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Nitrogen fixation (C_2H_2 reduction) in soil samples from rhizosphere of rice grown under alternate flooded and nonflooded conditions

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Summary In a greenhouse study the influence of alternate flooded and nonflooded conditions on the N_2-ase activity of rice rhizosphere soil was investigated by C_2H_2 reduction assay. The soil fraction attached to roots represent the rhizosphere soil. Soil submergence always accelerated N_2-ase and this effect was more pronounced in planted system. Moreover, rice plant exhibited phase-dependent N_2-ase with a maximum activity at 60 days after transplanting. The alternate flooded and nonflooded regimes resulted in alterations of the N_2-ase activity. Thus, the N_2-ase activity increased following a shift from nonflooded to flooded conditions, but the activity decreased when the flooded soil was returned to nonflooded condition by draining. However, the differential influence of the water regime on N_2-ase was not marked in prolonged flooded-nonflooded cycles. Microbial analysis indicated the stimulation of different groups of free-living and associative N_2-fixing microorganisms depending on the water regime.

Introduction

Nitrogen supply to the flooded rice soils through agents other than mineral fertilizers has long been recognized. Blue green algae, photosynthetic, free-living and associative heterotrophic bacteria have been implicated in contributing the major input of biological N to rice soils[2,13,24]. Heterotrophic N_2 fixation by bacteria in soil[2,6,17,25] and in association with rice roots[3,4,14,16,25] is of significance by virtue of high moisture content in the soil and nutrient availability in the vicinity of the roots. It has been clearly demonstrated that soil submergence has accelerated N_2 fixation[17,25] which was further enhanced by organic matter application[6,7].

In rainfed tropics rice fields are subjected to intermittent dry and wet conditions[8]. Alternate dry and wetland cropping systems have increased the availability of soil N to the higher plant[8,9]. Such alterations in the water regimes might influence soil microbial processes of importance to the soil fertility. Nayak and Rao[17] reported that soil N_2-ase was affected by alternate flooded and nonflooded conditions. Moreover, a differential influence of $(NH_4)_2SO_4$ and organic matter on soil N_2 fixation was noticed under continuous flooded and nonflooded conditions[6]. Under situations of alternating flooded and nonflooded conditions the decomposition of organic matter and mineralization of soil N would differ from that of a continuously flooded or nonflooded system. The present paper describes a greenhouse study of the N_2 fixation (C_2H_2 reduction) potential as influenced by the alternate flooded and nonflooded water regimes in planted and unplanted conditions.

Materials and methods

Greenhouse studies

An alluvial soil (pH 6.6, organic matter 1.8%, total N 0.02%, electrical conductivity 0.2 mmhos/cm, C.E.C. 18.6 meq/100 g) collected from the Institute field was filled (5 kg/pot) in porcelain pots with a lateral drainage hole padded with glasswool. The hole was plugged with rubber cork. Two series (planted and unplanted) of six sets of pots were arranged with three replicates for each water regime. The treatments included (a) a continuous flooded system, (b) a continuous nonflooded system, (c) a 15-day alternating flooded-nonflooded-flooded cycle, (d) alternating nonflooded-flooded-nonflooded cycle, (e) a 30-day alternating flooded-nonflooded-flooded cycle and (f) alternating nonflooded-flooded-nonflooded cycle.

Water regime

Water was added up to a column of 5 cm above the soil to provide submerged conditions. To achieve the nonflooded (60% W.H.C.) conditions in a submerged soil during the alternating cycles, the water was allowed to drain completely (by removing the cork) and after 4–5 days required amount of water was added to provide 60% W.H.C. (20% of moisture on a weight basis). It took 5 days for the drained soil to reach the moisture level (20%) of the normal nonflooded system. The level was maintained by periodical moisture determination and water was added when required to compensate for the evaporation loss. Water was changed at an interval of 15 and 30 days depending upon the treatment and the C_2H_2 reduction assay of the soil was conducted at least once during the cycle.

C_2H_2 reduction

The rhizosphere and non-rhizosphere (unplanted) soils were collected periodically from the pots. Rhizosphere represents the soil fraction attached to the root. The N_2-ase activity (C_2H_2) reduction was analyzed in 2 g (fresh weight) soil samples in six replicates for each treatment. The incubation of the soil samples and the nitrogenase analysis were carried out as described earlier[7,15,17].

