SUMMARY

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Azolla plants being small in size and aquatic in habit were found ideal material for basic physiological studies, under *in vitro* conditions. For the establishment of axenic stock cultures of *A. pinnata*, hydrogen perioxide (10% v/v) was found to be a suitable surface sterilizing agent. Sterile culture of *Azolla* once established, remained in healthy conditions under continuous culture.

Azolla cultured in nitrogen free Watanabe et al. (1977) medium, turned yellowish in colour after one week culture period. Regular weekly renewal of the nutrient medium was found necessary for optimal biomass production of *Azolla*, which maintained healthy condition of these plants. Renewal of the culture medium, maintained the pH of the nutrient medium above 5, which facilitated availability of required minerals to *Azolla* plants for their growth and development.

Out of three known nitrogen free culture media tested for optimal biomass production of *A. pinnata* under precisely controlled environmental conditions, Watanabe et al. (1977) medium was found most suitable. Highest biomass of *A. pinnata* in terms of fresh and

dry weights was obtained after an experimental period of three weeksat pH 5.5 to 6.5 of the medium. Moreover, there was no exogenous carbohydrate source added for optimal production of biomass of Azolla, thereby keeping the cost of its production under control. Anabaena azollae, isolated from Azolla cultured in media at pH 5.5 and 6.5, showed almost equal frequency of heterocysts and therefore showed almost identical capacity for nitrogen fixation.

It was observed that A. pinnata under in vitro conditions, responded to the mineral deficiencies of the culture medium by developing characteristic symptoms. Deficiency of potassium (less than 40 ppm) in the culture medium caused chlorosis on leaves. In addition, heterocysts frequency of the symbiont was reduced bringing corresponding decrease in acetylene reduction activity. Potassium at 40 ppm, supported all the growth parameters significantly higher than at other levels tested.

Magnesium was found to be required for optimal biomass production of Azolla. Its deficiency (less than 40 ppm) in culture medium resulted of chlorosis on leaves, and roots were detached from plants. At 40 ppm of Mg, heterocyst frequency coupled with maximum acetylene reduction activity were recorded in A. pinnata.

Azolla grown in calcium deficient (less than 40 ppm) medium showed reduction in its biomass production by 74%, when compared with plants grown in standard dose of calcium. At 40 ppm of calcium, all growth parameters recorded were significantly higher than in case of the plants grown in calcium deficient medium. A positive correlation was found in the biomass produced and its nitrogenase activity (r = 0.990).

The symptoms developed due to phosphorus deficiency in Azolla were decreased growth, decreased frond size, fragility of plants and marked browning of roots. Phosphorus at 30 ppm, above the level present in the standard Watanabe medium (20 ppm of phosphorus), was found to be the optimal level for maximum biomass production of Azolla.

Watanabe medium lacks cobalt, under in vitro conditions, biomass production and nitrogenase activity of A. pinnata were found to be improved by incorporating 0.1 ppm cobalt in the medium. Besides, addition of 10 ppm ascorbic acid to the culture medium increased biomass production of A. pinnata by 14% over the control value.

Presence of nitrogen source in the nutrient medium, reduced heterocyst frequency of isolated Anabaena azollae from Azolla, with simultaneous reduction in nitrogenase

activity of Azolla., But still, the biomass production of Azolla, chlorophyll, nitrogen and protein contents remained unaffected thereby indicating that in absence of nitrogen fixation by Anabaena, Azolla could fulfil its nitrogen requirement from the nitrogen source added to the culture medium. It was evident that Anabaena due to its position, within the dorsal leaf lobe of Azolla was well protected.

Azolla cultured on semi-solid medium, remained alive for three months, thereby its germplasm could be preserved. The biomass production of Azolla was rather slow but this method provided the means of preservation of its germplasm. Watanabe medium was modified by increasing phosphorus contents from 20 to 30 ppm and by the addition of 0.1 ppm of cobalt. The modified medium increased biomass production of A. pinnata by 30%, when compared to the plants grown in standard Watanabe medium. The findings of the physiological studies undertaken on A. pinnata, indicated the need for modification of Watanabe medium for supporting optimal biomass production.

Application of phytohormones such as kinetin, indole-3-acetic acid and 2, 4-dichlorophenoxyacetic acid were not beneficial in increasing biomass production of A. pinnata under in vitro systems. Gibberellic acid in

the low concentrations incorporated in the culture medium, increased biomass production of A. pinnata. But it appeared that Azolla cultures possessed sufficient amount of edogenous phytohormones, required for their growth and multiplication.

