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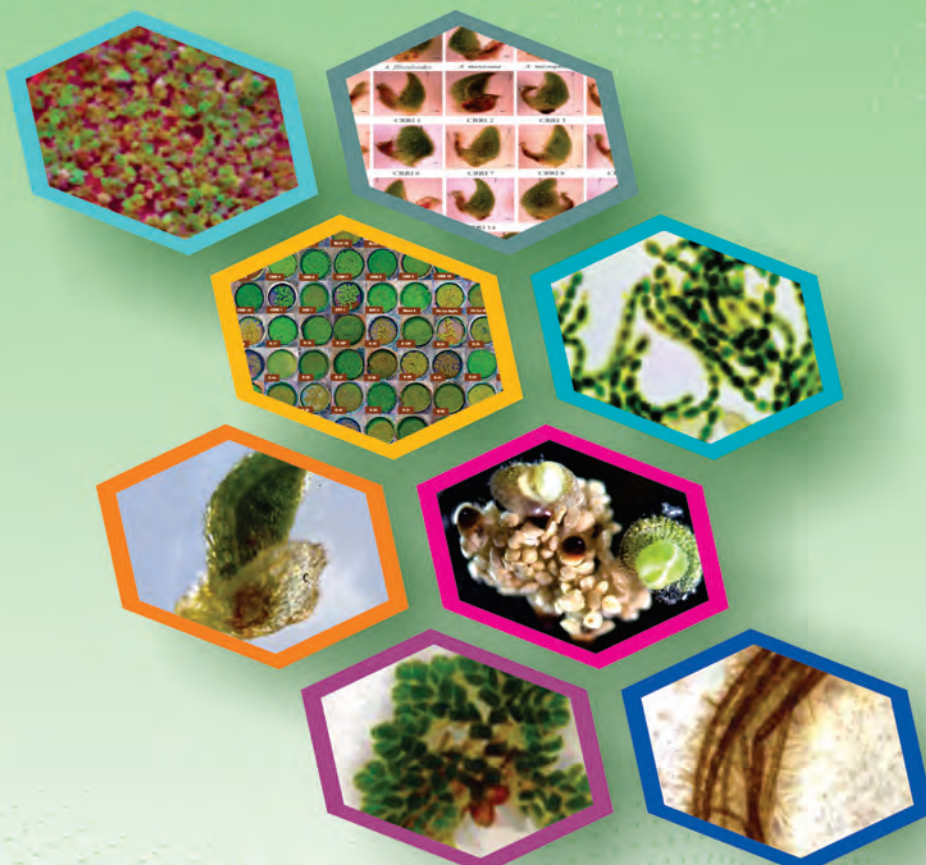
Glimpses of *Azolla* Research at NRRI: Current Status And Way Forward

Upendra Kumar | Laxmipriya Dash | AK Nayak



ICAR-NATIONAL RICE RESEARCH INSTITUTE
Cuttack-753006, Odisha, India

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Upendra Kumar, Laxmipriya Dash and AK Nayak

ICAR-National Rice Research Institute
Cuttack, Odisha-753006, India

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PREFACE

Azolla is a free-floating aquatic fern which possesses nitrogen-fixing cyanobionts in its dorsal leaf cavities. *Azolla*-cyanobionts association exists in a symbiotic relationship, in which the endosymbiont fixes substantial amount of atmospheric nitrogen, up to 600 kg ha^{-1} thus regarded as an ideal biofertilizer in agriculture, especially in rice. *Azolla* is considered as "green gold mine" owing to its broader applications as weed control, water purifiers, medicinal products, animal feedstock, biofuel production etc., besides being extensively used as a biofertilizer. *Azolla* also represents a component of the green revolution in agriculture, providing answers to pressing issues like nitrogen pollution, climate change, and food security.

We are maintaining, conserving and utilizing a total of 102 strains of *Azolla* germplasm at NRRI since 1976 and documented as NRRI Research Bulletin No. 19. The present research bulletin (***Glimpses of Azolla Research at NRRI: Current status and way forward***) has been framed to describe the chronological research work carried out on *Azolla* at NRRI, Cuttack since 1976 and it is useful for students, researches and other stakeholders who are planning to do work on *Azolla*.

Finally, we are expressing our sincere gratitude to all the researchers who have been associated with the research work on *Azolla* since its inception at the ICAR-National Rice Research Institute, Cuttack.

Authors'

TERMINOLOGY

Microsporocarp	Male spore producing structure
Megasporocarp	Female spore producing structure
Indusium	Tip or cap of the mega sporangium
Massula	A hardened layer of cytoplasm formed around the maturing microscope of *Azolla*
Frond	Leaf and leaf like part of *Azolla*
Sporophytic	A diploid phase in the life cycle of a plant and algae
Gametophytic	A haploid phase in the life cycle of a plant and algae
Rhizome	Planar stem from which leaves produced dorsally and roots ventrally
Chlorophyllous	Having or containing chlorophyll
Dorsal leaf lobe	The upper thick and photosynthetically active lobe among the two leaf lobes of *Azolla*
Ventral leaf lobe	The thin lower leaf lobe of *Azolla*

CONTENTS

1. Introduction

Azolla, an aquatic fern, boasts nitrogen fixing abilities, promotes rapid growth, and adaptability to thrive in rice fields. Rice (*Oryza sativa* L.), a vital cereal crop with global significance, is primarily cultivated throughout Asia. The temperate and tropical regions are the natural niches of *Azolla*. It maintains a permanent partnership with a cyanobiont, which sets it apart from other organisms (Kumar et al., 2019a; Kumar et al., 2017a). Cyanobiont and *Azolla* evolve continuously together generation after generation without any interruption inside the leaf cavity of vegetative plant body and the indusium cap of megasporocarps of gametophyte (Kumar et al., 2021a). The unique dorsal leaves of this fern contain cavities that house cyanobacteria, which can effectively convert atmospheric nitrogen (N) into usable forms (Yadav et al., 2014). *Azolla* serves multiple agricultural and environmental purposes, including functioning as a biofertilizer, a source of human food, feed for livestock and poultry, a natural weed and mosquito control agent, and as a means of enhancing water quality by removing pollutants from aquatic environments.

Azolla is a subspecies of Salviniaceae and has 7 major species (*Azolla microphylla, A.caroliniana, A.mexicana, A. filiculoides, A. rubra, A. pinnata* and *Azolla nilotica*). It is referred as "mosquito fern" or "duckweed fern" because it can quickly cover surfaces of still water bodies by forming a thick coating that resembles mats. Among ferns, *Azolla* is unique that it can float on water. In areas where rice is a staple crop, *Azolla* plays an important role in biological nitrogen fixation. It improves soil fertility and supports environment friendly farming methods. It is essential for improving plant health and productivity. Around 139 to175 million tons of nitrogen was added to soil by biological nitrogen fixation, and around 35 to 44 million tons were added through symbiotic associations including *Azolla*.

Azolla plays a crucial role in rice farming, which is commonly referred as "*Azolla* rice cultivation" or "*Azolla* rice farming". In this method, *Azolla* is introduced into rice fields to enhance soil fertility, boost productivity and decrease the dependence on chemical fertilizers. On the whole, the inclusion of *Azolla* in rice farming techniques represents a promising strategy for advancing agricultural sustainability and minimizing the environmental footprint of rice production. It showcases the potential of nature-based solutions to address the challenges of food security and sustainable agriculture. *Azolla* rice cultivation has a long history in some parts of Asia, particularly in countries like Vietnam, Philippines, Indonesia and India. In recent years, there has been a renewed interest in using *Azolla* as a sustainable agricultural tool, and research has been conducted to optimize its use in modern rice farming. *Azolla* dual cropping is an innovative and sustainable agricultural method that combines the cultivation of *Azolla*, with traditional crops like rice. This eco-friendly dual cropping strategy not only supports sustainable agriculture but also encourages responsible land management and thus making it an appealing choice for contemporary farming practices.

Azolla differs from many other ferns in its reproductive strategy, as it hardly produces sporocarps or typical spore-producing structures. Instead, *Azolla* reproduces asexually, through fragmentation and vegetative reproduction. Mutational studies in *Azolla* are a relatively less-explored, but they have the potential to reveal valuable insights into the genetic underpinnings of *Azolla*'s unique characteristics. These studies can provide knowledge on genetic diversity, the influence of environmental factors on the plant's genome, and how mutations might impact *Azolla*'s appearance, growth and its vital symbiotic relationships, especially with nitrogen fixing cyanobacteria. Additionally, these investigations can pave way for genomic research, facilitating gene mapping and leading to the development of improved *Azolla*'s role in sustainable agriculture.

A comparative study focusing on the growth, total chlorophyll content, and nitrogenase activity in various *Azolla* species holds significant importance in the field of plant science and agriculture. Such study would involve assessing how different *Azolla* species or strains perform in terms of their growth rates, photosynthetic capacity, and nitrogen fixing capabilities. The study encompasses multiple variables, including temperature, light, nutrient availability, and *Azolla* genotypes. By comparing these factors across different *Azolla* species, researchers can gain insights into which varieties are most efficient in growth, photosynthesis, and nitrogen fixation. Various biotic and abiotic factors affect the growth of *Azolla.* The abiotic factors which affect the growth of *Azolla* are of two types. One is structural habitat and another one is chemical factors. Structural habitat factors are like water, light intensity, temperature, humidity and chemical factors are pH level, CO_2 concentration, nutrient availability, dissolved oxygen, salinity and toxic substances. *Azolla* prefers to grow in free-floating conditions so it may be found on the surface of ponds, canal and lakes (Sadeghi et al., 2013).The response of *Azolla* to herbicide application under field conditions is a subject of concern in agriculture and environmental studies. *Azolla*'s role as a natural weed suppressor makes it a valuable component in rice paddies and water bodies. However, the use of herbicides in agricultural fields may unintentionally impact *Azolla*. Likewise there are numerous pests which damaged the *Azolla*, therefore, insect-resistance *Azolla* strains have been tested and identified.

Research work on *Azolla* at NRRI has been initiated since 1976. So far 102 *Azolla* germplasm belonging to six major species (*Azolla microphylla, A. caroliniana, A. mexicana, A. filiculoides, A. rubra* and *A. pinnata*) are being maintained, characterized and utilized (Kumar and Nayak, 2019). This bulletin provides a comprehensive overview related to research work on *Azolla* conducted at NRRI in the chronological order and also emphasizes the way forward for the futuristic work.

2. Chronology of *Azolla* research at ICAR-NRRI, Cuttack

(1976-1990)

- 1976: Initiation of *Azolla* research at NRRI, Cuttack
- Biochemical composition, sugars, chlorophyll and N contents in contrasting *Azolla*
- Heterocyst frequency in symbiotic alga from both wild and cultivated *Azolla*
- Distribution and abundance of *Azolla* across India
- Effect of *Azolla* on yield of rice varieties
- Effect of *Azolla* with or without N fertilizer in rice variety
- Role of *Azolla* as dual cropping in rice
- Effect of *Azolla* on rice in various intervals
- Effect of N fertilizer on growth of *Azolla*, N content, grain, straw yield and organic carbon in soil
- Cultivation of temperature-tolerant *Azolla*
- Testing of various *Azolla* isolates for insect resistance
- Use of *Azolla* as a green manure with high doses of N fertilizer
- International collaboration trial on the use of *Azolla* for rice crop
- Mineralization of *Azolla*
- Effect of phosphorous on growth, N, P contents of *Azolla*
- Comparative growth of *A. pinnata* and *A. filiculoides* in fields
- Effect of cow dung and cattle slurry on *Azolla* growth
- Effect of combined N sources on *Azolla* growth in planted field
- Effect of plant spacing on *Azolla* growth and rice yield

(1990-2005)

