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Growth and Nitrogen Fixation in *Azolla pinnata* under Field Conditions

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Abstract : The growth and nitrogen fixation of two indigenous and two exotic strains of *Azolla pinnata* under rice field conditions, were examined at Ambalantota, in the dry zone of Southern Sri Lanka where the terrain is undulating to flat, the rice soils are of the low humic gley type and the 75% expectancy value of annual rainfall is less than 500 mm. A seven to eight fold increase in biomass was recorded in 15 days, giving a doubling time of 4.8 days. *In situ* nitrogenase activity (measured by the acetylene reduction technique) gave values equivalent to 3.1 to 4.6 kgN/ha/day. These results lend strong support to the potential use of *A.pinnata* as a biofertilizer for rice in Sri Lanka.

1. Introduction

Azolla is a free floating aquatic fern which contains a nitrogen fixing blue-green alga (*Anabaena azollae*) as an endosymbiont. It grows well under flooded conditions and is used in certain Asian countries as an organic fertilizer for rice.[5,6] There are reports which indicate that *Azolla* performs better under low light and low temperature conditions,[1,3] but certain strains of *Azolla pinnata* the species native to Asia, have been found to grow well under conditions of relatively high light and temperature,[4,7] and such strains may have a greater agronomic potential in tropical habitats.

This paper reports on certain field investigations conducted on four strains of *Azolla pinnata* R. Brown, at the Rice Research Station at Ambalantota, in the dry zone of Southern Sri Lanka, where the terrain is undulating to flat, the rice soils are of the low humic gley type and the 75% expectancy value of annual rainfall is less than 500 mm.

2. Experimental

The four strains of *Azolla pinnata* examined, have been originally collected from Peradeniya, Debokkawa (a village 20 km away from Ambalantota), India and Bangkok (the latter two strains were obtained from the *Azolla* collection at the International Rice Research Institute, Los Banos, Philippines).

These strains were initially grown under laboratory conditions in the Department of Botany, University of Peradeniya, and then adapted to outdoor conditions at Peradeniya growing them in galvanized-iron trays in soil-water culture.

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A 2 cm layer of rice soil was added to each tray; 240 cm long, 60 cm wide and 10cm high and tap water was slowly added to fill up to 5 cm. These trays were inoculated with fresh *Azolla* and left outdoors, with periodic additions of concentrated superphosphate fertilizer. A bent glass tubing attached to one end of the tray was positioned in such a manner to provide drainage of water above the 6 cm level (Figure 1), in order to prevent *Azolla* spilling over during rainy weather. Material thus grown at Peradeniya was transported fresh to Ambalantota and gradually adapted to field conditions there. They were initially grown in small galvanized-iron trays in soil-water culture, under a partial shade and then transferred to 8m × 3m plots in the rice fields, and allowed to grow and adapt themselves for three months. Relatively heavy inocula, (1 to 2 kg fresh *Azolla* per m^2), were used at this stage, to ensure its proper establishment in the fields. The original 8m × 3m field plots were subdivided to smaller plots, using bamboo poles to keep the *Azolla* fronds together, because rapid fragmentation and separation of the fronds have been reported to retard the growth of *Azolla*.[1,3] As the *Azolla* carpet increased, the bamboo poles were moved gradually to increase the surface area of the plots (Figures 2 a, b, & c). The bamboo poles were placed in relation to the wind direction, the blowing of which would assist the *Azolla* cover to spread once a pole is removed. Initially, a large number of *Azolla* fronds turned red and many died, but certain fronds remained green and produced luxuriant growth, to cover the plots completely within three weeks.

These nursery cultures were maintained with periodic additions of triple superphosphate (TSP) powder to provide phosphorus and Carbofuran granules (3% a.i.) to prevent any insect attack. Material from these nurseries were used for the experimental evaluation of the growth and nitrogen fixation of *Azolla* in monoculture, under field conditions.

