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AN IMPROVIZED TECHNIQUE FOR MEASUREMENT OF NITROGEN FIXATION BY BLUE GREEN ALGAE AND *AZOLLA* USING MOIST SOIL CORES FROM RICE FIELDS

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SUMMARY

A modified technique for measurement of nitrogen fixation by blue green algae and *Azolla* sp. using intact, moist soil cores was developed for use in field studies in which chemical fertilizers, blue green algae and *Azolla* biofertilizers had been used in association with a rice crop. This method involves collection of fresh and moist soil cores (0–30 mm) using a soil auger, incubation with 10% acetylene in airtight glass vials under field conditions for three hours, and measurement of the ethylene produced using a gas chromatograph. Experiments carried out over a period of three consecutive years revealed that the estimates of nitrogen fixation were comparable with data of other researchers obtained through the use of soil water columns, sophisticated chambers and automated sampling devices. This simplified technique, therefore, can provide rapid and reliable measurements of nitrogen fixation in field experiments.

INTRODUCTION

Ecological studies of blue green algae (BGA) in submerged rice fields are often hampered by problems in methodology, primarily in estimating algal biomass (quantitatively and qualitatively) and in measuring biological nitrogen fixation by BGA and associations involving BGA. Nitrogen (N) is one of the key elements made available to the rice crop by BGA. This has been quantified using several methods (Watanabe *et al*., 1977). Accurate, reproducible measurements of the N-fixing potential of BGA as an estimate of N increment are extremely important in field studies, especially in rice cultivation (Roger and Ladha, 1992; Roger, 1996).

The significance of phototrophic N fixation in tropical rice fields was recognized by studies using acetylene reduction assay (ARA) and total N measurements (Rice and Paul, 1971). ARA is a sensitive tool to detect nitrogenase activity but its accuracy for quantification studies has been much debated (Lee and Watanabe, 1977; Roger, 1996). Watanabe and Cholitkul (1979) described various methods for field estimations of biologically fixed N. They observed that improvements and modifications in the methodology for ARA were needed for accurate measurements, especially with regard to the development of techniques for removing undisturbed soil samples for estimating

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N fixation in the surface soil. Most of the earlier techniques used tedious procedures for the collection of soil water columns or the placement of chambers and sampling devices in the field. None allowed for the collection of undisturbed soil samples. In this investigation, an attempt was made to evaluate the N-fixing potential of BGA and *Azolla* (a fern harbouring BGA in its cavities) in fresh, intact moist soil cores obtained from rice fields by using the acetylene reduction assay. The utility and reliability of this technique in field assessments of N fixation was demonstrated over a period of three consecutive years.

MATERIALS AND METHODS

A soil-based BGA biofertilizer (containing a mixture of *Anabaena variabilis*, *Nostoc muscorum*, *Tolypothrix tenuis*, *Aulosira fertilissima*, *Plectonema* and *Phormidium* strains) was prepared in a polyhouse following the usual protocols and inoculated at the rate of 15 kg ha^{-1} (Venkataraman, 1981; Prasanna and Kaushik, 1998). *Azolla microphylla,* also grown in a polyhouse, was obtained from the *Azolla* germplasm at NCCUBGA, and used at the rate of 1 t ha^{-1} (Singh, 1979).

The experiment consisted of treatments involving urea, applied at rates of 30, 60, 90 and 120 kg N ha^{-1}, alone and in combination with soil-based BGA biofertilizer and/or *Azolla microphylla*.

All the treatments were established in a randomized blocks design, with three replicates. Each plot measured 40 m^2, and the rice crop (a medium-duration variety PNR 381) was grown from July to October in 1999, 2000 and 2001. Urea was broadcast uniformly in split doses at the time of transplanting (50%), and at 30 (25%) and 60 days (25%) after transplanting. The biofertilizers were applied 7–10 d after transplanting by broadcasting over standing water. After transplanting, irrigation was applied daily for 90 d.

Soil core sampling

Fresh soil cores were collected from the field site using a steel coring device (20 mm diameter). The surface soil (0–30 mm) was removed from all intact soil cores held inside the coring device and placed in glass vials (55 × 150 × 20 mm). Similar vials with 20 ml of water served as the controls. The vials were sealed with airtight rubber stoppers.

Preliminary experiments were undertaken to optimize the number and depth (0–30, 0–40, 0–50 mm) of soil cores to be taken and the time of sampling (early morning, afternoon or evening). The 0–30 mm soil cores taken in the early morning were selected as the best because of the ease of sampling, maximum sensitivity and the similarity of values between replicates from each plot and treatment.