The samples were placed in B-D vacutainer (Becton-Dikinson, New Jersey) tubes (75 × 13 mm) stoppered, and the gas phase was replaced with high purity C_2H_2 (10% by volume) through a gas tight hypodermic syringe. The tubes were then incubated at 28°C for 24 h in the dark. At the end of the incubation a 0.5 ml sample of the gas phase from each tube was analyzed for C_2H_4 production on a GC fitted with a hydrogen flame ionization detector and a 1500 × 3 mm column filled with 100–120 mesh Porapak-R at a column temperature of 60°C. High purity N_2 at a flow rate of 30 ml/min served as the carrier gas. The N_2-ase activity was expressed as n moles of C_2H_4 formed/g dry soil/day. Tubes without C_2H_2 did not evolve endogenous C_2H_4 and the C_2H_2 reduction in the drained water was negligible. Organic C was estimated by modified Walkley and Black method.

Measurements of pH and redox potential (Eh)

The changes in pH and Eh were estimated thrice during the crop growth at 40, 65 and 85 days after transplanting. The redox potential was measured with a portable redox meter model RM-IF (TOA Electronics Ltd., Tokyo, Japan) fitted with a compound platinum and calomel electrode. Before measurement, a portion of the top oxidized layer (in flooded conditions) was scooped out carefully with minimum disturbance and the compound electrode was placed into the reduced zone. The potentials were measured after 2 to 3 min although the stabilization of the electrode was achieved within 10 to 20 sec. The electrode was checked against a standard of 0.0033 *M* potassium ferricyanide and 0.0033 *M* potassium ferrocyanide in 0.1 *M* KCl. The potentials are given in the table in millivolts based on the standard hydrogen electrode by addition of + 245 mV to redox readings. After measurement of potentials, pH values of the soil were also determined.

Enumeration of N_2-fixing bacteria

Determination of the numbers of aerobic (Azotobacter), associative (Azospirillum), facultative anaerobic (Bacillus) and anaerobic (Clostridium) heterotrophic N_2-fixing microorganisms was performed in freshly collected rhizosphere and nonrhizosphere samples from different treatments on 15 and 85 days after transplanting. Serial decimal dilutions of the soil samples were made and 1 ml amounts were transferred into N-free liquid media for MPN counts of Azospirillum and anaerobic N_2 fixers, and Azotobacter was counted on agar plates. Azospirillum was counted following the method suggested by Okon *et al.*[18] and population of anaerobic N_2 fixers and Azotobacter as per Rao *et al.*[20]. Results presented are the means of five replicates for Azospirillum and anaerobic N_2 fixers and three replicates for Azotobacter populations.

Results and discussion

Appreciable C_2H_2 reduction occurred under both flooded and nonflooded conditions, with higher activity under flooded conditions almost throughout the growing period of the plant (Table 1). Soil submergence accelerated N_2 fixation[6,25]. The activity was highest during 55–65 days after transplanting indicating the phase-dependent N_2-ase activity. This coincides with maximum tillering-panicle initiation stage of the plant growth. The rhizosphere effect also varied with the growth stage of rice plant with reference to the denitrifying activity[11]. Garcia[10] attributed the positive rhizosphere effect due to the development of anaerobic zones, and presence of root exudates in planted system. Perhaps these conditions in planted system favourably influenced the N_2-ase activity. There is considerable evidence that rice plant influences the rhizosphere soil N_2-ase activity[2,4,12,25] and the N_2-fixing potential was measured with an exogenous supply of carbon[1,5,15,17,22]. The low N_2-ase activity of the nonflooded soils might be due to higher oxygen tension, which is known to inhibit N_2 fixation[5]. There was a gradual decrease in the N_2-ase beyond 75 days both in planted and unplanted systems.

