CHAPTER - 3

MATERIALS & METHODS
3. Materials and Methods

All the experimental studies were conducted at the Department of Botany, University of Burdwan, and Crop Research Farm of Burdwan University.

Burdwan is a district town of West Bengal and is situated at $23^0 18'\ N$ latitude and $87^0 54'\ E$ longitude.

3.1 Meteorological conditions:

Usually seasons like the winter, spring, summer, rainy and autumn are referred to in the literature, but the meteorological data of the last 30 years reveal that Burdwan has three predominating seasons, the summer, monsoon and winter. The summer begins from the second half of April and continues upto the middle of June. It has average day/night temperature of around $30^\circ C$, high mean solar radiation ($545-610\ \mu\text{mol.m}^{-2}\text{s}^{-1}$), low amount of total rainfall ($3.7-4.29\ \text{mm d}^{-1}$) as well as average relative humidity of around 69%. The monsoon beginning from the middle of June continues upto the first half of September. It has comparatively high amount of total rainfall (about 70% of the total precipitation of the year), moderately high average day/night temperature ($28.0$ to $30^\circ C$), low mean solar radiation due to cloud and rains ($480-545\ \mu\text{mol.m}^{-2}\text{s}^{-1}$) high average relative humidity (75-80%). From the second half of November, the winter begins and continues upto the first half of February. It has comparatively low average day/night temperature (around $20^\circ C$), mean solar radiation between $375-425\ \mu\text{mol. m}^{-2}\text{s}^{-1}$, very low amount of total rainfall ($0.7\ \text{mm d}^{-1}$)
as well as low relative humidity (average 55-65%). Besides the three predominating seasons, the pre-summer (spring) and the autumn are also recognizable for comparatively shorter duration. The pre-summer (from second half of February till the end of March) and the autumn (from the second half of September to the middle of November) have average day/night temperature around 25°C, average relative humidity of slightly above 65%, low amount of total rainfall (1.5-2.5mm d⁻¹) and mean solar radiation between 425-475 μ mol. m⁻² s⁻¹.

The details of the meteorological data of different months of the experimental sessions, 1994-1999 have been produced in the Tables MD-1 and MD-2. The data of the table reveal that the average day/night temperature was comparatively high during May-June and low during December-January. Solar radiation remained low during July – August as the bright sunlight hours per day were lesser due to cloud and rains. During May – June, it remained high, fairly high during February – March and moderate during October – November. Total rainfall was maximum during July-August and the average relative humidity remained high during July-October.

3.2 Experimental plant materials:

Azolla is a free-floating pteridophytic genus of the monotypic family Azollaceae. The six species of Azolla such as A.pinnata, A.filiculoides, A.microphylla, Hybrid Azolla, A.caroliniana and A.mexicana are included in this investigation. The genus is divided into six species considering the presence of floats on the megaspores and the characteristics of gloclidia.
Table - MD-1. *Average Meteorological Data of Different Factors of Consecutive 5 years (1994–99).*

<table>
<thead>
<tr>
<th>Months</th>
<th>Rainfall (mm)</th>
<th>Humidity (%)</th>
<th>Temperature (°C)</th>
<th>Solar radiation mean average μ mol. m⁻²s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max.</td>
<td>Min.</td>
<td>Max.</td>
<td>Min.</td>
</tr>
<tr>
<td>January</td>
<td>1.07</td>
<td>90.11</td>
<td>41.86</td>
<td>25.43</td>
</tr>
<tr>
<td>February</td>
<td>1.39</td>
<td>86.75</td>
<td>41.88</td>
<td>27.06</td>
</tr>
<tr>
<td>March</td>
<td>1.15</td>
<td>79.60</td>
<td>36.40</td>
<td>34.20</td>
</tr>
<tr>
<td>April</td>
<td>2.33</td>
<td>85.40</td>
<td>44.00</td>
<td>35.40</td>
</tr>
<tr>
<td>May</td>
<td>2.73</td>
<td>85.80</td>
<td>50.40</td>
<td>37.00</td>
</tr>
<tr>
<td>June</td>
<td>7.82</td>
<td>87.60</td>
<td>63.20</td>
<td>34.20</td>
</tr>
<tr>
<td>July</td>
<td>8.97</td>
<td>90.60</td>
<td>73.80</td>
<td>32.60</td>
</tr>
<tr>
<td>August</td>
<td>13.85</td>
<td>93.00</td>
<td>79.20</td>
<td>31.80</td>
</tr>
<tr>
<td>September</td>
<td>8.46</td>
<td>90.40</td>
<td>72.80</td>
<td>31.44</td>
</tr>
<tr>
<td>October</td>
<td>2.49</td>
<td>89.00</td>
<td>67.20</td>
<td>30.94</td>
</tr>
<tr>
<td>November</td>
<td>0.61</td>
<td>87.32</td>
<td>50.40</td>
<td>28.80</td>
</tr>
<tr>
<td>December</td>
<td>0.20</td>
<td>85.98</td>
<td>40.58</td>
<td>19.93</td>
</tr>
</tbody>
</table>
Table – MD-2. Average Meteorological Data on Temperature and Solar Radiation in Partial Shade Conditions of Consecutive 5 years (1994 – 99) (Recorded by the Deptt. of Botany, Burdwan University).