Gas chromatographic analysis

The vials containing soil cores or water (control) were injected with 3.5 ml of acetylene (10% gas phase) after removing an equal amount of air by means of a disposable plastic syringe. The samples were incubated under field conditions for three hours. The time of incubation had been chosen as optimum after analysing

Table 1. Mean values for nitrogen fixation, measured as acetylene reducing activity (ARA, 10^4 η mole C_2H_4 g^{-1} soil h^{-1}) in soil cores taken 50 and 100 d after transplanting (DAT) from BGA and/or *Azolla*-and urea-treated rice fields over a period of three years (1999–2001).

	ARA	
Treatments	50 DAT	100 DAT
N_{30}	0.173 ± 0.017	0.021 ± 0.002
N_{30} + BGA	0.320 ± 0.025	0.023 ± 0.002
N_{30} + *Azolla*	0.530 ± 0.043	0.410 ± 0.084
N_{30} + BGA + *Azolla*	1.023 ± 0.153	0.058 ± 0.005
N_{60}	0.213 ± 0.017	0.019 ± 0.002
N_{60} + BGA	0.250 ± 0.010	0.046 ± 0.010
N_{60} + *Azolla*	1.663 ± 0.135	0.234 ± 0.024
N_{60} + BGA + *Azolla*	1.230 ± 0.020	0.210 ± 0.005
N_{90}	0.180 ± 0.010	0.063 ± 0.004
N_{90} + BGA	0.383 ± 0.050	0.029 ± 0.001
N_{90} + *Azolla*	1.143 ± 0.089	0.310 ± 0.090
N_{90} + BGA + *Azolla*	1.013 ± 0.048	0.390 ± 0.090
N_{120}	0.170 ± 0.010	0.046 ± 0.003
N_{120} + BGA	0.120 ± 0.006	0.029 ± 0.003
N_{120} + *Azolla*	1.393 ± 0.044	0.095 ± 0.007
N_{120} + BGA + *Azolla*	0.253 ± 0.035	0.031 ± 0.003

samples at intervals of 0.5 h following 1.5 h of incubation over a period of six hours. A gas sample of 1.00 ml was withdrawn from each incubated vial and injected into a Nucon Gas chromatograph fitted with an oven containing a 2 m column of stainless-steel tubing (2 mm internal diameter) packed with Porapak N (80–100 mesh). Nitrogen gas flowing at the rate of 35 ml min^{-1} was used as the carrier, while hydrogen and air were used to produce the flame for the Flame Ionization Detector. The oven, injector and detector were maintained at 100–120 °C to allow for ionization and detection of ethylene produced. The samples were taken in triplicate at the middle of the crop-growth period (45–50 d after transplanting) and before harvest (90–100 d) from each plot. After measuring the acetylene reduction, the soil cores were air-dried in the shade and the dry weights recorded. The ARA values were expressed per unit dry weight of soil.

For estimating the amount of N fixed it was assumed that one hectare of soil (top 0–30 mm) contained 2.2×10^6 g of air-dried soil (Alimagno and Yoshida, 1977; Carter, 1993).

Statistical analysis

The data were subjected to statistical analysis using the MSTAT-C package for calculation of standard error of the mean of the sample/treatment.

RESULTS

The ARA values of the soil cores decreased with increase in the rate of urea application (Table 1). Application of nitrogenous fertilizers at the rate of 60 kg N ha^{-1} gave

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maximal nitrogenase activity in the control and *Azolla*-treated plots at 50 d after transplanting. The ARA of plots inoculated with BGA biofertilizer did not show a consistent trend. For example, at 50 d after transplanting there was a sharp drop at 60 kg N ha^{-1} and an increase at 90 kg N ha^{-1}. There was a decrease of almost 50% in the ARA of soil cores taken from plots treated with BGA biofertilizers in combination with 120 kg N ha^{-1}. Sampling near harvest time, however, showed an obvious decreasing trend in ARA at N levels in excess of 60 kg ha^{-1} (Table 1). The BGA biofertilizer also performed better than did the control, but the ARA values were significantly below those of the *Azolla*- or BGA + *Azolla*-treated plots.

The ARA of *Azolla* did not decrease with increasing application rates of nitrogenous fertilizer, and a maximum value of 1.66×10^4 ηmoles C_2H_4 g^{-1} soil h^{-1} was obtained in plots that received 60 kg N ha^{-1}. The combination of BGA and *Azolla* showed a drastic decrease at 120 kg N ha^{-1} compared with the lower levels of N application.