Water management is one of the key factors involved in the productivity of crops, particularly for rice. The alternate flooding and drying cycles, common in rainfed cropping system in tropics[8], might exert influence on soil microbial processes of importance to soil fertility. In addition to continuous flooded and nonflooded conditions two different alternate flooded and nonflooded cycles of 15 and 30 day

Table 1. Influence of alternate flooded and nonflooded cycles on N_2-ase activity of the rhizosphere soil

	n moles of C_2H_4 formed/g soil/day							
	Days after transplanting							
Treatment	8	15	26	35	55	65	75	92
Flooded (continuous)	19^a	152^a	143^a	255^a	334^a	439^a	284^a	149^a
Nonflooded (continuous)	12^b	73^b	64^b	182^b	298^b	233^b	199^b	99^b
Alternate cycles (15 days)								
F + NF + F + NF	28^a	137^a	53^b	255^a	158^b	279^a	316^a	224^a
NF + F + NF + F	11^b	59^b	105^a	251^b	348^a	360^b	204^b	188^b
Alternate cycles (30 days)								
F + NF + F + NF	22^a	126^a	147^a	247^b	237^b	409^a	190^a	124^b
NF + F + NF + F	11^b	79^b	80^b	256^a	238^a	424^b	242^b	186^a
L.S.D. 5%	4	25	18	36	52	68	46	44
1%	5	34	25	49	72	94	64	61

Means of six observations for each treatment.
F = Flooded, NF = Nonflooded.
^{a} = Flooded conditions at the time of assay.
^{b} = Nonflooded conditions at the time of assay.

Table 2. Influence of alternate flooded and nonflooded cycles on N_2-ase activity of the unplanted soil

	n moles of C_2H_4 formed/g soil/day							
	Days of sampling							
Treatment	8	15	26	35	55	65	75	92
Flooded (continuous)	13^a	134^a	130^a	231^a	323^a	315^a	249^a	133^a
Nonflooded (continuous)	10^b	37^b	61^b	183^b	168^b	79^b	187^b	61^b
Alternate cycles (15 days)								
F + NF + F + NF	24^a	131^a	53^b	161^a	285^b	230^a	429^a	346^a
NF + F + NF + F	14^b	28^b	48^a	164^b	165^a	276^b	175^b	244^b
Alternate cycles (30 days)								
F + NF + F + NF	23^a	107^a	164^a	178^b	210^b	181^a	155^a	178^b
NF + F + NF + F	14^b	33^b	40^b	150^a	237^b	336^b	227^b	253^a
L.S.D. 5%	5	23	19	30	46	33	42	31
1%	6	31	26	41	64	45	58	43

Means of six observations for each treatment.
F = Flooded. NF = Nonflooded.
^{a} = Flooded conditions at the time of assay.
^{b} = Nonflooded conditions at the time of assay.

duration were included in the study. The alternate drained and flooded conditions exhibited profound influence on the C_2H_2 reduction activity. In the nonflooded-flooded cycle the N_2-ase activity of the nonflooded soil increased significantly upon submergence (Table 1). Conversely, the N_2-ase activity of the flooded soil decreased when the flooded soil was returned to nonflooded conditions. In flooded (15 d) + nonflooded (15 d) + flooded (15 d) cycle, N_2-ase activity in the soil decreased with a shift from flooded (15 d) to nonflooded (15 d) condition and then increased considerably upon subsequent flooding for 15 days (Table 1). Conversely, in a nonflooded (15 d) + flooded (15 d) + nonflooded (15 d) cycle, N_2-ase activity increased with a shift from a nonflooded (15 d) to a flooded (15 d) condition. However, subsequent alternate regimes did not significantly influence the N_2-ase activity. Changes in water regimes in 30 day cycles yielded similar effects on N_2-ase activity; however, after prolonged flooding (30 d) subsequent alteration to nonflooded (30 d) condition did not drastically affect the N_2-ase. Similar trends in the N_2-ase activity were observed in the unplanted system (Table 2). Unlike in planted system the nitrogenase activity was low in soil samples subjected to alternate flooded-nonflooded cycles. N_2-ase activity was negligible in flood and drain water.

The observed differences in the N_2-ase activity could be attributed to the changes in the rate of decomposition of native organic matter occurring concurrently with the alteration in water regimes. The aeration status of the soil has a marked effect on the organic matter decomposition and the rate of decomposition was reported to be faster in treatments with greater number of alternate aerobic and anaerobic periods[21]. Further, the rate and products of organic matter decomposition are different in flooded and nonflooded soils[1,23]. We found that the total organic carbon content in samples was little affected by the water regimes. Thus, the rate and products of organic matter decomposition among the treatments might have led to the alterations in the potential N_2-fixing activities.