<table>
<thead>
<tr>
<th>Months</th>
<th>Temperature (°C)</th>
<th>Solar radiation (μ mol. m⁻² s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum</td>
<td>Minimum</td>
</tr>
<tr>
<td>January</td>
<td>25.10</td>
<td>9.480</td>
</tr>
<tr>
<td>February</td>
<td>26.71</td>
<td>13.71</td>
</tr>
<tr>
<td>March</td>
<td>33.80</td>
<td>20.40</td>
</tr>
<tr>
<td>April</td>
<td>34.30</td>
<td>22.70</td>
</tr>
<tr>
<td>May</td>
<td>36.80</td>
<td>26.20</td>
</tr>
<tr>
<td>June</td>
<td>33.15</td>
<td>25.35</td>
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<tr>
<td>July</td>
<td>31.10</td>
<td>24.70</td>
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<tr>
<td>August</td>
<td>31.40</td>
<td>25.40</td>
</tr>
<tr>
<td>September</td>
<td>31.04</td>
<td>24.80</td>
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<tr>
<td>October</td>
<td>30.51</td>
<td>22.42</td>
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<tr>
<td>November</td>
<td>28.54</td>
<td>17.04</td>
</tr>
<tr>
<td>December</td>
<td>19.00</td>
<td>11.25</td>
</tr>
</tbody>
</table>
The genus possesses intrinsic interest in that its members are capable of assimilating atmospheric nitrogen, the actual agent of fixation is *Anabaena azollae* that is almost invariably present in cavities of their leaves. Fertile sporocarp of *Azolla* bear two types of sporocarp ---- the bigger one is the microsporocarp bearing fertile microsporangia with a larger sterile megasporangium while the smaller megasporocarp contains a large fertile megasporangium with sterile microsporangia. Spores of *Azolla* are dark in colour. Megaspores are larger than microspores.

The work has been broadly divided into two parts:-

A. Sporulation capacity of six *Azolla* species under this agro-ecological condition.

B. The viability and germination capacity of spores throughout the year particularly under the extreme temperature condition during April-June (Summer).

Development of sporelings (plantlets) during different seasons in both controlled and field conditions and manipulation of growth pattern by the application of potential growth regulators like Phytonol, GA₃, IBA and NAA in relation to important environmental factors.

A. The important parameters for the study of sporulation:-

i) **Sporulation Index (SI):**

It indicates the percentage of sporocarp bearing plants over the total number of plants studied. It is expressed as follows:-

\[
\text{Sporulation Index (SI)} = \frac{\text{Number of sporocarp bearing plants}}{\text{Number of plants studied}} \times 100
\]
ii) Sporocarp Output (SO):-

It indicates the total number of megasporocarps and microsporocarps produced by the plants studied and SO is expressed as –

\[
\text{Total number of mega}/\text{and microsporocarps of the plants studied} \\
\text{Sporocarp Output (SO) = } \frac{\text{Total number of mega}/\text{microsporocarps of the plants studied}}{\text{Total number of plants}}
\]

iii) Ratio of mega/ and microsporocarp :-

It indicates the number of megasporocarps out of 10 microsporocarps of the plants studied.

3.3 Azolla sporocarp (spore) inoculum production.

The highly sporulating and higher biomass producing culture of Azolla was selected for spore inoculum production. In a well-drained area, pit (3m x 5m x ½m) was built. In it harvested Azolla was placed and allowed to undergo decomposition for a period of two weeks or more. After decomposition Azolla was air dried for 24 hours. Then in a coarse screen (about 1mm) Azolla compost ( decayed Azolla) was passed. The particles that passed through the coarse screen were dried. Dried spores then were packed in plastic bags or glass jars and labeled to indicate species, date of harvest and place of collection.