Anatomical studies of A. pinnata showed, that Anabaena azollae was present in the dorsal leaf lobe. The epidermal lining of the dorsal leaf cavity possessed two branched hairs on the path of the foliar trace, and their number remained constant. The other outgrowths were unbranched epidermal hairs and their count increased linearally from 3rd leaf onwards, highest being in the 15th to 20th leaf. Anabaena trichomes consisted of vegetative cells and heterocysts, the latter being the site of nitrogen fixation, its frequency affected the nitrogen fixing capacity of Azolla. In the 15th leaf onwards upto 20th leaf, the heterocysts frequency was optimal (30%). In Anabaena, both, the vegetative and heterocysts cells possessed chlorophyll and phycocyanin pigments, but their quantities differed.

A. pinnata could tolerate 10 mM sodium chloride effects on its growth and nitrogenase activity. Sodium chloride incorporation in the culture medium for Azolla

decreased its chlorophyll contents thereby its photosynthetic activity was decreased. Since there is a close relationship between photosynthesis and nitrogen fixation, reduction in phytosynthetic activity reduced nitrogen fixation. Presence of sodium chloride in the culture medium decreased heterocyst frequency bringing proportionate decrease in acetylene reduction activity. The protein contentswere also reduced by the presence of higher levels of sodium chloride in the medium.

As regards the ammonia assimulating enzymes, presence of sodium chloride in the culture medium reduced glutamine synthetase (GS) proportionately while glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT) remained less affected.

Ionic constituents of A. pinnata grown in sodium chloride containing media, showed that Azolla accumulated sodium and chloride ions. Their quantities increased with the increase in sodium chloride level in the culture medium, indicating that Azolla could be used for partial removal of salinity from the media or water. However, further research in this direction may give fruitful results. Similarly Azolla could absorb large quantities of potassium from the culture medium and thereby it could

be used as potassium source to rice plants if Azolla is incorporated in rice soil.

The phycocyanin contents present in the vegetative cells and heterocysts of Anabaena, isolated from salt treated Azolla, showed gradual decrease. The chlorophyll contents of vegetative and heterocysts cells disappeared at 40 mM sodium chloride level present in the culture medium. But at this level, phycocyanin contents were still retained in both the cell types, thereby indicating its relative resistance to salinity. Phycocyanin has been reported as protein reserve and that might be the cause of retention of phycocyanin contents by these cells.

The findings of basic physiological studies conducted on A. pinnata, under in vitro systems were tested in the application studies. Azolla was grown on large scale in plastic trays in modified Watanabe medium. About 12-fold increase in its biomass production was achieved.

The rates of ammonification of Azolla in its vegetative and sporulating stages of development showed that they were slow nitrogen releasing fertilizers. At the end of eighth week, about 60% of ammonification occurred from both the types of Azolla. The chemical constituents of Azolla were potassium, phosphorus, magnesium and other

elements. The application of Azolla to rice soil not only added nitrogen fertilizers, it enriched the soil by adding all other minerals of Azolla, is rich in. In addition, the decaying of Azolla improved soil texture by rendering it porus. Azolla application to rice variety IR 28, significantly increased rice grain and straw yield. When Azolla was combined with ammonium sulphate and used as fertilizer, the rice grain and straw yield improved further as the effect was found to be synergestic.

Thus these studies on A. pinnata provided basic information needed for its optimal biomass production. Application of A. pinnata to the rice plants increased yield of rice grains of variety IR 28, in both the seasons.

The high lights of this study conducted on Azolla pinnata R.Br., the Indian species are enumerated below :

1) Use of hydrogen peroxide as surface sterilizing agent for raising axenic cultures was found suitable.
2) Weekly renewal of culture medium facilitated biomass production under in vitro and in vivo systems.
3) Modification of medium containing 40 ppm K, 40 ppm Ca, 40 ppm Mg, 30 pp P, 2 ppm Fe and 0.1 ppm Co along with the other micronutrients supported

significantly higher biomass production.

4) Semi-solid-culture medium was devised for maintaining germplasm.

5) Anatomically, the 15th leaf onwards upto 20th leaf, the heterocyst frequency was optimal.

6) Presence of combined nitrogen source in the culture medium, diminishes nitrogen fixing capacity of the symbiont, Anabaena azollae.

7) Establishment of fluorescent method to interpret the impact of salinity of water/medium, so that salinity resistant strains could be isolated.

8) Establishment of in vivo methods of mass production.

9) Rate of ammonification indicative of Azolla to be a slow nitrogen releasing biofertilizer.

10) Beneficial effects as biofertilizer on rice strain IR 28 showing increased yield of straw and grain.

11) Its incorporation enriched the soil with mineral nutrients and also improved the texture.

The present research has indicated the necessity of undertaking on a more massive scale and at a National Level, a programme for cultivating Azolla pinnata, the Indian species, for its use as biofertilizer thus aiding the economy of the poor farmer and also reducing the pollution potential due to chemical fertilizer.