- Dual cropping of *Azolla* with rice varieties of different durations under spacing management
- *Azolla* dual cropping in absence and presence of superphosphate, animal dung and response to rice crops
- Response of *Azolla* to herbicide application under field condition
- Cytological studies in *Azolla*
- Mutational studies in *Azolla*
- Hybridization studies in *Azolla*
- Tissue culture studies in *Azolla*
- Effects of pesticides and herbicides on *Azolla*
- Economical aspects of *Azolla* dual cropping
- Decomposition of fresh *Azolla* in soil and its effect on soil microflora
- Mineral nutrition, effect of biocides and nitrogen fixation in *Azolla* sp.
- Comparative study of growth, total chlorophyll and nitrogenase activity in *Azolla* sp.
- Growth and nitrogen fixation of *Azolla*

at varying periods of inoculation in rice fields and its effect on rice yield

- ❖ Effect of varying levels of *Azolla* inoculum on growth, yield and N uptake of rice
- ❖ Effect of levels of phosphorous and *Azolla* inoculum on its growth, nitrogen fixation and rice yield
- ❖ Effect of plot area and its dual cropping with rice
- ❖ Effect of rice crop canopy on growth and nitrogen fixation of *Azolla*
- ❖ Nitrogenase activity of *Azolla pinnata* isolates in different seasons
- ❖ Effect of P, Mo and Fe application on growth and nitrogen fixation by *Azolla* and rice yield
- ❖ Growth and nitrogen fixation of *Azolla caroliniana* and rice yield at varying levels of urea
- ❖ Studies on sporocarp germination in *Azolla caroliniana*
- ❖ Effect of P-enrichment of *Azolla caroliniana* inoculum on its growth, nitrogen fixation and rice yield
- ❖ Effect of N, Fe and Mo on growth and nitrogen fixation of *Azolla* and rice yield
- ❖ Effect of varying nitrate levels on growth, and nitrogenase activity of *Azolla caroliniana* and *Azolla pinnata*
- ❖ Mineralization by different *Azolla* sp.
- ❖ Isolation and characterization of alga-free *Azolla*
- ❖ Photosynthetic studies on alga-free and alga- containing *Azolla*
- ❖ Effect of alga-free and alga- containing *Azolla* on rice
- ❖ Effect of *Azolla* inoculum density and N-fertilizer on growth and nitrogen fixation of *Azolla* and rice yield
- ❖ Effect of calcium carbonate and urea on growth and nitrogen fixation of *Azolla* and rice yield
- ❖ Effect of natural and synthetic pesticides on growth and nitrogen fixation of *Azolla* and rice yield
- ❖ Mineral composition of *Azolla* sp.
- ❖ Characterization of *aphanothece* containing *Azolla*
- ❖ Interactions of nitrogen fertilizer and karanj oil in *Azolla*
- ❖ Effect of metacid on morphology, growth and nitrogen fixation of *Azolla*
- ❖ Effect of benthiocarb on morphology, growth and nitrogen fixation of *Azolla*
- ❖ Studies on growth hormone and benthiocarb interactions in *Azolla*
- ❖ Macro nutrient interactions in *Azolla*
- ❖ Response of *Azolla* to zinc, lead and chromium
- ❖ Studies on morphology and physiology of *Anabaena*-free and *Anabaena*-containing *Azolla*
- ❖ Effect of calcium on growth of *Anabaena*-free and *Anabaena*-containing *Azolla*
- ❖ Effect of time of transplanting on

growth and nitrogen fixation of *Azolla* and rice yield
- Influence of pesticide on growth and nitrogen fixation of *Azolla* and rice yield
- Response of *Anabaena*-free and *Anabaena*-containing *Azolla* to magnesium
- Time of rice transplanting and performance of *Azolla* intercropping
- Effect of moist soil culture of *Azolla* inoculum on growth and nitrogen fixation of *Azolla caroliniana* and rice yield
- Effect of temperature on germination of *Azolla* sporocarps
- On-farm trial on *Azolla* utilization and feedback from farmers

(2005-2008)

- Biochemical basis of grain quality enhancement through organic management practice

(2012-2017)

- Nutrient profiling of six species of *Azolla*
- Plant-growth promoting bacteria associated with different species of *Azolla*
- Cyanobiont diversity and their interaction with nutrient profiling in *Azolla*
- Cyanobacteria diversity in six species of *Azolla* through metagenomic approach

(2017-2024)

- Development of sporocarp-based *Azolla* formulation for wet land rice cultivation system
- Use of *Azolla* as livestock feed and microbial growth medium
- Induction of *Azolla*-sporocarp germination
- Identification of physiological markers in *Azolla* strains
- Development of low-cost *Azolla* pellet making machine
- Role of nitrate as antioxidant to alleviate salt stress in *Azolla*
- Screening protocol standardized for identification of salinity-tolerant *Azolla* strains
- Effect of salinity stress on growth, chlorophyll fluorescence, antioxidant enzymes and nutrient content in *Azolla* spp.
- Combined effects of elevated CO_2 and nitrogen fertilizers on plant physiological variables in two contrasting *Azolla* spp.

3. Maintenance, cultivation and body parts of *Azolla*

Like all other Pteridophytes, *Azolla shows two* distinct phases *in* the heteromorphic *life* cycle (sporophytic and gametophytic) which follow each other in regular succession. In sporophytic phase, *Azolla* reproduces vegetative by fragmentation under normal condition. But during adverse condition, it produces heterosporocarp (micro and mega-sporocarps) which enters into gametophytic phase. Each frond consists of leaves, rhizome and roots **(Fig.1).** Leaves occur in two rows alongside the rhizome and each leaf has a thin ventral and thick dorsal lobe. Dorsal leaf lobe contains chlorophyll a, chlorophyll b and carotenoids which is responsible for photosynthesis. These pigments imparts green colour to the plants. Red colour of dorsal and ventral leaf lobes of *Azolla* is mainly due to the presence of red pigment (anthocyanin). Dorsal leaf lobe is hydrophobic and the roots are hydrophilic and these are hanging downwards into the water. These roots are adventitious and are chlorophyllous during the early stages but when they mature they turn brown due to partial decomposition and eventually detaches from the plant (Kumar and Nayak, 2019).

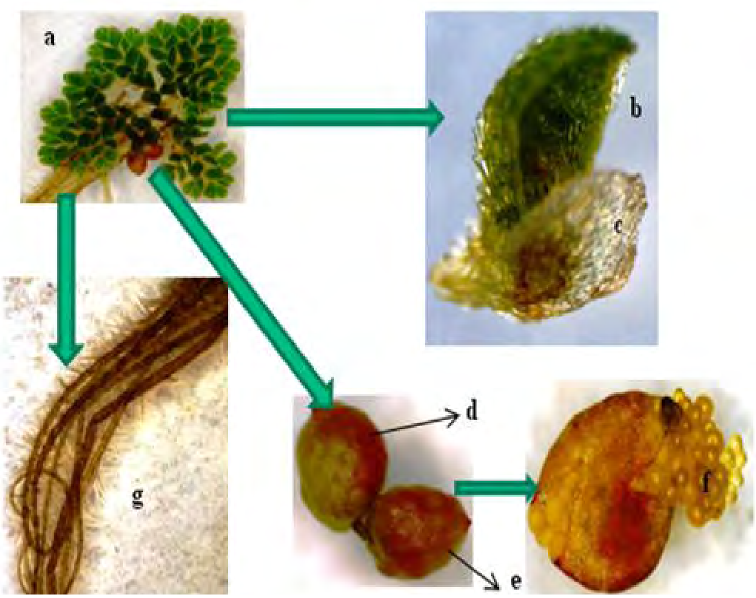

Fig.1.a. Whole gametophytic frond of *Azolla*; b. Dorsal leaf lobe of single leaf; c. Ventral leaf lobe of single leaf; d. Megasporocarps; e. Microsporocarp; f. Spores; g. Root with root hairs.

3.1. Culture maintenance of *Azolla* at NRRI

For the culture maintenance of *Azolla*, many media have been tested for better growth rate under natural condition. N-free Hoagland's and IRRI media were found more effective compared to others (Kumar and Nayak 2019). *Azolla* are maintained in hydroponic condition in open beakers or jars kept under wire netted chamber. Research showed that an inoculum of 0.5 g of fresh *Azolla* might yield 32 g within 20 days irrespective of *Azolla* species under natural condition. Phosphorous is the limiting nutrient of *Azolla* growth and nitrogen fixation and research also showed that phosphate content in the harvested *Azolla* is directly proportional to the phosphate level in the medium. Maximum growth of *Azolla* is obtained at slightly acidic medium (pH 6) and decreased at acidic (<pH 5) and alkaline medium (>pH 9). Interestingly, total N content in *Azolla* increased from 2.5 to 170.2 mg within 20 days due to cyanobiont N_2 fixation (Subudhi and Singh, 1978).

3.1.1. Effect of sodium chloride

By increasing the concentration of sodium chloride from 2.5 to 95 mM in 2/5 strength of N-free Hoagland's medium, the growth and chlorophyll contents of the fern decreased gradually. However, a gradual increase in amino nitrogen and soluble sugar contents was noticed. In the concentrations of 5, 15 and 20 mM NaCl, the increase in generation time to 3.32, 10.63 and 13.28 %, soluble sugar to 4, 0.6 and 8.33% and amino nitrogen to 50, 85 and 100% over the control (0 dose of NaCl) was marked respectively (Subudhi and Singh, 1978).

3.1.2. Effects of combined nitrogen sources

The growth of the fern was slow in calcium nitrate supplemented medium than in the N free medium. The fresh matter yield, chlorophyll, soluble sugar and amino nitrogen decreased gradually in N treated *Azolla*. The fresh matter yield in presence of ammonium chloride and calcium nitrate was 61.54 and 7.52% less than the control, whereas the decrease in 29.5 and 27.5% chlorophyll content was noticed. However, in ammonium chloride and calcium nitrate treated *Azolla,* increase of 257 and 201% amino nitrogen and 16 and 20% soluble sugar contents were observed, respectively. *Azolla* cultured in N free and nitrate-containing media showed a linear increase of heterocyst frequency from apical to basal leaf. The heterocyst frequency of alga present from 3rd to 9th leaf was in the range of 12.8 to 31.0 % in N free medium and 6.94 to 16.95 % in 4 mM NO_3 treated medium. Thus, there was reduction in heterocyst frequency of alga in N treated *Azolla* (Subudhi and Singh, 1978).

3.2. Cultivation of *Azolla*

Azolla is primarily cultivated in an aquatic environment which involves provision for water, temperature, light and nutrient levels. *Azolla* prefers nutrient rich water. In case where water lacks nutrients, incorporating organic matter or an aquatic fertilizer is to be taken into consideration. The fern requires sunlight for photosynthesis, but avoid exposing it to direct sunlight for extended periods. It grows on warm temperature ranging from 20-30 ˚C. Continuously monitoring the quality of the water, ensuring that the pH levels are maintained. Harvesting *Azolla* at regular interval is necessary to prevent excessive growth when it extends beyond 50-75% of the water surface. *Azolla* can be successfully multiplied in soils below pH 8.5. When introduced in the paddy fields, the multiplication was rapid after 20 days of planting. So as long as light penetrates the canopy, *Azolla* can be grown simultaneously with rice plants. An inoculum of 1 kg/m^2 generates around 12 tonnes of green matter per hectare in 15-18 days; the N content of *Azolla* is about 4-5% N on dry weight basis. During winter, the multiplication was very rapid. Recently, *Azolla* production is done by increasing biomass from inoculums which is the easiest way to cultivate. As *Azolla* multiplies faster, its cultivation becomes easy and comfortable for an ordinary farmer. There are mainly two methods of *Azolla* multiplication: (i) standing water method and (ii) nursery method (Kumar et al., 2020).

3.2.1. Standing water method

Different *Azolla* cultivation methods are adopted for the growth of *Azolla*. In standing water method, a meadow or a field having shallow standing water is selected. In this method up to 5-10 cm depth of water is required for *Azolla* cultivation.4-8 kg P_2O_5/ha of super phosphate is applied for the rapid growth of *Azolla*. An amount of 100-200 g of *Azolla* inoculum is added into the standing water. Within three weeks, *Azolla* is rapidly multiplied and formed a carpet on the water surface that can be collected and utilized right away or dried and stored for later use.

3.2.2. Nursery method

Azolla can grow in a small nursery plots (50-100 m^2) with strong bunds all around so that water can stand up to a height of 5-10 cm **(Fig. 2)**. Due to higher percolation rate of water in the newly constructed nursery plot, puddling is usally done. Another method of controlling water percolation is by compacting the soil. Plastering the bottom and sides with a mixture of cow dung and fine clay is yet another effective method of controlling percolation. Permanent *Azolla* nurseries can be constructed with brick and cement. Small nursery beds are advantageous compared to large plots as wind causes drifting of *Azolla* towards one side in large plots. Spreading polythene sheets at the bottom of the nursery beds can also control percolation (Kumar and Nayak, 2019). At NRRI, Cuttack, the fern was multiplied throughout

Fig. 2 Cultivation and maintenance of *Azolla* at NRRI, Cuttack, Odisha

the year in 32 plots each of 8 m^2 since 1977-78. The study was also conducted to assess the biomass of *Azolla* and it has been reported that annual production of *Azolla* is around 420 t/ha containing 1.09 t N where average day-night mean water temperature is 30.5-24.5 ˚C **(Table 1).** Although fern multiplied mostly by vegetative mean, but reproductive structures (sporocarp) are also being formed during November to December.

Table1 Fresh yield and Nitrogen contribution of *Azolla* in rice

Year	Month	Mean water Temperature (˚C day/night)	Amount inoculated (t/ha)	Amount harvested (ha)	Increased amount (t/ha)
	Jan	24-19	15.0	55.5	40.5
	Feb	29-20	11.2	50.9	39.7
	Mar	30-24	14.9	49.5	34.5
	Apr	32-27	14.9	50.0	35.0
	May	37-28	14.9	44.4	29.4
	Jun	34-27	18.7	60.8	42.0
1978	Jul	32-29	14.9	48.6	33.6
	Aug	32-27	14.9	47.7	32.7
	Sep	32-28	14.9	39.9	24.9
	Oct	31-26	7.40	28.1	20.6
	Nov	29-23	18.7	67.8	49.0
	Dec	25-17	11.2	49.7	38.4
	Average yield	30.5-24.5	14.3	49.4	35.0

Source: Annual report, 1978

3.3. Mineral composition of *Azolla* species

Four *Azolla* species namely *A. caroliniana, A. filiculoides, A. pinnata* and *A. nilotica* were grown for 25 days in the net house in earthen pots under flooded conditions and their mineral composition was analyzed by using Atomic Absorption spectrophotometer. calcium, magnesium, potassium, iron, manganese and zinc contents of different *Azolla* species on dry weight basis ranged from 0.89-1.17, 1.32-1.56, 1.66-2.06, 0.19-0.57, 0.02-0.14 and 0.05-0.06%, respectively. Copper content of all the species were 0.0004% whereas, mercury and lead were not detected. *A. pinnata* recorded maximum level of calcium and potassium,while *A.caroliniana* recorded maximum level of iron and manganese and *A. nilotica* recorded maximum level of magnesium and zinc (Singh, 1980).