A hybrid Azolla developed by International Rice Research Institute, Manila was introduced by Kushari (1994) in India and performed better in sporocarp
production than other species. It has wide tolerance to the agro-ecological conditions in West Bengal. It is a hybrid of two species i.e., \textit{A.microphylla} (0*) and \textit{A.filiculoides} (0+).

3.4 Measurement of Hybrid \textit{Azolla} sporocarp

\textit{a)} Megasporocarp measurement

Length of the spore = 35.68\(\mu\)m
Breadth of the spore = 27.287\(\mu\)m

\textit{b)} Microsporocarp measurement

Length of the spore = 8.396\(\mu\)m
Breadth of the spore = 6.297\(\mu\)m

3.5 Maintenance of \textit{Azolla} culture

The biomass based spores of Hybrid \textit{Azolla} (1kg) were cultured in concrete tank with soil and pond water under partial shade for use of sporelings. The different species of the genus \textit{Azolla} were properly cultured in concrete tanks with pond water and soil under partial shade at the Net House before using as experimental materials.

Intercultural practices like application of phosphate fertilizers, removal of algae, and application of predator controlling biocides were done once a week to maintain the plants of the stock cultures green and healthy.
B. Study on development of sporelings:

3.6 Layout and designing of experiments:

I. Pot culture experiment

The experiment was done under particular level of illumination i.e., under partial shade condition. The meteorological condition is referred in table MD-2. The culture was maintained under a 2 meter high movable shed covered with two layers of opaque polythene sheets and the pots received approximately 50% of the total solar radiation, normal atmospheric temperature and normal relative humidity.

II. Field culture experiment

The experiments were conducted in the Crop Research Farm of the Botany Department, Burdwan University, for field application of biomass-based spores.

3.7 Parameters used:

Seasonal parameters: The experiments were conducted in four seasons such as $S_1$, $S_2$, $S_3$ and $S_4$. $S_1$ April to June, $S_2$ July to October, $S_3$ December to January and $S_4$ February to March. 2gms of hybrid Azolla spores were cultured to develop Azolla sporelings of various sizes such as 0-0.5mm, 0.5-1.0mm, 1.0-2.0mm and 2.0-3.0mm, which were used for different treatments. Different growth parameters like size of the sporelings, leaf number, leaf size, root number and root size of sporelings, chlorophyll content of phytomass,
heterocyst frequency of the endosymbiotic algae and biomass of *Azolla* sporelings were calculated after specific period of incubation.

**Estimation of biomass:-**

Dry Weight (mg) biomass was determined by drying a known amount of fresh tissue in hot air oven at 80°C for 48 hours or more for getting a constant weight.

**Determination of heterocyst frequency of *Anabaena* symbiont:-**

Heterocyst frequency of the algal symbiont (*Anabaena azollae*) was determined by counting the number of heterocysts per one hundred algal cells. Leaves were taken from apex, middle position and base of a plant; teared apart separately on glass slides, mounted properly and the number of vegetative cells as well as heterocysts were counted separately observing through a light microscope. Approximately 100 algal cells from the leaves of each of three positions were counted, average was taken and the heterocyst frequency (percent) was expressed as number heterocysts per one hundred algal cells.

**Estimation of chlorophyll:-**

Chlorophyll content of *Azolla* plants was determined following Arnon’s method (1949). 100mg fresh *Azolla* biomass (excluding roots) were immersed in 5ml acetone and kept at low temperature in a deep freeze for 24 hours. The supernatant was decanted off and the plant tissues were rinsed repeatedly with a little volume of acetone until they were completely free from green colour. The final volume of acetone was made to 10ml and the intensity of the green
colour was measured at 665nm in a spectro-photometer, (Model ECGS 5700A). The chlorophyll content was expressed as milligram per gram dry tissues. (mg.g\(^{-1}\) dry tissue).

**Preparation of IRRI's growth medium for the culture of Azolla**-

The IRRI's medium has been introduced by Watanabe (1977a). For the preparation of IRRI's medium the following reagents are used.