2.1 Growth and nitrogen fixation

This experiment was conducted in 1m × 5m plots with independent irrigation and drainage facilities. These plots had bunds 20 cm wide and 15 cm high, and the flood water level in them was maintained between 5 to 10 cm. There were four replicates for each *Azolla* strain tested and these were arranged in a randomized-complete-block design.

One week after preparation, the plots were inoculated at the rate of 180 g (fresh *Azolla*) per m^2 (i.e. 900 g per plot or 1.8 t/ha), using material from the field nurseries. Each inoculum was mixed with 6 g/kg (fresh *Azolla*) of TSP powder and 1 g/kg (fresh *Azolla*) of Carbofuran. TSP powder at the rate of 3 g/m^2 was broadcast over the *Azolla* cover, every three days and Carbofuran was applied at the rate of 0.5 g/m^2 at the initial sign of any pest attack. Every 3 days, the fresh weight of *Azolla* was measured separately in each plot. The few weeds that showed up were hand removed. Within 15 days, most of the plots were completely covered by *Azolla* and further biomass measurements were stopped at this stage. Nitrogen fixing activity of these 15-day old material was measured by the acetylene reduction assay.

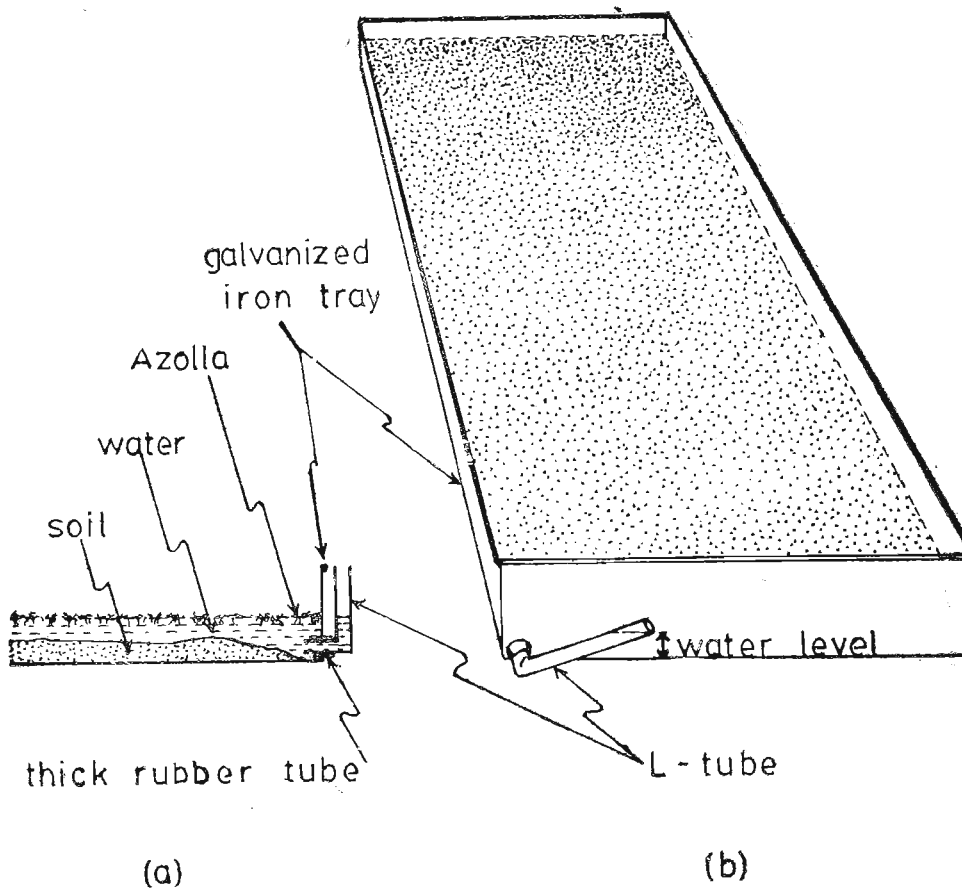

Figure 1. Outdoor, soil-water culture of *Azolla pinnata* in a galvanized-iron tray.

(*a*) Cross sectional view of the draining system.

(*b*) View of tray showing drain tube attachment.