3.4. Effect of fertilizers on growth of *Azolla*

The impact of fertilizers on *Azolla* growth showed that phosphate was an important requirement for its quick multiplication. The fresh weight of *Azolla* was increased when 10 to 100 g of superphosphate per plot were applied. The colour of *Azolla* transitioned from reddish brown to greenish. The application of muriate of potash (MOP) indicated a small improvement on growth whereas ammonium sulphate did not promote growth. The growth could improve slightly when ammonium sulphate was added with superphosphate. The addition of potash, Mo and Co along with phosphate did not increase the growth as obtained with the superphosphate. By increasing the dose of superphosphate and potash the amount of chlorophyll content increased while the addition of ammonium sulphate did not enhance the chlorophyll formation (Singh,1980).

3.5. Growth on soil in varied pH

The fern grew poorly in Hyderabad soil of pH 8.1 **(Fig. 3)** but grew better in slightly acidic soils of Cuttack (pH 5.9). Growth was not supported in the extremely acidic soils of Potakali (pH 3) and Kattampalli (pH 3.6). In Potakali soil the plants died within 24 hours of incubation and about 10 days in Kattampalli soil in waterlogged condition. The iron is more readily available at acidic and neutral pH than the alkaline pH. Therefore, growth of *Azolla* was slow in alkaline soil.

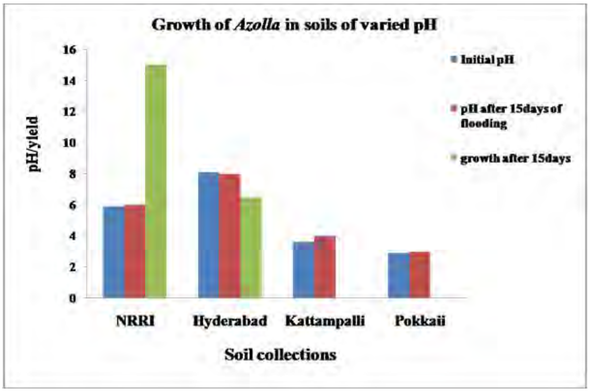

Fig. 3 Growth of *Azolla* in soils of varied pH

3.6. Ecological aspects

The growth of *Azolla* was not affected by frequent rain; infact, growth was optimal throughout the monsoon season. However, plants are washed into channels in intense rains and get accumulated when wind and wave action were high and therefore raising of bunds was helpful to avoid accumulation. *Azolla* could grow within the temperature range from 15 to 40 ˚C, although its best growth occurred at temperature range from 20-30 ˚C. Among the three seasons, winter was comparatively more conducive for its multiplication. During summer green coloured plants changed to reddish brown but when the temperature dropped the green colour reappeared. The change in colour from green to brownish was brought about by the bright sun light and availability of right nutrients. Although higher water levels did not seem to have a negative impact on development, the standing water in field from 2 to 10 cm appeared to be necessary.

3.7. Insect pests

During the hot and rainy seasons insects frequently attack *Azolla* crop. The insect larvae belonging to orders Lepidoptera and Diptera roll the leaves of various *Azolla* plants and feed upon them which give rise to brown patches. At this point the pest management is crucial; otherwise, the entire crop of *Azolla* would be destroyed within a few days. Successful control could be achieved by the use of Savidol and Furadon. Furadon was applied at the rate of 3 kg/ha which is most effective in supressing the pests. The application of Furadon along with inoculum encouraged the growth of *Azolla*. These pesticides have no negative effect on algal symbiont (Anonymous,1980).

4. Basic studies on *Azolla* at NRRI

4.1. Cytological studies

Azolla collected from different nations varied in terms of shape, N content, growth, pigmentation and tolerance to temperature and pests. therefore, shoot-tip squashing procedures were used to conduct cytological tests. Results indicated that the species and varieties have different somatic chromosome number (2n) which is reported as 46, 64, 44 and 40. The cells of each species were spherical to cylindrical with normal cell division and heterochromatic chromosome.The comparative karyotypes were asymmetric.

4.2. Studies on *Azolla* sporocarp germination

A.pinnata and *A.caroliniana* sporocarps, collected from fresh *Azolla* plants, decomposed plants and from soil surface were germinated in pots with flooded soil under the net house conditions. The sporocarp collected from fresh and decomposed *Azolla* plants showed 4 and 11% germination in *A.pinnata* and 18 and 23% germination in *A. caroliniana*, respectively. The sporocarps collected from soil surface recorded 56 and 34% germination in *A.pinnata* and *A.caroliniana* respectively. The sporulation frequency and number of sporocarps per plant in *A. microphylla* and *A. pinnata* were the highest when they were grown under continuously flooded condition. Flooding for 3 days and then no flooding for a day had no effect on their sporulation but flooding for a day followed by no flooding for a day significantly reduced the sporulation frequency in *A.microphylla* and number of micro and mega sporocarps in both the species. The sporulation frequency and sporocarp number were the lowest when *Azolla* was grown under continuously non flooded condition. However, the percentage of megasporocarps in total sporocarps did not differ significantly among different treatments.

The biomass production and intensity of sporulation in *A.microphylla* were significantly influenced by the rate of inoculation. *Azolla* fresh weight, sporulation frequency and number of microsporocarp increased with increasing inoculum level upto 7 g/pot and the number of megasporocarps increased upto 5 g/pot. The rate of inoculation had no effect on the percentage of megasporocarps. Wider variation in sporulation was noticed among twenty one *A.pinnata* strains, irrespective of whether they were grown without or with application of phosphorus. Phosphorus application inhibited sporulation in all the strains. The number of megasporocarps was decreased more than that of microsporocarp resulting in the lower percentage of megasporocarps. The formation of sporocarps was less affected by treatment with phosphorus than in the others. Nevertheless, strains 10 and 14 with the highest sporulation frequency and sporocarp number in both the treatments without and with P could be used for sporocarp production. The germination was 42.7% at 25 ˚C and 8.7% at 15 ˚C. It was observed that high temperature treatment was not effective in improving the germination. It was rather detrimental.

5. *Azolla* as dual cropping

5.1. *Azolla* as dual cropping with rice varieties using different parameters

Azolla was dual cropped with three rice varieties (Annapurna, Ratna and Jaya) during dry season of 1982 with and without N fertilizer. After seven days of transplanting rice, 0.5 t/ha of fresh *Azolla* was inoculated. After 20 days of incubation, full cover of *Azolla* was observed resulting in 9.9-11.7 t/ha fresh weight containing 29-31 kg N, which decomposed by itself after 15-20 days of further incubation. In *Azolla* dual cropping treatment an increase in percentage of grain yield in the ranges from 4.5-13.0, 27.2-45.8 and 27.2-39.1 over control, with 30 kg N/ha, 60 kg N/ha and 30 kg N/ha+*Azolla*, respectively (Singh, 1982).

5.2. Unveiling the impact: superphosphate vs. animal dung

To know the impact of dual cropping of *Azolla* along with superphosphate and animal dung, two varieties of rice (CR109 and Supriya) were studied during *Rabi* and *Kharif* season of 1982. An amount of inoculum of 0.5 t/ha fresh *Azolla* was applied which covered the field within15-20 days and started degrading after 15-20 days. The nitrogen contribution was 24.0-29.2 kg/ha, while growth of *Azolla* was 6.5-9.7 t/ha in the *Rabi* season and 6.3-9.7 t/ha during *Kharif* season. In *Azolla* dual cropping, rice yield was assessed under three treatments (with super phosphate (40 kg/ha), with cow dung (1 t/ha) and without super phosphate)) . The yield of rice in case of super phosphate was 31.8%, in case of cow dung 30% and in case of without super phosphate 18.1% higher than the control during *Rabi* season and 10.5, 10 and 5.3% during *Kharif* season, respectively. Thus, it was concluded that super phosphate can be replaced by animal dung. In the treatment of applying 30 kg N/ha as urea in field the increase in grain yield was 31.8 and 14.0% in *Rabi* and *Kharif* seasons while with 60 kg N/ha as urea the increase in grain yield was 63.6 and 29.3 % in *Rabi* and *Kharif* seasons respectively. Dual cropping of *Azolla* has not only saved the amount of chemical fertilizer (30 kg N/ha) to be applied but also resulted in extra grain yield. *Azolla* dual cropping produced a noticeably higher number of panicles/m^2 (Singh, 1982).

5.3. Economical aspects of *Azolla* dual cropping

The economical calculation has been made on *Azolla* dual cropping cultivation and it has been shown that the cost incurred was Rs. 38 (as per year 1980) and that was mostly on *Azolla* inoculum production, superphosphate and furadan application. Whereas the equivalent rice grain yield was obtained after application of 30 kg N (as urea) whose cost was 140.00 (as per year1980). Therefore, the study revealed that the use of *Azolla* as a N-fertilizer instead of urea resulted in a net increase of 268.15% per hectare in rice cultivation (Singh, 1980).

6. Impact of *Azolla* for sustainable crop enhancement

During *Rabi* season, the increase in grain yield over control was 25 and 47% with two different rice varieties (IR 8 and Supriya), respectively. During *Kharif*, it was 27 and 7.8% with another rice variety (Jaya) when 10 tons of *Azolla* which contained roughly 25 kg N/ha, were added. Straw yield was also more in the *Azolla* incorporated treatments than in the control (Table 2) (Singh, 1978).

Table 2 Effect of various doses of *Azolla* incorporation in soil before planting (variety -Jaya)

Treatments	*Rabi*		*Kharif*	
	Grain yield (kg/ha)	Straw yield (kg/ha)	Grain yield (kg/ha)	Straw yield (kg/ha)
Control	3943	2783	2900	2872
Azolla (5 t/ha)	4077	2866	3600	3111
Azolla (10 t/h)	4139	3100	3300	2605
Azolla (15 t/h)	4400	3350	3900	3450
Azolla (20 t/h)	4637	3416	4100	2994
Azolla (10 t/h)+25 kg N	5348	4450	4500	2705
25 kg N	4658	3600	4100	3150
50 kg N	5824	4783	3800	2821

Source: Annual report,1978

Table 3 Effect of *Azolla* in various combinations with N fertilizer on the rice yield

Treatments	*Rabi*	
	Grain yield (kg/ha)	Straw yield (kg/ha)
Control	1884	1866
Azolla basal (10 t/ha, incorporated)	2180	1883
Azolla basal (10 t/ha, unincorporated)	2408	2150
Azolla basal (10 t/ha, incorporated)+30 kg N/ha basal through A.S.	2312	1700
Azolla basal (10 t/ha, unincorporated) +30 kg N/ha basal through A.S.	2416	1933
30 kg N/ha basal through A.S.+*Azolla* (10 t/ha,incorporated)T.D.	3056	2600
30 kg N/ha basal through A.S.+*Azolla* (10 t/ha, unincorporated) T.D.	2760	2366
Azolla basal (10 t/ha, incorporated) T.D.	2108	2016
Azolla basal (10 t/ha,unincorporated) T.D.	2100	1950
30 kg N/ha through A.S. basal	2556	1950
60 kg N/ha through A.S. (two splits)	3412	2950
Azolla compost basal (30 kg N+ 30 kg N/ha through A.S. as T.D.)	3112	2433

*T.D. = Top dressing. Source: Annual report, 1979

Table 4 Effect of *Azolla* on growth, N uptake and soil organic C content of variety Vani

Treatments	N uptake (kg/ha)	% of soil organic carbon
Control	36.5	0.61
Incorporated *Azolla* (5 t fresh wt/ha)	44.8	0.63
Incorporated *Azolla* (10 t fresh wt/ha)	54.5	0.76
Incorporated *Azolla* (15 t fresh wt/ha)	63.4	0.73
Incorporated *Azolla* (20 t fresh wt/ha)	65.8	0.85
AS-20 kg N/ha (basal)	50.4	0.67
AS-40 kg N/ha (basal)	73.5	0.66

AS-60 kg N/ha (basal)	83	0.66
AS-80 kg N/ha (basal)	86.4	0.63
Incorporated *Azolla* (5 t fresh wt/ha) + 20 kg N/ ha as AS)	61.9	0.68
Incorporated*Azolla* (7.5 t fresh wt/ha)+30 kg N/ ha as AS)	60.7	0.76
Incorporated*Azolla* (10t fresh wt/ha)+40 kg N/ ha as AS)	85.2	0.86
Unincorporated *Azolla* (3 t fresh wt/ha)	58.9	0.69
Unincorporated *Azolla* (4.5 t fresh wt/ha)	59.5	0.73
Unincorporated *Azolla* (6 t fresh wt/ha)	64.5	0.76

* AS= Ammonium sulphate. Source: Annual report, 1979

7. Ecological significance of *Azolla*

It has been possible to cultivate *Azolla* at NRRI throughout the years under field conditions with an annual 333 tons/ha green material corresponding to about 840 kg N. Besides beneficial effects of incorporation of *Azolla*, dual cropping of *Azolla* in a field growing rice and inoculated with small amounts of the fern after transplanting of rice has been found to meet the nitrogen requirement of the crop at later growth stages. During the *Kharif* season the performance of four different *Azolla* species- *A.caroliniana, A.rubra, A.filiculoides* and *A.pinnata* were evaluated. These species were inoculated at the rate of 1.0 t/ha after 10 days of rice planting (CR 1018). When urea was not applied, fixed N and the fresh weight of all the species were highest. The fresh weight of different species reduced when urea was applied at 40 kg and 80 kg N/ha. Among these *Azolla* species, urea has higher inhibitory effect on *A. pinnata* whereas, *A.caroliniana* performed better at all treatments of urea application. The treatment of *A. caroliniana* resulted highest grain yields while *A. pinnata* caused the lowest.