- **a)** NaH\(_2\)PO\(_4\)  \(\rightarrow\) 20ppm
- **b)** K\(_2\)SO\(_4\)  \(\rightarrow\) 40ppm
- **c)** CaCl\(_2\)  \(\rightarrow\) 40ppm
- **d)** MgSO\(_4\)  \(\rightarrow\) 40ppm
- **e)** FeSO\(_4\). 7H\(_2\)O  \(\rightarrow\) 0.5ppm
- **f)** EDTA  \(\rightarrow\) 26.1gm
- **g)** Trace element

Trace element component includes the following elements:

- **i)** Mn  \(\rightarrow\) 0.5ppm
- **ii)** Mo  \(\rightarrow\) 0.15ppm
- **iii)** B  \(\rightarrow\) 20ppm
- **iv)** Zn  \(\rightarrow\) 0.01ppm
- **v)** Cu  \(\rightarrow\) 0.01ppm
- **vi)** Co  \(\rightarrow\) 0.01ppm

To prepare 1000ml IRRI's medium, 1ml of each reagent but 0.1ml Fe. EDTA (0.1ml) was used and the final volume was made with distilled water.
3.8 Preparation of different growth regulators which have been used for the treatment of sporelings:

1) **Phytonol**

Phytonol is a pure n-tri acontanol. Phytonol is totally a non-toxic bioregulator of growth which can be recycled in plant systems for the benefit and growth of the tea bushes and other plants. 5ppm Phytonol was diluted to 0.05ppm for use in *Azolla* culture.

2) **Gibberellins (GA$_3$)**

The Gibberellins are a large family of closely related to tetracyclic diterpenoid compounds, which are all carboxylic acids. GA$_3$ is the most abundant in nature, and its commercial form was used in the present study. Its chemical formula is C$_{19}$H$_{22}$O$_6$. The effectiveness of Gibberellins in regulating growth and quantitative change in growing tissues suggests that they are natural growth regulating substance in higher plants. 100ppm was prepared for the experiments.

3) **Indole butyric acid (IBA)**

It is a synthetic auxin. Its chemical formula is C$_{12}$H$_{13}$NO$_2$. The most characteristic effect of this auxin is to promote growth by cell enlargement. 100ppm was prepared for experiment.
4) **Naphthalene acetic acid (NAA)**

It is also a synthetic auxin. Its chemical formula is C$_{12}$H$_{10}$O$_{2}$

10ppm was prepared for experimental purpose.

3.9 **Experimental Set up**

I. **Pot Culture Experiment**

a) **Sporulation of Azolla**-

Six Azolla species including one Hybrid (*A.pinnata, A.filiculoides, A.microphylla, A.caroliniana, A.mexicana* and one Hybrid) were used as experimental materials. Sporulation was studied with reference to Sporulation Index, Sporocarp Output and ratio of mega - and microsporocarps of six species during different seasons of the year starting from June 1994 to May 1995. Azolla samples collected from 8 – 10 places in the pot culture and 10 – 15 places in the field culture were used for the measurement of sporulation. The number of sporulating plants as well as mega – and micro sporocarps on each plant were counted in 100 randomly selected plants using a magnifying glass.

b) **Germination of spore**-

To study the germination and viability of spores, the experimental material was biomass based sporocarps of *A.microphylla, A.pinnata* and Hybrid, which were well adapted in ecological condition of West Bengal. The study includes the germination behaviour of spores as well as development of sporelings. The study was
done after 5, 10 and 15 days of germination and this was continued just after the harvesting months of February (1995) to April (1995). The biomass based spores collected in February were partially decomposed in small open pits for 30 days. They were dried in shade for 7 days and packed in polythene bags. The germination study was continued since March after one month's storage. 2gm spores of each species were cultured in 250ml IRRI's medium in a pot of 176.625 sq. cm. under partial shade condition.

To study the viability of spores and germination behaviour of fertilized megaspores of Hybrid *Azolla*, experiments were performed in the month of March 1995 and continued upto April 1996.

To study the effect of temperature on viability of Hybrid *Azolla* spores, three beakers of 49.06cm were taken containing soil of 0.3cm and 1.0cm depths. Viable spores were sown on the soil surface, below the soil depths of 0.3cm and 1.0cm and incubated in the oven at the temperatures of 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 and 100°c individually for three days having exposure of 8 hours per day. In case of viability study, 2gm of particular temperature treated spores were cultured in 250ml IRRI's medium to record the days required for germination and number of sporelings.

c) Growth study of the sporelings (plantlets):-

To study the growth behaviour of sporelings having various sizes the experiment was conducted from the month of April (1994) to March (1995) under the partial shade condition of the net-house. The Hybrid *Azolla* sporelings of different size ranges such as 0 – 0.5, 0.5 – 1.0, 1.0 – 2.0 and 2.0 – 3.0mm were identified after culturing in 250ml IRRI's medium based on the parameters like leaf size, leaf
number, root number, root length, heterocyst frequency of the symbiont and time required to attain the particular size after emergence. Such sporelings were used as inoculum.