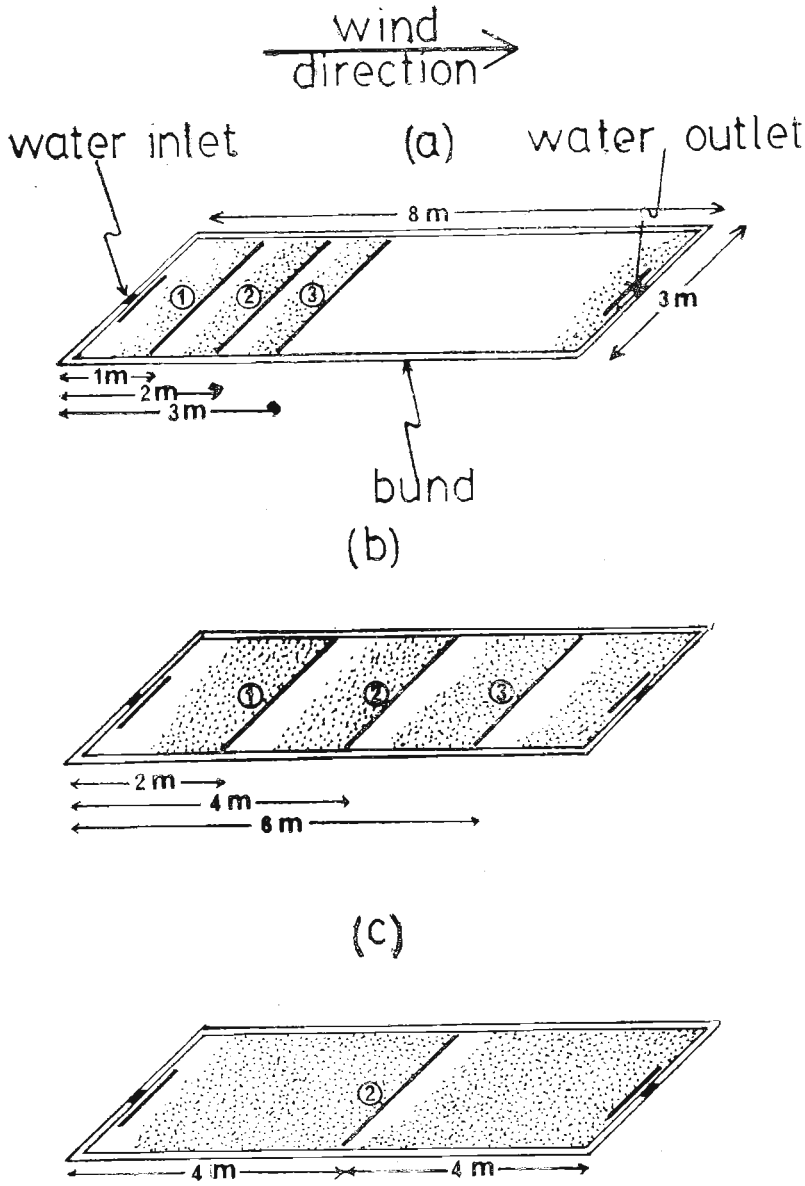

Figure 2. Nursery culture of *Azolla pinnata* under field conditions.

(*a*) 1st stage : Bamboo poles (1), (2) & (3) placed at 1m intervals.

(*b*) 2nd stage : Bamboo poles moved to double the area of each sub - plot.

(*c*) 3rd stage : Bamboo poles (1) & (3) removed as *Azolla* forms a complete cover.

2.2 Measurement of Acetylene Reducing Activity (ARA)

ARA measurements on field grown *Azolla* are frequently conducted in glass bottles, conical flasks and other transparent containers. Although such measurements are sometimes carried out in the field, it is not possible to incubate the samples in a floating position together with the natural *Azolla* cover. In the present study, a novel method of incubation was adopted, whereby it was possible to keep the samples floating *in situ* with the rest of the *Azolla* mat. In this case, transparent, plastic baby feeding bottles of approximately 300 ml were used as sample containers. Short pieces of flexible PVC tubing stoppered at one end with subaseal caps, were firmly fixed to the lids of the bottles, so as to permit the injection and withdrawal of gases. Each feeding bottle was filled upto the 100 ml mark with *Azolla* and water from the plot and this was allowed to float in a horizontal position with the rest of the *Azolla* in the field (Figure 3).