7.1. Biological N_2 fixation

The dual cropping of *Azolla pinnata* (Vietnam) with three rice varieties namely (Annapurna, Ratna and Jaya) was conducted during the wet and dry seasons with the inoculum of 500 kg fresh *Azolla* per ha after a week of transplanting. After 20 days of incubation period a production of 10-15 t/ha green matter containing 28-33 kg N/ha was obtained. There was an increase in grain yield in all the varieties with *Azolla* alone which was comparable to 60 kg N/ha. Ratna responded better among the three varieties. In the *Azolla* experiment with those rice varieties, the crop responded better in *Azolla* basal + once dual cropping treatment than the *Azolla* dual cropping twice.

7.2. Effect of *Azolla* on rice fields at varying time periods

Azolla pinnata was dual cropped with rice at weekly intervals (0-6 weeks) after transplanting in addition to the split application (50:25:25) of urea at a rate of 30 kg N/ha, using the inoculum of *Azolla* (500 kg weight/ha) during the dry season. Both biomass and N yield of *Azolla* were observed to decrease with the delay in its application up to 4 weeks after transplanting (WAT), but further delay had little effect on *Azolla*. Use of *Azolla* up to 4 WAT increased (13.6-21.5%) the N yield of rice over 30 kg N/ha urea treatment. *Azolla* applied up to 1WAT gave more grain yield than that obtained with urea at 60 kg N/ha (Table 5). Higher grain yield in treatments where *Azolla* was applied up to 1 WAT was due to increase in number of panicles/m^2 and grains/panicle (Manna and Singh, 1989).

Table 5 Effect of varying periods of *Azolla* inoculation with different doses of urea on its growth, N_2-fixation and rice yield

Treatments	*Azolla* biomass (t/ha)	N_2-fixed (kg N/ha)	Rice grain yield (t/ha)
No N, no*Azolla*			2.77
30 kg N/ha			3.31
60 kg N/ha			3.69
Azolla 0WAT	11.6	30.4	4.02
Azolla 1 WAT	11.0	28.6	4.01
Azolla 2 WAT	9.0	23.6	3.80
Azolla 3 WAT	7.6	18.2	3.70
Azolla 4WAT	5.9	14.5	3.50
Azolla 5 WAT	5.0	12.2	3.40
Azolla 6 WAT	4.9	11.7	3.40
C.D. (0.05)	1.2	4.90	0.25

* WAT- weeks after transplanting. Source: Annual report, 1979

7.3. Variations in *Azolla* inoculum on growth, yield and N uptake of rice

Fresh cultures of *Azolla* at a rate of 0.5, 1.0, 2.0 and 3.0 t/ha 7 days after rice transplanting along with the split application of urea at the rate of 30 kg N /ha during the dry season was tested. *Azolla* growth and N_2-fixed were measured at 10, 20 and 30 days after inoculation

(DAI). It was observed that the increasing inoculum levels covered the fields in shorter time and produced more biomass and N yield accordingly. *Azolla* used at 3.0 t/ha reduced leaf area index (LAI) and dry matter of rice at 17 days and number of tillers upto 27 days after transplanting (DAT). Rice growth at flowering stage was better when 2.0 t/ha *Azolla* was applied as compared to inoculum up to 1.0 t/ha and 60 kg N/ha urea treatment. *Azolla* inoculum levels produced similar or more grain and N yields to those obtained with urea at 60 kg N/ha, while *Azolla* inoculated at 3.0 t/ha gave more yield than the inoculum up to 1.0 t/ha (Singh and Singh, 1989).

7.4. Nitrogenase activity of *Azolla* in different seasons

The nitrogenase activities of four isolates of *A.pinnata* were studied at different hours of the day during months of April, July and January in fallow rice fields.The enzyme activity varied from 8.7 to 29.54, 8.88 to 25.45 and 2.57 to 26.6 n moles C_2H_5/mg in April, July and January months, respectively, at different hours of the day in those isolates. The nitrogenase activity was more at 12 hours of the day than at 7 and 17 hours, irrespective of the month and *Azolla* isolates (Singh and Singh, 1989).

7.5. Heterocyst frequency and nitrogenase activity in *Azolla* species

The heterocyst frequency and nitrogenase activity in different *Azolla* leaves from stem apex were studied in seven *Azolla* species grown in pots under net house conditions both in absence and presence of single super phosphate (6 kg P_2O_5/ha). In all the species, heterocyst frequency and nitrogenase activity were minimum in first leaf and maximum in last leaf. Addition of super phosphate increased the heterocyst frequency and nitrogenase activity in all the species. Under no P treatment, *A. microphylla* showed highest heterocyst frequency (19.9 %) and *A. filiculoides* had maximum nitrogenase activity, whereas under super phosphate treatment, *A. filiculoides* had highest heterocyst frequency of 22.9% and maximum nitrogenase activity of 14.1 n moles C_2H_4/mg in the last leaf. The enzyme activity was more when measured on whole plant basis than on individual leaf basis. On whole plant basis, *A.microphylla* recorded the maximum nitrogenase activity of 22.3 and 17.3 n moles C_2H_4 /mg with P and without P treatments, respectively (Singh and Singh, 1989).

7.6. Effects of phosphorous and *Azolla* on its growth, N_2-fixation and rice yield

This study involved 4 levels of *Azolla* (100, 200, 400 and 600 kg/ha) and 4 levels of phosphorus (10, 15, 20 and 25 kg P_2O_5/ha). *Azolla* biomass and N_2-fixed were determined at 20, 30 and 40 days after inoculation (DAI). Increase in inoculum upto 400 kg/ha and in phosphorous upto 20 kg P_2O_5/ha was found to encourage growth and N_2-fixation of *Azolla* at 20 and 30 DAI. However, inoculum of 200 to 600 kg/ha irrespective of phosphorous level during dry season and at 25 kg P_2O_5/ha during wet season fixed similar amounts of nitrogen at 40 DAI. Phosphorous levels had little effect on biomass and N yields of *Azolla* supplied at 600 kg/

ha. The N_2-fixation at 40 DAI in treatment of 200 kg/ha inoculum at 25 kg P_2O_5 level was comparable to that 600 kg/ha inoculum irrespective of phosphorous level during the wet season. Inoculum of 200 to 600 kg/ha produced similar grain yields of rice during the wet season, while grain yield in 600 kg/ha inoculum treatment was more than the inoculum of 200 kg/ha during the dry season. Phosphorous levels of 15 to 25 kg P_2O_5/ha did not differ with respect to rice yield (Singh et al., 1988).

7.7. Effect of moist soil culture of *Azolla* inoculum on growth, N_2-fixation and yield of rice

The effect of moist soil culture of *Azolla* inoculum was evaluated using CR 749-20-2 as a test crop. *Azolla caroliniana* inoculum was initially grown under flooded conditions and then subjected to moist soil for 2, 4 and 6 days, prior to inoculation to rice fields. *Azolla* grown throughout under flooded conditions served as the control. The inoculum from different moist soil culture treatment was applied to rice at 1.0 t/ha 10 days after transplanting and grown with 3.33,6.66 and 9.9 kg P_2O_5/ha applied through single super phosphate in 1, 2 and 3 splits respectively with N at 30 kg/ha uniformly. The moist soil culture for 2 days increased the growth and N yield of *Azolla* in different P treatments significantly by 13.9 to 121.4 and 13.3 to 78% over the control during the wet and dry season, respectively. The increase was maximum where 3.33 kg P_2O_5 /ha was applied once. In terms of *Azolla* biomass and N yield, 2 days moist soil culture was almost equal to 3.33 kg P_2O_5/ha. In both the seasons, this treatment recorded significantly higher grain and straw yields than the control. However, longer durations of moist soil culture did not further improve *Azolla* growth, N yield and rice yield. Increasing the rate of P application increased the growth and N yield of *Azolla* as well as the grain and straw yield of rice, irrespective of the moist soil treatment.

7.8. Combined nitrogen sources and nitrogenase activity of *Azolla* in rice field

Field experiments were conducted to study the effects of different nitrogen sources, rates and methods of application on growth, N_2-fixation rate of *Azolla* and their response to rice yield. Among different sources of nitrogen, urea super granule and farmyard manure (FYM, 90 kg N/ha) applications showed less inhibitory effect than ammonium sulphate and urea on N_2-fixation. The rate of N_2 fixation by *Azolla pinnata* in unfertilized fields was maximum and the values on 14^{th} day of incubation (DAI) were 0.89 kg N /ha /6 h of a day in wet and 0.83 kg N/ha/8h of a day in dry seasons, respectively. Whereas, the N_2-fixation rates were reduced to 80, 87, 34 and 60% in wet and 77, 87, 45 and 58% in dry season in AS (Ammonium sulphate), U (urea), USG (urea super granule) and FYM (farmyard manure) treatments, respectively. Use of *Azolla* with 90 kg N/ha of these fertilizers increased rice yield by 8.9-13.2% and 10.6-16.4% during wet and dry seasons respectively, over fertilized (non *Azolla*) treatments.

7.9. *Azolla* decomposition in soil and its effect on soil microflora

After being incorporated of *Azolla* into damp soil it has been reported that the significant amount of the *Azolla* biomass was decomposed as organic matter within a week. Soil microbial population were assessed in the decomposed *Azolla* and it was reported that the number of bacteria and fungi increased quickly during the first week of incorporation and then there was a noticeable decline in these population. Whereas, the less variability in actinomycetes was observed and these were declined in second and third week of *Azolla* incorporation. In *Azolla* plant decomposition the soil moisture played an important role. After a week, when the soil was flooded, the number of decomposing fungi, bacteria and actinomycetes rapidly decreased and then reappeared after the drainage of water. Study showed that the soil microflora is more abundant in damp soil than in flooded soil and moist soil experienced the highest rate of organic matter decomposition. When *Azolla* was added to the soil the C:N ratio of soil was 24.8-28.0 but after 25 days, it dropped to11.2-11.7 (Singh, 1982).

Table 6 Effects of plot area and *Azolla* inoculum on its growth, N_2-fixation and rice yield

Plot area(m^2)	*Azolla* biomass (t/ha)			N_2-fixed (kg N/ha)			Rice grain yield (t/ha)		
	A1	A2	Mean	A1	A2	Mean	A1	A2	Mean
46.8	7.5	11.2	9.4	18.9	27.9	23.4	2.93	3.07	3
93.5	6.4	9.1	7.8	16	22.6	19.3	3.13	2.95	3.04
187	4.9	6.1	5.5	12.4	15.4	13.9	3.11	3.26	3.19
280.5	5.3	6.9	6.1	13	17	15	3.14	3.06	3.1
Mean	6	8.3		15.1	20.7		3.08	3.09	

A1=0.5 t *Azolla* /ha, A2=1.0 t *Azolla* /ha. Source: Annual report,1980

Table 7 Effects of growth and N_2-fixation of *Azolla* by spacing and shading to them

Nitrogen level	Mean reduction in light intensity(%)			*Azolla* biomass(t/ha)			N_2-fixed (kg N/ha)		
	S1	S2	Mean	S1	S2	Mean	S1	S2	Mean
No Nitrogen									
A1	51.9	48.9	50.4	7.6	7.4	7.5	16.4	16	16.2
A2	51.9	49.4	50.7	7.9	7.7	7.8	17	16.6	16.8

40 kg N/ha									
A1	60.1	56.2	58.2	5.4	6.5	5.9	11.5	13.8	12.7
A2	59.6	55.5	57.6	6.2	6.8	6.5	13	14.4	13.7
80 kg N/ha									
A1	65	60.6	62.8	4.5	4.9	4.7	9.5	10.2	9.8
A2	56.8	60.8	63.3	5.1	5.6	5.3	10.8	11.7	11.2

S1=15*20 cm, S2=15*7, 15*40cm, A1=0.5 t *Azolla* / ha, A2=1.0 t *Azolla* /ha
Source: Annual report, 1982

7.10. *Azolla* as compost

Biomass from *Azolla* plants is applied straight to the field or dried. It breaks down quickly in 7 to 10 days when added straight to the soil. On the other hand, nitrogen availability lasts for duration of one to ten weeks. According to experiments, two weeks after inclusion, 34% of the total nitrogen is accessible, followed by 63% after four weeks, 76% after six weeks, and 85% after eight weeks. Application of *Azolla* in the green form produces better results than dry form. Green manuring supplied 20-40 kg N ha[1] (Singh et al. 1981).