d) Inoculation of *Azolla* sporelings:-

Inoculum sporelings of different size ranges such as 0 – 0.5mm, 0.5 – 1.0mm, 1.0 – 2.0mm and 2.0 – 3.0mm were developed for experimental purposes. Such sporelings were also used as inoculum. Before inoculation, sporelings of various sizes were superficially sterilized by 0.01% aqueous mercuric chloride solution for about 30 seconds, washed several times in tap water to eliminate the disinfectant and other adhered soil particles if any. The plants and sporelings were blotted separately, superficial water was removed.

e) Treatment with growth regulators:-

To study the effect of phytonol on growth behaviour of sporelings, the experiment was conducted from April 1996 to March 1997 in different seasons. 100 sporelings of each sizes were pretreated with 0.05ppm phytonol solution of particular volume for 2 days and then cultured in 250ml IRRI’s medium for 10 days. The culture of sporelings in IRRI’s medium without pretreatment of phytonol served as control. The different volumes of 0.05ppm phytonol solution such as 5ml, 10ml, 20ml, 25ml, 30ml, 40ml and 50ml were used in separate pots for particular size range of the sporelings. The leaf number, size of leaf, root number, root length and heterocyst frequency of the symbiont were recorded after 10 days of culture in each set. The whole experimental setup was placed in a partial shade condition of the net house.
To study the effect of IBA, different volumes of 100ppm IBA such as 1ml, 5ml, 10ml, 20ml, 30ml, 40ml, 50ml, and 60ml were used in the investigation. After pretreatment with the particular volume of IBA for two days the sporelings were cultured in IRRI’s medium as usual and the same growth parameters were recorded after 10 days. The culture of sporelings in IRRI’s medium without pretreatment of IBA served as control.

The different combinations of IBA and Phytonol were used for searching suitable volume mixture of IBA and Phytonol. For such purpose 10ml IBA and 30ml Phytonol, 10ml IBA and 40ml Phytonol, 20ml IBA and 30ml Phytonol, 20ml IBA and 40ml Phytonol, 30ml IBA and 30ml Phytonol, 30ml IBA and 40ml Phytonol were used. The same procedure was adopted. To maximize the growth behaviour of sporelings, the foliar treatment of growth regulators was done. Phytonol, IBA and mixtures of IBA and Phytonol were used on 2.0 – 3.0mm Hybrid sporelings. The growth regulators were sprayed as mist over the surface of the sporelings. The foliar treatment of phytonol (40ml), IBA (30ml) and mixture of IBA (30ml) and phytonol (40ml) was done weekly during 3 – 4 consecutive weeks or more in equal split volumes. The same growth parameters were noted until the 2.0 – 3.0mm sporelings attained maturity in every season.

To study the effect of foliar spray of Gibberellin (GA₃) and Naphthalene Acetic Acid (NAA) on 2.0 – 3.0mm Hybrid Azolla sporelings, the extreme season of April – June was selected. Sporelings were pretreated with GA₃ and NAA in concentration controlled condition in contrast to the treatment of phytonol and IBA in quantity controlled condition. The concentrations of GA₃ were 10ppm, 20ppm, 40ppm, 80ppm and 100ppm but the concentrations of NAA were 0.1ppm, 0.2ppm,
0.4ppm, 0.8ppm and 1.0ppm. The same procedure was followed for spray treatment on 2.0 – 3.0mm sporelings during April – June.

To study the effect of foliar treatment of growth regulators in different combinations, 0.05ppm phytonol, 100ppm IBA, 40ppm GA₃ and 0.4ppm NAA were used on 2.0 – 3.0mm sporelings during April – June. The treatment was done weekly during 3 – 4 consecutive weeks in equal split volumes.

After scheduled days of incubation, the cultured sporelings were harvested with the help of polythene strainers; the water was allowed to trickle down and then the sporelings were placed on dry absorbent papers for removing the surface water. Different growth parameters were measured.

II. Field Culture Experiment

During the year 1998 – 1999 four plots of 1m x 2m² area were prepared according to split-plot design. In each plot 1Kg Hybrid Azolla spores were sown and after germination of spore different growth regulators such as Phytonol, IBA and mixtures of IBA and Phytonol were sprayed on different plots. The study was continued during November 1998 through October 1999. Fertilizer of 250gm superphosphate, 25gm foratox 10G and 25gm furadan 3G were applied weekly. The observation was recorded at particular intervals in order to obtain mature Hybrid Azolla plants.

3.10 Statistical analysis

All the data collected in this investigation, were statistically analyzed for significance test at the treatment levels. In appropriate places, they have been incorporated (Gomez and Gomez, 1984).