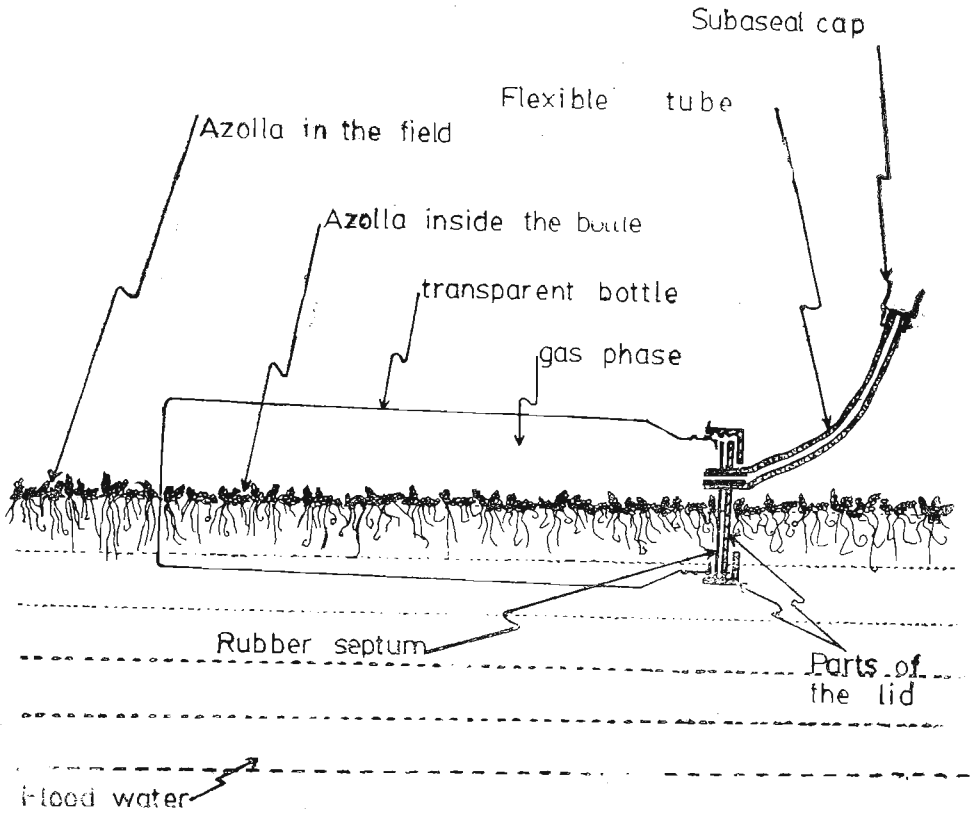

Figure 3. Use of a 'baby feeding ' bottle for the field incubation of *Azolla* with acetylene.

Using a 50 ml graduated syringe, a volume of air equivalent to 10% of the gas phase in the bottle was first withdrawn and an equal volume of acetylene was then injected. In this manner, the test *Azolla* samples were incubated under 10% acetylene in air. Eight bottles were randomly thrown into each plot, and the values given in Table 1 are the mean of eight such readings. A set of 'blank samples' containing *Azolla*-free paddy water was also included, to monitor any background nitrogenase activity. Incubation with acetylene was carried out for 20 minutes, under a light intensity of 90 klx and a temperature which fluctuated between 34°C to 37°C. At the end of the incubation period, 2 ml gas samples were withdrawn and stored in vacutainers for subsequent gas chromatographic analyses. The samples were analysed at 80°C, in a Perkin-Elmer Sigma-4 gas chromatograph, fitted with a 2m, 80/100 mesh column of Poropak T. Detection of ethylene and acetylene was done on a Hydrogen-Flame-Ionization detector, with nitrogen at a flow rate of 30 ml per minute serving as the carrier gas.