8. Effects of macro, micronutrients, pesticides and climate change on the growth and nitrogen fixation of *Azolla*

8.1. Effect of phosphorous, iron and molybdenum application on growth and N_2-fixation by *Azolla* and rice yield

Molybdenum or iron application significantly increased the fresh weight and fixed N during the *Rabi* season. During the *Kharif* season, molybdenum application showed no significant effect on the fresh weight, but increased the fixed N whereas effect of iron was not significant on both fresh weight and fixed N. The grain yield of rice without phosphorous, molybdenum and iron application was 2.8 and 3.6t/ha in *Kharif* and *Rabi* seasons, respectively. In both seasons, phosphorous application significantly increased the grain yield (12.0-14.4% in wet season and 8.6-9.5% in dry season) whereas effects of molybdenum and iron were not significant. Phosphorous application at 8 and 16 kg P_2O_5/ha produced similar grain and straw yields (Singh and Singh, 1988).

8.2. Effects of phosphorous, insecticide and fungicide application on growth and N_2-fixation of *Azolla* and yield of rice

Azolla caroliniana was inoculated at 1.0 t/ha after 10 days of transplanting and subjected to 3 treatment combinations involving phosphorus (superphosphate, 10 kg P_2O_5/ha), an insecticide (carbofuran, 2.5 kg/ha) and a fungicide (carbendazim, 2 kg/ha). Fresh weight and nitrogen yield of *Azolla* and grain yield of rice in different treatments ranged from 9.8 to 13.3 t/ha, 20.5 to 32.3 kg/ha and 3.9 to 4.7 t/ha, respectively. Treatment involving combined application of phosphorus application alone produced higher *Azolla* fresh weight and nitrogen yield as compared to application of either the insecticide or the fungicide (Anonymous, 1992).

8.3. Interactions of N fertilizer and karanj oil on *Azolla*

Karanj oil was mixed with urea and applied in three equal splits. Fresh weight and nitrogen yield of *Azolla* and grain yield of rice in different treatments ranged from 7.2 -13.4 t/ha, 14.4- 29.7 kg/ha and 3.8-5.7 t/ha, respectively. *Azolla* fresh weight and N yield gradually decreased with increasing levels of urea. Application of karanj oil reduced the inhibitory effects of urea on *Azolla* and consequently increased the grain yield at varying nitrogen levels by 6 to 9% (Anonymous,1991).

Table 8 Growth, N_2-fixation of *Azolla* and yield of rice as influenced by karanj oil and nitrogen fertilizer application

	Dry season			Wet season		
Treatment	*Azolla* fresh wt	N_2-fixation (kg N/ha)	Rice yield (t/h)	*Azolla* fresh wt	N_2fixation (kg N/ha)	Rice yield (t/ha)
0 kg N/ha	12	25.7	3.8	10.8	23.8	4.1
0 kg N/ha + karanj oil	13.4	29.7	4.1	13.1	29.3	4.5
30 kg N/ha	10.9	21.9	4.2	9.3	20.4	4.6
30 kg N/ha + karanj oil	12.6	28	4.5	12	27.7	4.8
60 kg N/ha	8.8	17.9	4.7	8.9	18.9	5.3
60 kg N/ha + karanj oil	13.4	25.2	5	11	24.3	5.4
90 kg N/ha	7.2	14.4	5.2	8.3	18	5.6
90 kg N/ha + karanj oil	10.3	22.6	5.7	10.7	24	5.6

Source: Anonymous, 1991

8.4. Response of *Azolla* to chromium, zinc and lead

Azolla species was grown in the mineral medium supplemented with 0.1 to 5 ppm of chromium. All the *Azolla* species recorded the highest fresh and dry weights at 15 DAI irrespective of the treatment. An increase in concentration of chromium caused varying degree of growth inhibition on different dates. The percentage of fresh and dry weight reductions over control at 15 DAI were 8.2 to 29.3, 4.3 to 29.3, 6.4 to 27.3 and 6.0 to 39.7, respectively. In *Azolla* spp., *A. pinnata* produced higher fresh and dry weights than other species with all the chromium levels, despite a comparatively higher growth reduction with 1.0-5.0 ppm of chromium. In all the treatments the fresh and dry weights of *Azolla* were the highest at 15 DAI. Increasing concentrations of zinc and lead showed significantly decreased fresh and dry weight on all the dates. Out of the two elements tested, zinc was more inhibitory than lead. The percentage of growth reduction over control at 15 DAI was 7.1 to 55.9 with zinc and 11.1 to 32.1 with lead. However, *Azolla* continued to grow, with as high as 250 ppm of zinc and lead although it's fresh and dry weights were substantially decreased (Anonymous,1992).

8.5. Herbicide and insecticide interactions in *Azolla*

Different herbicides and insecticides were applied at their recommended doses at the days after transplanting of rice variety (IR-36) seedlings during wet season. In different treatments the fresh weight and N-yield of *Azolla* ranged from 7.2 to 12.3 t/ha and 13.3 to 25.8 kg N/ha, respectively. Herbicide application significantly dropped the fresh weight and N-yield. Comparatively thiobencarb was more effective than butachlor. Due to application of different insecticides, the fresh weight and N-yield of *Azolla* in untreated, butachlor-treated and thiobencarb-treated plots were increased. Interactions between the herbicide and insecticide were not significant. Applications of the insecticides also increased the grain and straw yields of rice which were lower in thiobencarb-treated plots than in untreated plots (Singh et al., 1988).

8.6. **Climate change**

Green manures are widely used in rice production and may influence methane efflux. Influence of application of *Azolla* (*Azolla caroliniana* wild), on methane efflux from a flooded alluvial soil planted to rice, and selected soil and plant variables were investigated in a field experiment at NRRI, Cuttack. *Azolla* was either incorporated as green manure at the beginning of the experiment or grown as dual crop in the standing water along with rice. Methane efflux varied considerably among different treatments with one peak each at vegetative and maturity stages of rice. Methane emission was low in all the plots during the 1st two weeks after transplantation with the exception of *Azolla* @ 16 t/ha, to provide 30 kg N/ha, resulted in a high methane efflux during the first 20 days after transplantation. Methane efflux was considerably low in treatments where *Azolla* was grown as a dual crop either in conjunction with urea or following *Azolla* incorporation. Application of urea alone at 60 kg N/ha also stimulated methane efflux from flooded fields planted to rice. Dual cropping of *Azolla* in conjunction with urea effected lowest methane efflux (89.29 kg methane/ha). Dual cropping of *Azolla* in conjuction with urea considerably reduced methane efflux without affecting the rice yields and can be used as a practical mitigation option for minimizing flux from flooded rice (Bharati et al., 2000).

9. Unraveling the *Azolla-Anabaena* symbiosis: morphological and physiological insights

Azolla microphylla and *Azolla filiculoides* were made *Anabaena* free by treating them with a mixture of antibiotics. *Azolla* was grown for 3 days in +N medium supplemented with antibiotics mixture and then transferred to-N medium without antibiotics and grown for 7 days. This sequence was repeated twice. The *Anabaena* free *Azolla* thus obtained required N supplementation for its normal growth. The morphology and physiology of *Anabaena* free and *Anabaena* containing *Azolla* were studied after growing them in +N and –N medium, respectively. *Anabaena* free *Azolla* was smaller in size with fewer branches, less green in color and possessed longer and more number of roots as compared to *Anabaena* containing *Azolla*. Its growth rate was lower than that of *Anabaena* containing *Azolla*. The total chlorophyll content of *Anabaena* free and *Anabaena* containing *Azolla* was 0.840 and 0.985 mg/g fresh weight for *A.microphylla* and 0.724 and 0.898 mg/g fresh weight for *A. filiculoides*, respectively. The ARA (acetylene reduction activity) of the *Anabaena* containing *A. microphylla and A. filiculoides* was 9.42 and 8.27 n mol C_2H_4/g fresh weight/min whereas the corresponding *Anabaena* free *Azolla* showed a negligible ARA (acetylene reduction activity) of 0.09 and 0.04 n mol C_2H_4/g fresh weight /min respectively (Anonymous, 1992).

9.1. Isolation and characterization of alga-free *Azolla*

The *Azolla* species namely *A. caroliniana, A. pinnata* and *A. nilotica* were free from the symbiotic alga, *Anabaena azollae*, after treatment with antibiotics (streptomycin sulphate, Dicrysticin-5 and Tetracycline). The concentration of antibiotics varied for the *Azolla* species depending on their tolerance. The alga free *Azolla* did not grow on N-free medium and required N supplementation. The relative growth rates of alga free *A. caroliniana, A. pinnata and A. nilotica* in N supplemented medium were 44.6, 52.2 and 79.5% lower than that of corresponding alga containing species in N-free medium. The alga free *Azolla* did not exhibit any acetylene reduction activity.

9.2. Effect of alga-free and alga containing *Azolla* on rice

In a pot experiment, alga free and alga containing *A. caroliniana* and *A. pinnata* were grown with urea at 0 and 30 kg N/ha after 7 days of transplanting *Azolla*. The alga containing *A.*

caroliniana and *A. pinnata* recorded the maximum fresh weight of 60.3-75.3 g/pot in no N treatment during wet and dry seasons at 14 days after inoculation (DAI). Alga free *Azolla* species recorded the maximum fresh weight of 15.2-21.2 g/pot in no N treatment at 14 DAI and 50.2-61.4 g/pot in 30 kg N treatment at 21 DAI during the two seasons. The N yields of *Azolla* are different in the two seasons. Urea at 0, 30 and 60 kg N/ha recorded the grain yields of 7.1, 11.5 and 18.3 g/pot during wet season and 11.8 18.2 and 23.8 g/pot during dry season, respectively. In no N treatment, alga free *Azolla* showed no significant effect on grain yield, whereas alga containing *Azolla* species increased grain yield by 47.4-73.2% during two seasons. Both alga free and alga containing *Azolla* species along with 30 kg N/ha urea recorded higher grain yield (13.7-32.4) than urea alone (30 kg N/ha) during wet and dry seasons. However, grain yields were significantly higher with alga-containing *Azolla* species than with alga free *Azolla* species.

9.3. Effect of calcium on growth of *Anabaena* free and *Anabaena* containing *Azolla*

Anabaena free and *Anabaena* containing *Azolla filiculoides* were grown under laboratory conditions with varying levels of calcium (10 to 40 ppm) in +N and –N medium, respectively. In both the cases, *Azolla* grown without calcium showed the lowest fresh weight, dry weight, chlorophyll content and the highest anthocyanin content. The fresh weight, dry weight and chlorophyll content of *Anabaena* free and *Anabaena* containing *Azolla* increased with an increase in calcium level up to 30 ppm and thereafter declined. This indicated that presence or absence of *Anabaena* had no significant impact on the calcium requirement of *Azolla*. The acetylene reduction assay (ARA) of *Anabaena* containing *Azolla* was also increased with increasing calcium levels up to 30 ppm. Calcium at 10 to 30 ppm increased the fresh weight, dry weight and chlorophyll content over the control by 1.0 to 3.7, 1.0 to 3.5 and 1.0 to 3.3, 1.0 to 3.0 and 1.0 -1.7 fold in *Anabaena* free *Azolla*, respectively (Anonymous, 1993).

10. Latest research on *Azolla* at NRRI

10.1. Development of soil-based sporocarp formulation of *Azolla* for wet land rice

There are 102 strains of *Azolla* available at ICAR-NRRI, Cuttack. Among these, 23 strains of sporocarp producing *Azolla* (CRRI 1, CRRI 2, CRRI 3,CRRI4,CRRI5,CRRI 6, CRRI 7,CRRI 8,CRRI 9,CRRI 10,CRRI 11,CRRI 12,CRRI14,CRRI15, CRRI16, GSMI1, IEPI1, IEPI4, R86, R94, *P. assam, A. Pinnata and A. microphylla*) were identified. Out of these, one strain (CRRI-1) of *A. pinnata* was standardized for the development of soil-based sporocarp formulation with the count of 50 spores per 10 g soil **(Fig. 4).** This research has led to a development of a novel formulation based on *Azolla* sporocarp, which significantly minimized the *Azolla* primary inoculum needed for wet land rice cultivation (Kumar et al., 2018).

Fig. 4 Soil based sporocarp formulation of *Azolla*

10.2. Development of morphological marker to identify different *Azolla* species

Despite a great deal of research on taxonomy of *Azolla;* its identification based on morphology remains unclear. Therefore, using six known *Azolla* species as a references (*Azolla microphylla*, *Azolla caroliniana*, *Azolla mexicana*, *Azolla rubra*, *Azolla filiculoides* and *Azolla pinnata*), an attempt was made to differentiate *Azolla* strains based on morphological traits. Out of 96 strains,the results indicated that 21 strains including two identified species *i.e. A. microphylla* and *A. Pinnata* were sporulated during December to February, 2018 **(Fig. 5A).** Except for GSMI 1 all strain showed morphological-resemblance with *A. pinnata*, whereas GSMI 1 was resembled with *A. microphylla* **(Fig. 5B).** The presence of rounded dorsal leaf lobe in *A. caroliniana* has made sporulating its unique marker in Euzolla sub-section and hence, differentiated from other four species (*A. microphylla*, *A. mexicana*, *A. rubra* and *A. filiculoides*) **(Fig. 5C) (**Kumar et al., 2021b)**.**

Fig. 5 (A) Coat colour of micro sporangia in different *Azolla* strains. (a) Red tinted and (b) translucent yellow colour. (B) Shapes and imbrications of leaves in different *Azolla* strains. (a) Star shaped and high imbrications, (b) star shaped and medium imbrications, and (c) triangular shaped and slight imbrications. (C) Shapes of dorsal leaf lobe (DLL) in different *Azolla* strains. (a) Highly acuminated (sharp pointed), (b) slightly acuminated (less pointed), and (c) rounded.

