging from 0.1ppm to 10ppm was effective than control. But 0.4ppm NAA was most effective over control. 32 days of four successive treatments at an interval of 8 days were enough for getting mature \( \text{Azolla} \) plants from 2.0-3.0mm sporelings during April-June season.

The efficiency of different combinations of growth regulators was further testified by the interactive effects of growth regulators. The different combinations of two growth regulators showed varying effects during April-June season. NAA and \( \text{GA}_3 \) combinations showed most pronounced effect where 20 days were required to obtain mature plant during April-June. This was followed by the combinations of phytonol + \( \text{GA}_3 \), IBA + \( \text{GA}_3 \), NAA + phytonol and IBA + phytonol. The efficiency of various combinations of the growth regulators was further testified by the interactive effects of three or more growth regulators. The combination of NAA, phytonol and \( \text{GA}_3 \) was most conducive in promoting the growth of sporelings. This was followed by the combination of IBA+phytonol+\( \text{GA}_3 \). April-June was selected as the test season as this was unfavourable season for \( \text{Azolla} \) growth. 20 days of four successive treatments at an interval of 5 days were enough for obtaining mature \( \text{Azolla} \) plants. But most pronounced effect was found with the combined treatment of IBA+phytonol+ \( \text{GA}_3 \)+NAA in which 19 days were required to raise the 2.0-3.0mm sporelings into mature plants after four successive treatments at 5 days interval. So the combined effect of the four growth regulators not only enhanced the growth but also hastened the maturity of sporelings. So it was found that 5ml each of 0.05ppm phytonol, 100ppm IBA, 40ppm \( \text{GA}_3 \) and 0.4ppm NAA after combined foliar application showed maximum growth promoting effect even in unfavourable period of summer (April-June).