3. Results

The growth patterns of the four *Azolla* strains are shown in Figure 4. It can be seen from this figure that all the four *Azolla* strains have followed a similar pattern of growth; after an initial, short lag phase, they have multiplied quite rapidly until the 15th day, when further measurements were stopped, because limitation of space would have retarded the rate of growth in the plots that were completely covered by this time. It is clear from this curve that the biomass of *Azolla*, has increased 7 to 8 fold within 15 days. Although there is not much difference in the final biomasses produced, by the different strains, the strain from Debokkawa appears to have performed marginally above all the others. Nitrogenase activity (ARA) of the 4 *Azolla* strains of 15 day, are in Table 1. There are no significant differences in ARA among the four strains.

TABLE 1. Biomass and Acetylene Reducing Activity (ARA) of 15 day old monocultures of *Azolla pinnata* strains grown in 5m² field plots at Ambalantota, in the low - country dry zone of Sri Lanka. Growth conditions are the same as in Figure 4.

Azolla strain	Fresh weight of *Azolla* [a] (g/plot)	ARA [b] (μ mol C_2H_4 / g (f. wt. /h)	Rate of N_2-fixation [c] (kgN/ha/day)
Debokkawa	8000	2.59 ± 1.50	4.64
Bangkok	7892	2.44 ± 1.36	4.30
Indian	7600	1.82 ± 1.18	3.10
Peradeniya	7125	2.41 ± 1.36	3.84

(*a*) Mean value of four replicates.

(*b*) Mean value of eight samples incubated with 20% acetylene in air from 1330 to 1430 CST under 90 klx 34 to 37°C.