10.3. Use of *Azolla* as livestock feed

Even though *Azolla* is a good source for livestock feed it is not widely adopted because of problems associated with its shelf life, transportation and storage facilities. An attempt was made to prepare *Azolla* pellet for livestock feed by using a pellet making machine. *Azolla* pellet was prepared by mixing suitable additives and bringing its moisture content to a range that prolongs its shelf life **(Fig. 6) (**Kumar et al., 2020a)**.**

Fig. 6 NRRI-*Azolla* feed pellets for livestock feed

10.4. Development of low-cost *Azolla* pellet making machine

Even though *Azolla*, a freshwater fern, is a valuable feed source for livestock, issues with its shelf life, transportation, and storage facilities prevent it from being widely used. *Azolla* pellet was attempted to be developed with a pellet making machine for use as animal feed. *Azolla* pellet was made by combining the right ingredients and adjusting the moisture content to a level that would increase the product's shelf life. The equipment proportion of ingredients to make pellets is manually controlled and measures 310 mm in length, 100 mm in width, and 610 mm in height. There is only one hopper, and it has a 2 kg capacity. This machine produces briquettes that are 30 to 50 mm in size and 10 mm in diameter **(Fig. 7)** **(**Kumar et al., 2020b)**.**

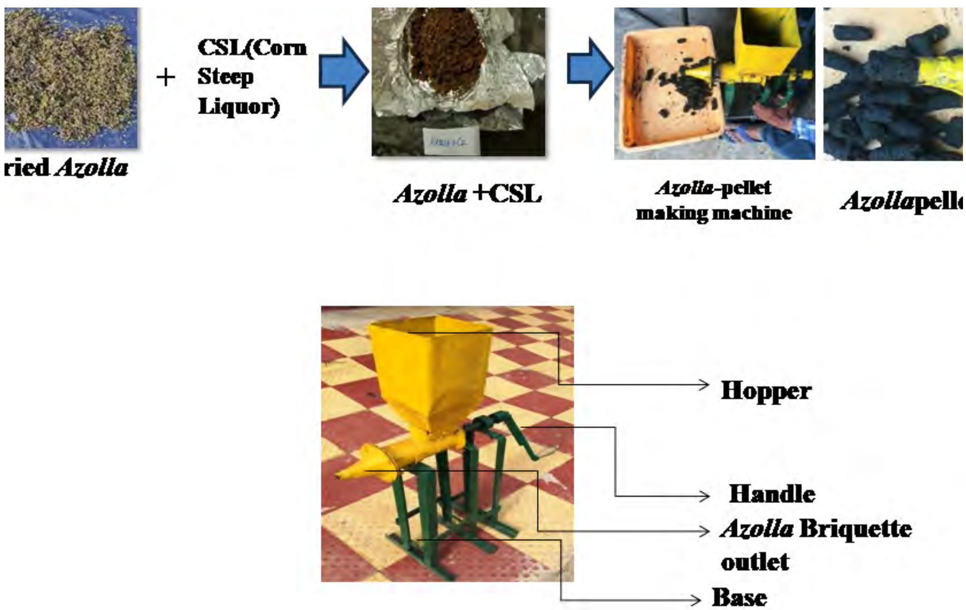

Fig. 7 Making of *Azolla* pellet for livestock feed

10.5. Use of *Azolla* as microbial growth medium

Preparation of microbial-growth culture media from plant extracts is becoming popular now-a-days. Plant-extracts are useful and well suited for microbial-growth because they contain all necessary nutrients, growth factors, amino acids, vitamins and minerals. Therefore, *Azolla*-based microbial culture media "NRRI-AzoMedia" (NAM) was developed to assess the microbial-growth and to evaluate their functional attributes and compared with respective commercial media **(Fig. 8).** *Azolla* is a known aquatic fern and having rich source of nutrients like crude protein (18.6-25.9%), lipid (ether extract, 2.9-4.6%), total sugar (6.27-7.66%), antioxidant (53.2-76.9%), calcium (0.62-2.21%), phosphorous (0.65-

1.03%) and all essential micronutrients. The fast multiplication rate (doubled within 2-3 days) of *Azolla* is another important and additional feature making it suitable for developing microbial-growth media. The medium can be used for cultivating and maintaining microorganisms and this can also be used for purity checking prior to biochemical testing. This is comparatively simple and cost effective as compared to several commercial media. The NAM can be used for the microbial studies of diverse environmental samples. It could also be used for the cultivation and enumeration of specific macro and micro-nutrient utilizing bacteria by addition of particular substrates. NAM provides the necessary nitrogen compounds, carbon, vitamins and also some trace ingredients necessary for the growth of microorganism. This media can be useful for day-to-day microbiology laboratory research, diagnostic work and can able to reduce 30-60% expenditure from the known commercial media (Kumar et al., 2021c).

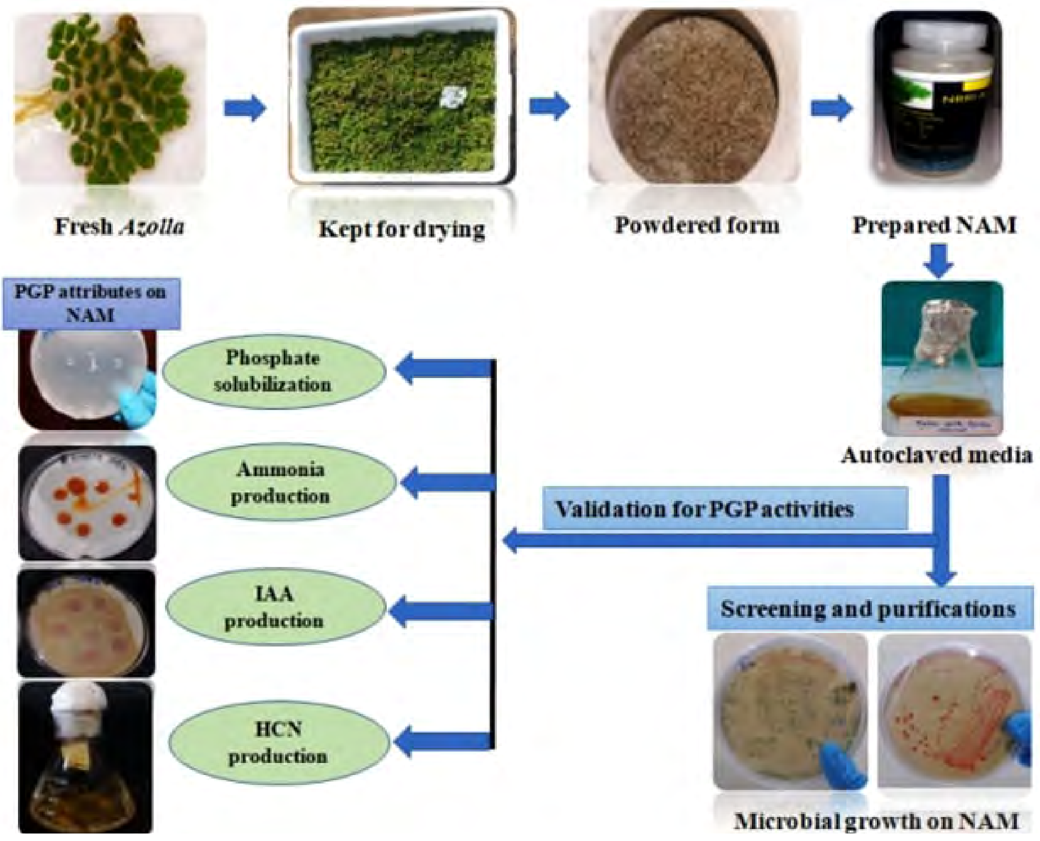

Fig. 8 NRRI *Azolla* medium for microbial growth

10.6. Use of *Azolla* sporeling as source of inoculum

Azolla microphylla sporocarps were germinated in ordinary tap water, sterilized distilled water, unsterilized and sterilized soil and –N and +N IRRI Medium to find out suitable medium for germination. The percentage of germination was the highest in +N medium (82.0) and the lowest in tap water (39.0), indicating that +N medium was the best for sporocarp germination. In another experiment, growth pattern of *Azolla microphylla* sporeling grown in –N and +N medium was compared. The sporelings grown in +N medium produced 22.2-125.7% higher fresh weight than those in –N medium up to 30 days after inoculation (DAI). Those sporelings grown under N medium maintained substantially higher relative growth rate than in –N medium, indicating necessity of N supplementation. The trend was almost reversed at the later stages. In both –N and + N medium, the relative growth rate was the highest during 0-5 DAI and then there was a gradual decline as the sporeling growth progressed, with a few aberrations.

10.7. Farm trial on *Azolla* and feedback from farmers

The performance of *Azolla caroliniana* dual cropping with rice was evaluated during the wet season in farmers' fields at five locations each in Ersama block of Jagatsinghpur district and Astarang block of Puri district. Rice varieties CRM 839, CRM 870, CRM 880, Swarna, Gayatri and Padmini were used in both the blocks. *Azolla* grew well at all the locations and produced average fresh biomass of 10.7-14.3 and 10.8-15.1 t/ha in Ersama and Astarang,

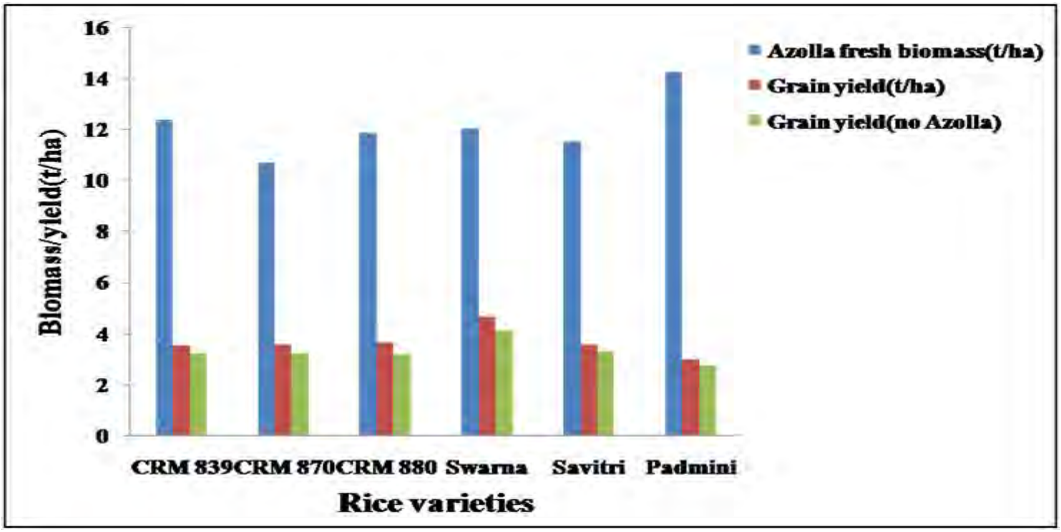

Fig. 9 Effect of *Azolla* dual cropping on rice yield on farmer's field in Ersama block

respectively. The grain yield of different rice genotypes in *Azolla* dual cropping +30 kg N/ha (urea) treatment at Ersama and Astarang sites was in the range of 2.99-4.66 and 3.66-4.67 t/ha, as against 2.75-4.10 and 3.42-4.25 t/ha in 60 kg N/ha (urea) treatment,respectively. Hence, *Azolla* dual cropping not only resulted in a saving of 30 kg N/ha of the chemical fertilizer but also produced an additional yield of 0.24-0.56 t/ha at Ersama and 0.16-0.42 t/ha at Astarang. Among the rice varieties, Swarna at Ersama and Gayatri at Astarang produced the highest grain yield, while the lowest grain yield was recorded by Padmini at both the sites. The cultures CRM 839,CRM 870 and CRM 880 recorded comparable yield. Interaction between the rice varieties and treatments was not significant, suggesting that all the varieties showed similar response to *Azolla* application **(Fig. 9, Fig. 10).**