DISCUSSION

Nitrogen-fixing cyanobacteria are known to be abundant in rice fields where they are responsible for the inherent fertility of these ecosystems. Although a number of investigations have been undertaken to evaluate the N-fixation rates (Watanabe *et al*., 1977; Venkataraman, 1981), very few reliable data are available and there is a definite need for *in situ* measurements of nitrogenase activity (Watanabe and Cholitkul, 1979).

The present investigation was undertaken to evaluate the N-fixing potential of BGA and *Azolla* in moist soil cores taken from a field experiment involving different doses of urea alone or in combination with BGA biofertilizer and *Azolla*. It was observed that, in general, increasing the level of urea application decreased nitrogenase activity, and *Azolla*- or *Azolla* + BGA-treated plots exhibited significantly higher ARA values compared with the control or BGA-treated plots.

Earlier studies (Dommergues and Rinaudo, 1979) indicated that BGA might become dominant anytime during the cultivation cycle, exhibiting one or several peaks of ARA. Lee *et al*. (1977) found that from 4–6 weeks after transplanting ARA in the rhizosphere of the rice cv. IR 26 increased to reach a maximum value at the heading stage, followed by a rapid decrease. This is similar to the present results. Also, with BGA the synthesis and activity of nitrogenase is normally inhibited at high concentrations of NH_4^+-N (>300 mg/l), especially in paddy fields where the organic matter content is extremely high and nitrogenous fertilizers are applied in large doses (Rowell *et al*., 1977). In the present investigation, however, a gradual increase in ARA was observed at N application rates up to 90 kg ha^{-1}.

The symbiotic association of *Azolla-Anabaena* spp. has been shown to retain significant nitrogenase activity when grown with nitrate or urea (Peters and Mayne, 1974), although contrary reports on decreasing biomass and ARA in *Azolla* spp. with increasing levels of urea also exist (Manna and Singh, 1990). In those experiments, *Azolla pinnata* and *A. caroliniana* were used while *A. microphylla* was used in the present study.

Azolla has been reported to perform better with short- and medium-duration rice varieties (such as PNR 381) than with long-duration varieties, and its incorporation into the soil has been shown to be more beneficial to the rice crop than growing it in association with the rice crop (Singh, 1979). In the experiment reported here, the plots inoculated with *Azolla* or *Azolla* + BGA together with applications of 30, 60 and 90 kg N ha^{-1} (as urea) exhibited high ARA at 100 d after transplanting. This may be a result of the slower rate of mineralization of *Azolla* (Singh, 1989) when compared with the quicker decomposition/mineralization of BGA/urea.

Over three years, the incubation of fresh, moist soil cores with BGA, *Azolla* or both on the surface under field conditions, provided a uniform, reproducible value for each particular treatment combination. The estimated maximum amount of N fixed (at 50 d after transplanting) by BGA, *Azolla* or BGA + *Azolla* ranged between 6.1 and 54.1 kg ha^{-1} and exceeded the 4.8–5.6 kg ha^{-1} in plots that received urea alone, for one cropping season. Alimagno and Yoshida (1977) obtained a similar trend when they evaluated the rates of N fixation in fields treated with 40–50 kg N ha^{-1} in the form of urea over the entire rice-cropping season. Values ranged from 2.3–5.7 kg N ha^{-1}, compared with 18.5–33.3 kg ha^{-1} in fields without chemical fertilizer application. In their review of field studies on N fixation in paddy soils, Watanabe and Cholitkul (1979) concluded that N-fixation rates associated with algae in wetland rice, where no chemical nitrogenous fertilizers had been used, may not exceed 10 kg ha^{-1}. In this investigation, fertilizer N was applied at rates up to 120 kg ha^{-1} with or without BGA and/or *Azolla*, and a wide range of N-fixation rates comparable with earlier estimates were obtained. The technically improved methodology developed in this study has immense potential in that it is relatively simple and needs no sophisticated equipment. The earlier techniques involving soil-water-column sampling and the use of portable chambers and automated samplers (Lee and Watanabe, 1977; Watanabe *et al*. 1977; Roger and Ladha, 1992) are tedious and time consuming. This improvised novel technique is able to provide reliable estimates comparable to earlier data and can be valuable, therefore, for the rapid analysis of N fixation in soil cores. Also, it can be employed effectively for dependable estimates of quantitative variations existing in rice field experiments involving BGA and/or *Azolla* as biofertilizer inputs.

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