In addition to the changes in the organic matter decomposition, severe N loss has been reported to occur in soils subjected to a period of alternate drained and flooded conditions[19]. Even, changes in the oxygen concentration during the alternating water regimes may influence the N_2-ase activity. Nayak and Rao[17] attributed the changes in the N_2-ase activity in alternate flooded-nonflooded systems to both the rate of decomposition of organic matter and N stress.

Changes in pH and redox potential (Eh) indicated that planted soils had a lower Eh compared to that of unplanted system despite the

Table 3. Changes in Eh and pH in the soil subjected to alternate flooded and nonflooded conditions

	Days after transplanting					
	40		65		85	
Treatment	Eh	pH	Eh	pH	Eh	pH
Planted						
15 day cycle						
F + NF + F + NF	– 26	6.4	+ 50	7.4	+ 123	6.3
NF + F + NF + F	– 20	6.3	+ 53	7.7	+ 143	7.0
30 day cycle						
F + NF + F + NF	– 20	6.3	+ 30	7.1	+ 130	7.0
NF + F + NF + F	– 20	7.1	+ 36	7.1	+ 130	6.9
Unplanted						
15 day cycle						
F + NF + F + NF	+ 27	7.1	+ 56	7.1	+ 126	7.0
NF + F + NF + F	+ 20	6.4	+ 30	7.1	+ 133	6.9
30 day cycle						
F + NF + F + NF	+ 23	7.5	+ 30	6.8	+ 130	6.6
NF + F + NF + F	+ 20	7.4	+ 40	7.0	+ 143	6.8
Planted						
Flooded (continuous)	– 26	7.0	+ 40	7.1	+ 133	6.9
Nonflooded (continuous)	– 26	6.8	+ 30	6.8	+ 140	6.5
Unplanted						
Flooded (continuous)	+ 20	7.1	+ 40	7.1	+ 140	6.9
Nonflooded (continuous)	+ 20	6.3	+ 40	6.8	+ 143	6.5

variation in water regimes up to 40 days. However, the differences were not marked during the subsequent samplings on 65 and 85 days (Table 3). The pH was consistently lower in continuous nonflooded than in continuous flooded system and there was no effect of plants on pH. The alternate water regimes also had no effect on the soil pH.

Microbiological analysis showed that the effects of water regimes on the population of N_2 fixing microorganisms were related to the flooded-nonflooded cycles and different groups of N_2 fixers (Table 4). In general planted soils exhibited higher numbers of Azospirillum and Azotobacter after 85 days under both flooded and nonflooded conditions. The population of anaerobic N_2 fixers and Azotobacter, in particular, was stimulated as a result of alternate flooded-nonflooded (15 d) cycles while Azospirillum was not affected. These results

Table 4. N_2 fixing microorganisms as influenced by alternate flooded and nonflooded conditions in paddy soil

Treatment	Population of N_2 fixers/g dry soil					
	Azospirillum (× 10^6)		Anaerobic (× 10^5)		Azotobacter (× 10^4)	
	After 15 days	After 85 days	After 15 days	After 85 days	After 15 days	After 85 days
Planted						
Flooded (continuous)	1.2	2.1	0.24	1.7	5.3	0.5
Nonflooded (continuous)	2.2	2.8	0.01	1.2	2.8	3.4
Flooded (15 day cycle)	N.D.	2.4	N.D.	3.4	N.D.	12.0
Nonflooded (15 day cycle)	N.D.	2.0	N.D.	3.6	N.D.	43.2
Unplanted						
Flooded (continuous)	0.46	2.1	0.20	2.4	0.73	0.3
Nonflooded (continuous)	1.4	1.4	0.20	2.4	4.1	2.2
Flooded (15 day cycle)	N.D.	2.4	N.D.	3.0	N.D.	17.5
Nonflooded (15 day cycle)	N.D.	3.4	N.D.	3.7	N.D.	26.2

N.D. = Not determined

demonstrate differential effects of water regimes on specific groups of N_2 fixers which perhaps would partly account for differences in N_2 fixation in paddy soils subjected to alternate flooded and nonflooded cycles. Although these studies demonstrate the differential response of water regime on the soil N_2-ase and N_2 fixing populations more information is needed on the rate and products of organic matter decomposition and N mineralization in situations of alternating water regimes.

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