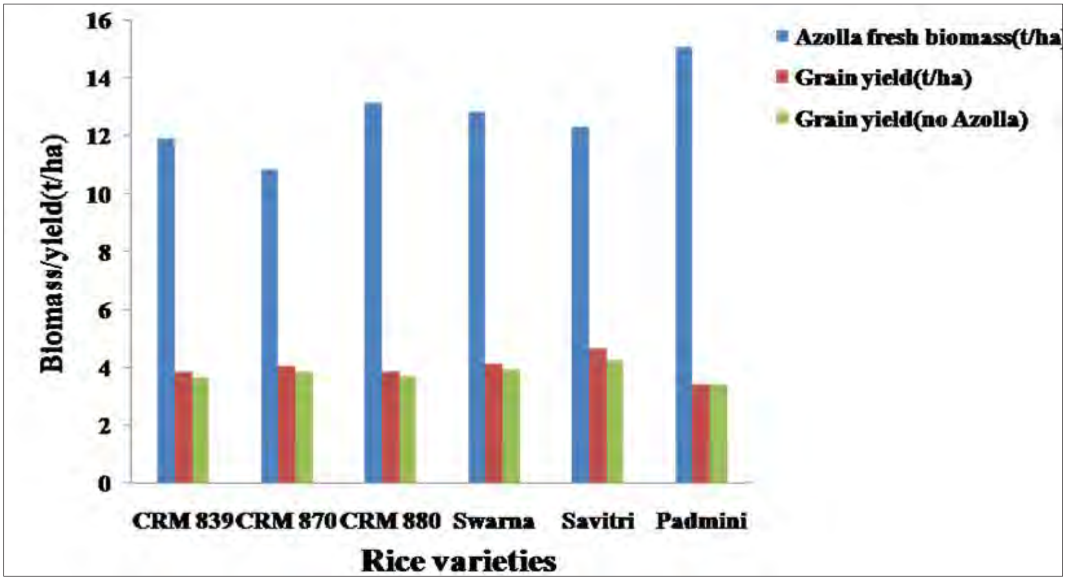

Fig.10 Effect of *Azolla* dual cropping on rice yield on farmer's field in Astarang block

10.8. Plant-growth promoting bacteria associated with different species of *Azolla*

It has been determined that there are abundant plant growth-promoting bacteria from several *Azolla* species, including *A. microphylla*, *A. mexicana*, *A. filiculoides*, *A. caroliniana*, *A. pinnata*, and *A. rubra*. Almost 70% of the isolates with characteristics that promoted plant growth were chosen. Twelve isolates (AM1, AM2, AM3, AM4, AM5, AP1, AP2, AP3, AF1, AF2, AF3 and AC1) tested positive for nitrogen fixation based on *nifH* gene amplification. Furthermore, all nitrogen-fixing bacteria showed preference for utilizing three substrates: casein, gelatin, and nitrate (Kumar and Panneerselvam, 2016).

10.9. Cyanobiont diversity and their interaction with nutrient profiling in *Azolla*

Using the Illumina-Miseq® sequencing technology, the cyanobiont diversity of six *Azolla* species (*Azolla microphylla*, *A. mexicana*, *A. filiculoides*, *A. caroliniana*, *A. pinnata* and *A. rubra*) was evaluated. Nutrition profiling and biomass analysis of *Azolla* species were also carried out, and the results were associated with cyanobiont diversity. Data from operational taxonomic units (OTUs) showed that the Nostacaceae family was more prevalent across all *Azolla* species, and *Cylendrospermopsis* was the most abundant relative member of the Nostacaceae in the majority of *Azolla* species. *Cylendrospermopsis* was identified in much higher relative abundance in *A. pinnata* compared to *A. microphylla*, *A. mexicana* and *A. caroliniana*. The primary productivity of various *Azolla* spp. revealed that *A. pinnata* had considerably ($p<0.05$) greater biomass (6.15 t/ha/cycle), followed by *A. mexicana* and *A. filiculoides*, while *A. caroliniana* had the lowest biomass (3.49 t/ha/cycle). The nutrient profile data showed that *A. microphylla* had considerably ($p<0.05$) greater nutrient content values (25.86 and 76.87%, respectively) in terms of crude protein and total antioxidants than *A. mexicana*, *A. pinnata* and its cyanobiont sequence (OTUs) were positively correlated with nutrient detergent fiber (NDF) and acid detergent fiber (ADF), according to a biplot analysis based on the nutrient profiles of six species of *Azolla*. This suggests that these factors may be crucial in the cyanobiont associations in *A. pinnata* and vice versa. Other *Azolla* species, however, did not show a favourable association. Overall, regardless of *Azolla* spp., *Cylendrospermopsis* was determined to be the dominant cyanobiont. Additionally, based on nutrient profiling, *A. microphylla* may be regarded as adequate feed for animals (Kumar et al., 2019a).

10.10. Induction of *Azolla*-sporocarp germination

Azolla sporocarp rooted in soil was induced to germinate using a novel technique. Using 753, 353, and 180 micron sieves, the compost form of developed *Azolla* sporocarp, which contained megasporocarps and microsporocarp, was sieved in this procedure. Two of the sieved composts that were less than 753 microns were retained in pots with water, while the other one was disposed. After 1-2 days, megaspore connected to massulae was seen in 353 micron sieved compost, and these germinated in water under the right conditions after 13–15 days **(Fig. 11)** (Kumar et al., 2020c, 2021a).

10.11. Identification of physiological markers in *Azolla* strains

The objective of the study was to assess changes in physiological variables in different *Azolla* species. These parameters included growth, estimation of photosynthetic pigment, and chlorophyll fluorescence imaging using ImageWin software in sporocarp-producing *Azolla* strains. Based on physiological factors, we found that GSMI 1 was closely related to *A. microphylla*. When comparing GSMI 1 and *A. microphylla* to other *Azolla* strains, the primary root architecture parameters-such as length, root volume, surface area, average diameter, and projectile area-were noticeably higher. The strains were not distinguished by chlorophyll florescence imaging data because the majority of them had Fv/Fm values that were almost similar **(Fig. 12).** Fv/Fm values were highest in CRRI 4 and lowest in *A. rubra*, indicating a markedly different pattern between CRRI 4 and *A. rubra*. Furthermore, to distinguish GSMI 1 and *A. microphylla* from other *Azolla* strains root imaging could be

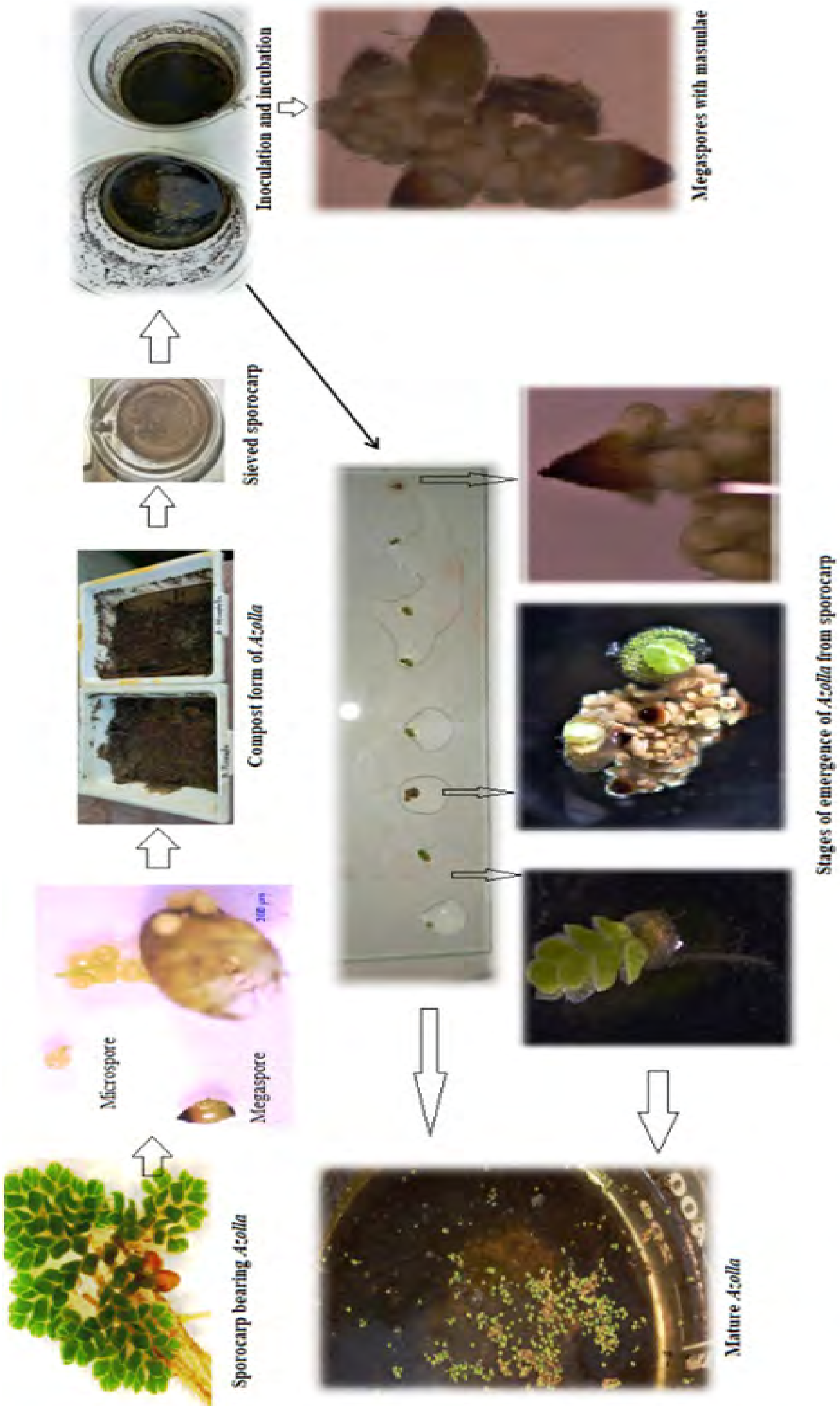

Fig. 11 Protocol for *Azolla*-sporocarp germination

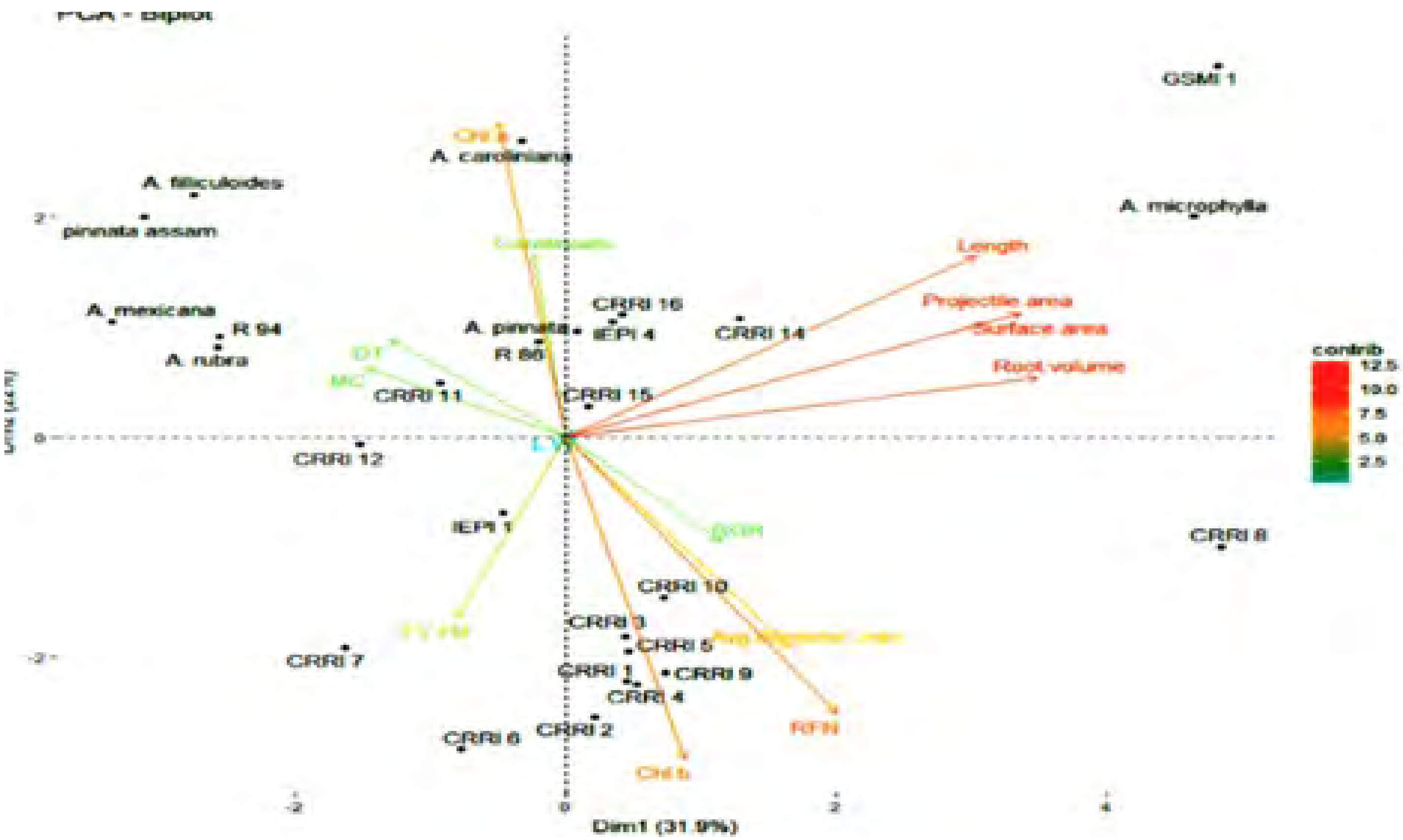

Fig.12 Principal coordinate analysis (PCoA) of *Azolla* strains on the basis of different morphological and physiological traits. L: length of the root (mm); RV: root volume (mm^3); RSA: root surface area (mm^2); ARD: average root diameter (mm); RPA: root projectile area (mm^2). RGR: relative growth rate ($g\ d^{-1}$); RFN: relative leaf (frond) number ($no.\ d^{-1}$); DT: doubling time (d); L:W: ratio of length and width; M%: moisture content in %; Chla: chlorophyll a ($\mu g\ mL^{-1}$); Chlb: chlorophyll b ($\mu g\ mL^{-1}$); C: carotenoid ($\mu g\ mL^{-1}$); F_v/F_m: maximum efficiency of photosystem II (PSII).

used as a physiological marker, while *A. rubra* could be identified from other strains using chlorophyll florescence (Kumar et al., 2021b).