(*c*) $N_2 : C_2H_4 = 1 : 3$

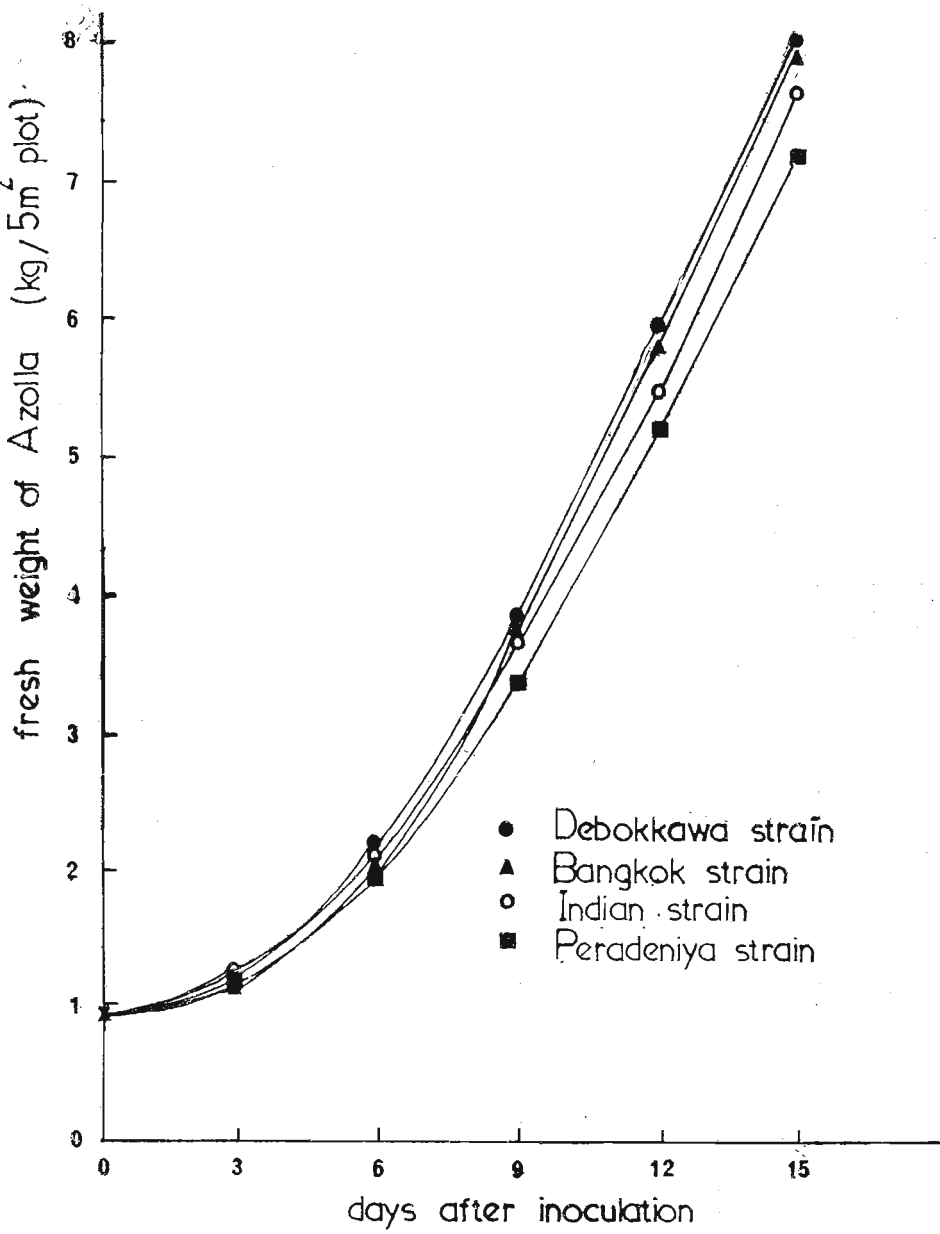

Figure 4. Growth patterns of the different strains of *Azolla pinnata* in 5 m² field plot at Ambalantota. Each plot was initially inoculated with 900g (f. wt.) of *Azolla* together with 6 g/kg (fresh *Azolla*) of Triple - Super Phosphate (TSP) and 1 g/kg (fresh *Azolla*) of Carbofuran (3% a. i.). Subsequently, TSP powder (3 g/m²) was broadcast over the *Azolla* cover every 3 days and Carbofuran (0.5 g/m²) was added at the initial sign of any pest attack. (Diurnal light intensity : 5 to 125 klx; daily temperature : 25° to 37°C).

4. Discussion

The results of these experiments clearly show that all the four strains of *A.pinnata* tested, are capable of rapid growth under field conditions at Ambalantota, where light intensity reaches values upto 125 klx and the temperature goes upto 37°C. These results confirm the previous reports[4,7] that certain strains of *Azolla* are capable of growing under tropical field conditions. ARA values (Table 1) around noon time ranged from 1.8 to 2.6 μ moles of ethylene per gramme fresh weight of *Azolla* per hour. These values when converted on a 1:3 nitrogen fixed: ethylene produced ratio and extrapolated in relation to their biomass, on a 12h/ 12h light/ dark cycle per day, give a range of nitrogen fixation equivalent to 3.1 to 4.6 kgN/ha/day. If this rate of activity is presumed to continue for 14 days, it would be equivalent to 43 to 64 kgN/ha. This extrapolation may appear to be an overestimate since it is based upon the presumption of a constant rate of fixation for 12 hours a day, for 14 days. Nevertheless, it is perhaps indicative of the potential maximum which may be available for exploitation under optimal conditions. Also the assumption that there is no fixation during the 12 h dark period is an under estimate, because it has been shown that 25 to 30% of the light activity may continue in the dark.[2] It is therefore reasonable to assume that an initial growth of *Azolla* for two weeks in a rice field has the potential to provide an input of 40 to 60 kgN/ha.

Although the rate of growth and nitrogen fixation among the four strains tested were not very different from one another, the strain from Debokkawa has performed best (Table 1). This may be a reflection of its original habitat which is only 20 km away from Ambalantota and falling within the same agroecological zone. From these results, it can be concluded that *A.pinnata* is suitable for use, even in the dry zones of Sri Lanka, provided that water supply is not a serious limitation.

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