10.12. Role of nitrate as antioxidant to alleviate salt stress in *Azolla*

Potassium nitrate (KNO_3) acts an antioxidant agent in plants in case of salt stress via maintaining ion balance at interior of cell. To validate this, different NaCl treatments (0 mM, 50 mM and 100 mM) were given to *Azolla* growing N-free IRRI medium for 15 days. The treatments were conducted in the presence or absence of KNO_3 (4 mM). After the incubation, it was found that, in absence of KNO_3, the electrolytic leakage was increased by 0%, 13.36% and 35.76% in 0 mM, 50 mM and 100 mM NaCl treatments, respectively where as in presence of KNO_3 the frond number and relative growth rate was increased by 57.05%, 52.38%, 33% and 78%, 50%, 14%, respectively in comparison to control. In case of 50 mM salt treatment in presence of KNO_3, the fluorescence chlorophyll imaging-based minimal fluorescence (F0), maximal fluorescence (Fm), maximal PSII quantum yield (Fv/Fm) and quantum yield of non-regulated energy dissipation in PSII (Y(NO)) were increased by 16.66%, 88.88%, 52.23% and 29.33% respectively in comparison to control **(Fig. 13)**.. Overall, the present study suggested that 4 mM KNO_3 acts as antioxidant in *Azolla rubra* under salt stress (Anonymous, 2019) .

10.13. Screening protocol standardized for identification of salinity-tolerant *Azolla* strains

A rapid protocol to screen *Azolla* for salt-tolerance by using modified methodology (earlier used for screening for rice cultivars for salt-tolerance) was standardised. *Azolla microphylla* and *Azolla rubra* were used as plant material for salt-tolerance screeing along with rice cultivars (Luna Sankhi and IR 64) as check. Two saline treatments such as 80 mM and 120 mM NaCl were imposed in pots along with control (0 mM NaCl). Parameters like soil pH, EC, antioxidant enzymes (catalase, SOD and ascorbate peroxidase) and relative growth rate were observed and results indicated *at par* in *Azolla* as compared to rice cultivars. Overall , our observaton showed that the modified protocol for sality-tolence is robust in *Azolla* and it could able to screen large number of *Azolla* strains for salt-tolence effectively (Narayan et al., 2021, 2023).

10.14. Effect of salinity stress on growth, chlorophyll fluorescence, antioxidant enzymes and nutrient content in *Azolla* spp.

Effect of salinity stress on morpho-physiological, biochemical characteristics, photosynthetic efficacy, nutrient and high affinity potassium transporter (*HKT)* genes in *Azolla* has been studied. The results indicated that out of 102, 8 *Azolla* (*A. microphylla*, BLCC 5, BLCC 18, BLCC 28, Pa Car WTY, R 18, R 54 and R 59) were found tolerant to 80 mM NaCl. The best species for salt tolerant (80 mM NaCl) was *A. microphylla*, whereas the least-tolerant

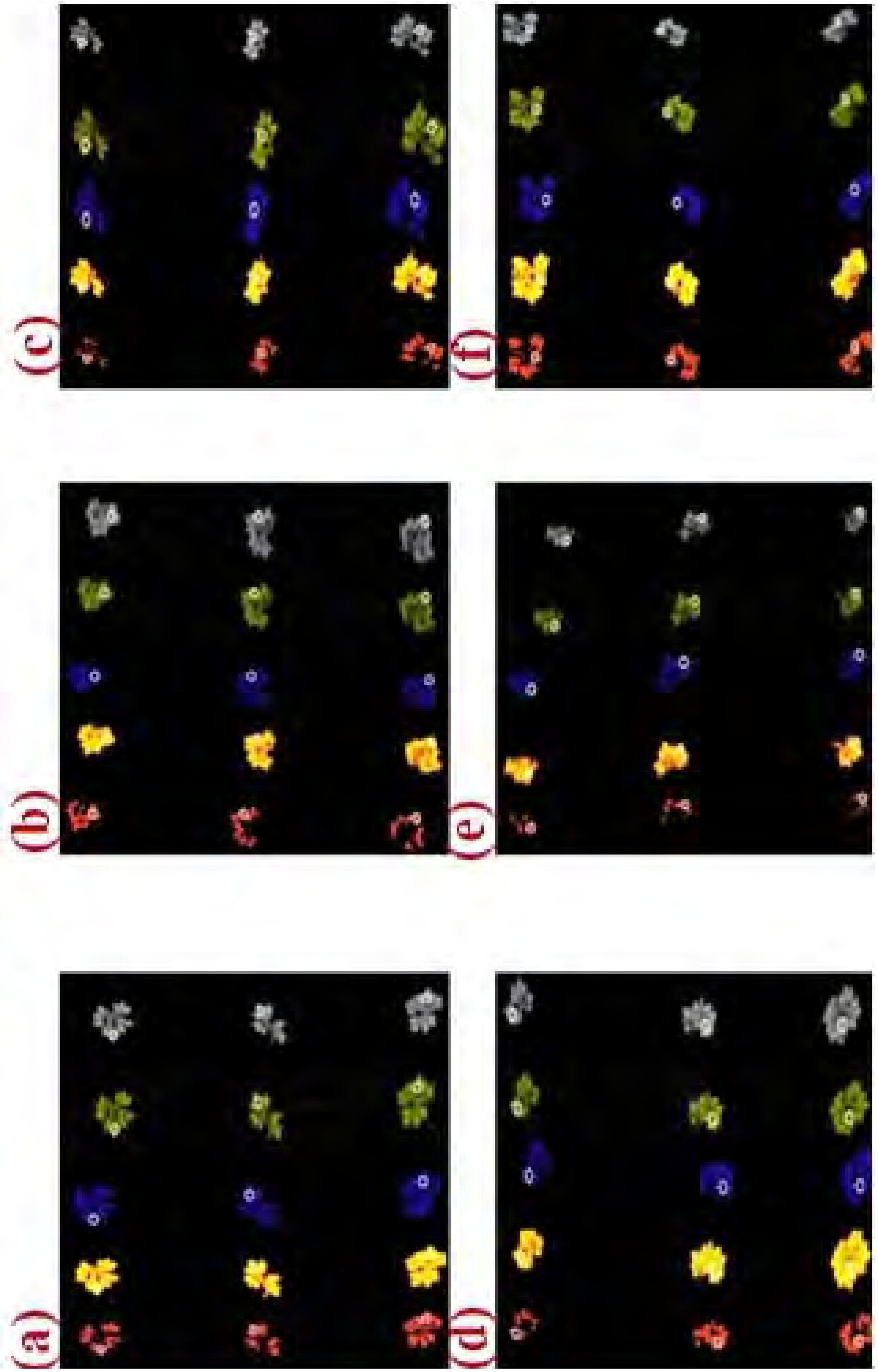

Fig.13 Fluorescence chlorophyll imaging data of *Azolla rubra* under salt (NaCl) stress condition treatment imposed with a. Control; b.Only nitrate (NO_3); c. 50 mM NaCl; d. 50 mM NaCl +NO_3; e. 100 mM NaCl; and f. 100 mM NaCl +NO_3.

was *A. rubra*. Fresh biomass production, frond length and width in *A. microphylla* were significantly ($p<0.05$) higher in *A. microphylla* than *A. rubra* in both 40 and 80 mM NaCl. Moreover, chlorophyll a/b ratio, carotenoids and chlorophyll fluorescence (CHF)-derived F_O, F_m, F_v/F_m and root architecture were higher in *A. microphylla* than *A. rubra* under 40 and 80 mM NaCl. Contents of Na^+ and Ca^{2+} increased in both *A. microphylla* and *A. rubra*, which can interfere with the uptake of essential macronutrients; however, these were accumulated comparatively less in *A. microphylla* than *A. rubra*, whereas a reverse trend was observed in cellular accumulation of K+ content. *A. microphylla* had higher SOD, APX, and proline activities in 40 and 80 mM NaCl than *A. rubra*. Three HKT genes (*Req 6, Aeq14,* and *Aeq16*) were amplified in *A. microphylla* under NaCl stress, while their amplifications were not observed in *A. rubra* (salt susceptible). In *A. microphylla*, the expression of the *Req6* (*HKT*) gene were more under NaCl stress. Moreover, further research is needed to discover the biochemical and molecular processes that confer salinity tolerance in *Azolla* plants (Narayan et al., 2024).

10.15. Combined effects of elevated CO_2 and nitrogen fertilizers on plant physiological variables in two contrasting *Azolla* spp.

The combined effect of eCO_2 and N-fertilizer on growth and physiological parameters of *Azolla* was conducted in OTC to assess these parameters under combined influence of eCO_2 (550 ± 20 µmol mol^{-1} as compared to ambient CO_2 (aCO_2) *i.e.* 400 ± 10 µmol mol^{-1}) and higher dose of N-fertilizer (60 kg N ha^{-1}, 50% higher than recommended dose of N for duel cultivation of *Azolla* and rice *i.e.* 40 kg N ha^{-1}) along with control (no N-fertilizer) in two *Azolla* species (*Azolla microphylla* and *A. pinnata*). The results indicated that growth, chlorophyll, caroteneoids, cyanobiont heterocyst frequency, nitrate reductase, *cya* and *nifH* gene copy numbers were significantly higher under eCO_2 treatments without N-fertilizer, irrespective of *Azolla* species. The addition of N-fertilizers reduced all parameters except antioxidants in both aCO_2 and eCO_2. Interestingly, between two *Azolla* species, these parameters were observed higher in *A. microphylla* compared to *A. pinnata*. Treatments with aCO_2 separated from eCO_2 under influence of N-fertilizers, implied the significant interaction of CO_2 and N-fertilizers in both *Azolla* species. Moreover, two-way interactions of eCO_2 and higher N-fertilizer dose, showed a significant difference of all parameters in both *Azolla* species. Overall, the present study suggested that combination of eCO_2 and N-fertilizer, nullify the negative effect of higher dose of N-fertilizer on physiological variables in both *Azolla* species under CO_2-maintained-OTC (Kumar et al., 2024).

11. Research and development needs

- Quantification of nitrogen fixing gene organization study inside the *Azolla* is one of the best alternatives to assess nitrogen fixing ability of *Azolla* under various environmental conditions.

- ❖ Next generation sequencing (NGS) analysis of *Azolla* is another solution to understand the co-evolution pattern of *Azolla*-cyanobiont.
- ❖ Identification of cost-effective protein rich *Azolla* stains for livestock, fish and poultry feeds.

12. Way forward

- ❖ Presently, to meet the target of food and feed security for the increasing world population, proper scientific research with technological developments is urgently required.
- ❖ Molecular markers to be identified through meta and transcriptome sequence of *Azolla* for better understanding of *Azolla*-cyanobiont and other bacteriobionts interactions. Extension of recombinant plant and microbial protocols would facilitate the validation process and the development of stress-tolerant *Azolla* strains.
- ❖ Overall, the long-term and in-depth studies on *Azolla*-Cyanobionts-Bacteriobionts interactions are still needed to mitigate the elevated food and feeder demands in the era of global climate change.
- ❖ *Azolla* has the potential to contribute to environmental sustainability by sequestering carbon dioxide, purifying water and wastewater treatment should be further explored.
- ❖ Though *Azolla* is a popular biofertilizer for rice growers, its use in other crops is very limited. The status of soil health in field after incorporation of *Azolla* in combination with other traditional fertilizers is also limited.
- ❖ As *Azolla* has invasive nature, it moves into canals and other water bodies where eutrophication typically occurs, which causes algal blooms and harms the aquatic ecosystem as well as human life. Hence, a robust management practice should be developed to minimize over growth of *Azolla* in an aquatic environment.

13. Conclusion

Azolla, with its diverse ecological benefits and agricultural applications, holds immense promise for sustainable agriculture and environmental conservation. As ongoing research continues to unveil its potential, *Azolla* stands as a beacon for a green revolution in agriculture, offering solutions to address critical challenges such as nitrogen pollution, climate change and food security. Whole genome sequence database of *Azolla filiculoids* is now available, hence it is easy to understand *Azolla* cyanobacteria nitrogen fixation process and biomass production under rice-based cropping system. Superior strains for huge biomass and bio energy productions as well as suitable feed for livestock are need to be identified. It is also essential to provide suitable knowledge among farming community on *Azolla*-based bio energy, manure and livestock feed production. There are limited reports to deciphere the exact biochemical pathway and also systematic molecular approach to identify different metabolite production in *Azolla*.

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ICAR-National Rice Research Institute
Cuttack - 753006, Odisha, India
Phone: 0671-2367757, Fax: 0671-2367663
Email: director.nrri@icar.gov.in
directorcrricuttack@gmail.com
Website: http://www.icar-nrri.in

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