GROWTH OF RICE VARIETY BW 267-3 AS AFFECTED BY DIAZOTROPHS, INOCULATED UNDER DIFFERENT CONDITIONS

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ABSTRACT

Inoculation with diazotrophs was tested to supplement at least a part of the N requirements of a rice crop. Rice variety BW 267-3 was inoculated repeatedly with high densities of *Azospirillum irakense* KBC1 in un-autoclaved and autoclaved soils in a pot experiment. The rhizosphere population of the inoculated bacterial strain reduced drastically with time showing that its competitive ability was poor. Competition from native microorganisms for various factors including energy sources apparently affects this process. However, under *in vitro* conditions the diazotrophs supported plant growth probably via contribution of fixed nitrogen. Effect of malate was different on different rice-bacterial combinations in the presence of N.

Key words

Rice, *Azospirillum*, Inoculation, Competition, C substrate

INTRODUCTION

Rice, the world's most important cereal crop for human consumption, is the staple food of more than 3 billion people (Cantrell and Reeves, 2002). By 2025 rice yield should be increased by at least 60% to feed the increasing population (Dobermann and White, 1999). Increased cereal production resulting in the 'green revolution' was mainly due to genotypes highly responsive to chemical fertilizers, particularly N (Boddey *et al.*, 1995). Replenishment of soil N most commonly involves extensive application of chemical fertilizers (Peoples *et al.*, 1995), which is associated with economical and environmental problems. A better way to supply at least a part of the N requirements of rice plants is associative biological dinitrogen fixation (BDF). A research programme formulated during an IRRI Think-Tank workshop in 1992 was confirmed and refined in 1996 (Ladha *et al.*, 1997). The long-term objective of this programme was to enable rice plants to fix their own N. Study of non-nodular associations to improve the association between rice and dinitrogen fixing soil bacteria was one of the short term approaches of this endeavour.

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The *Azospirillum*-rhizosphere association for cereals and grasses was considered the principal alternative to the legume-*Rhizobium* symbiosis (Dobereiner, 1992). Agronomically significant quantities of N can be derived from associative BDF in wetland rice (Boddey and Dobereiner, 1994). However, the effect of bacterial inoculation has not been always positive, rather different investigators have found (1) no effect (2) inconsistent effects among successive trials with time or various simultaneous treatments and (3) negative effects (Roger *et al.*, 1993). Vande Broek *et al.*, (1993) reported that establishment of *Azospirillum* on growing plant roots is well recognized as a critical step towards an effective plant growth promotion. Presence of a large number of indigenous microorganisms may prevent the root colonization by *Azospirillum* cells (Boddey and Dobereiner, 1994). In the rhizosphere, supply of C substrates to the bacteria and fixed N to the plant is likely to be inefficient and one of the reasons for this is competition from other rhizosphere microorganisms (Boddey and Dobereiner, 1995).

The present study focused on testing whether the introduction of a diazotroph in high numbers by repeated inoculation under a low competitive environment could strengthen the rhizospheric association of a selected rice-diazotroph combination and whether a pure C substrate as an energy source under controlled conditions could improve the contribution of the bacterium to the host.

MATERIALS AND METHODS

The rice variety BW 267-3 used was a moderate fertiliser responsive, 3.5 month maturity type, with a maximum yield potential of $8x10^3$ kg ha^{-1}. Three bacterial strains: *Azospirillum irakense* KBC1, a rice root isolate from Iraq (Khammas *et al.*, 1989); *A. irakense* KBC1 (P 0.2), IAA excreting mutant of the above strain and *Alcaligenes faecalis*, a rice root isolate from China were used in these studies.

Pot experiment

Paddy soil (reddish brown latosol) collected from a field in the mid-country wet zone of Sri Lanka, was dried, crushed and passed through a 2 mm mesh and samples were removed and subjected to chemical analysis (pH-H_2O: 6.33, total N: 0.073% and organic C: 0.776%). Two sets of pots were filled with 3.5 kg soil pot^{-1}, of which one set was sterilized by autoclaving. The soil was submerged for 2 weeks. A basal dressing of 25 and 20 kg ha^{-1} (Department of Agriculture recommendations) of P_2O_5 and K_2O, respectively were incorporated into the soil the day before transplanting. Nitrogen was not given since it masks the effect of inoculation (Omar *et al.*, 1989). Plants of similar growth raised in an aseptic nursery were transplanted to pots (plastic buckets), at 2 hills per pot. Pots were placed in a 30 cm high tank with water up to soil level of the pots to minimise temperature fluctuations. The pots were kept 30 cm apart from each other in order to avoid cross contamination. The following treatments were given: no inoculation (T0); 50ml pot^{-1} of 1.2×10^9 CFU ml^{-1} bacterial suspension sprayed to the flood water 2 weeks after transplanting

(WAT) (T1); 50ml pot^{-1} of 8×10^8 CFU ml^{-1} bacterial suspension mixed with the flood water and the top soil was puddled (10-12 cm depth) the day before transplanting (T2) and a combination of both above T1 and T2, (T3) were employed. The plantlets of T2 and T3 treatments were also inoculated by dipping their roots in a 8×10^8 CFU ml^{-1} broth for 3 h just before transplanting. All uninoculated controls were given autoclaved broth to provide the same amount of nutrients as in the inoculum broth.

Cut plant acetylene reduction activity (ARA) of washed roots, plant dry weight and total N (%) of plant and soil were monitored on samples harvested at 30 and 80 days after transplanting (DAT). Most probable number (MPN) count of a randomly selected sample for each treatment of macerated root and rhizosphere soil was determined.

In vitro inoculation experiment

A spermosphere (Thomas-Bauzon *et al.*, 1982) with some modifications was used. A test tube of size 200 x 23 mm filled to 1/3 with neutral gravel (22 g, 1-2 mm size), cotton plugged and covered with aluminium foil, was sterilised. Surface disinfected pre-germinated seeds (one seed $tube^{-1}$) were transferred to these tubes under aseptic conditions. The seed was placed at subsurface level in the gravel in order to avoid it floating on the nutrient solution and rising up with root growth. Seven millilitres of sterile MPCL medium (Lavigne, 1987) with 1 ml inoculum broth (autoclaved broth in the case of non-inoculated controls) were added carefully to submerge the gravel. The MPCL medium was modified to suit the treatments as with 0.07% w/v of malate as a source of carbon for the bacteria, or without malate. The inoculum density used was 10^7 CFU ml^{-1} and 6 replicates were maintained for each treatment. Each tube was covered with aluminium foil upto the gravel level to prevent light reaching it. This set up was placed in a water circulating system, with water up to the gravel level of tubes to minimise temperature fluctuation, in a growth chamber (ambient temperature 26° -36°C). Sterile tap water was added periodically under aseptic conditions, to the spermospheres to keep the gravel submerged. The ARA of intact plant and plant dry weight were measured after 30 days of growth. Survival of the bacteria was tested by spreading a drop of plant nutrient solution at sampling, on to N-free malate plates.

Aseptic plant nursery for pot experiment

Incubation jars of 1200 ml capacity with an aperture on the lid were filled upto 3-4 cm level with neutral gravel. The MPCL medium was added to its field capacity. The lid was closed, the aperture was cotton plugged in order to allow aeration, and autoclaved. Surface disinfected, pre-germinated (on nutrient agar plates) and uncontaminated seeds (15-20 jar^{-1}) were transferred aseptically to these jars (Fig. 1) and placed in a water circulating system to minimize temperature fluctuations. These were allowed to grow for 2 weeks before transplanting to pots.

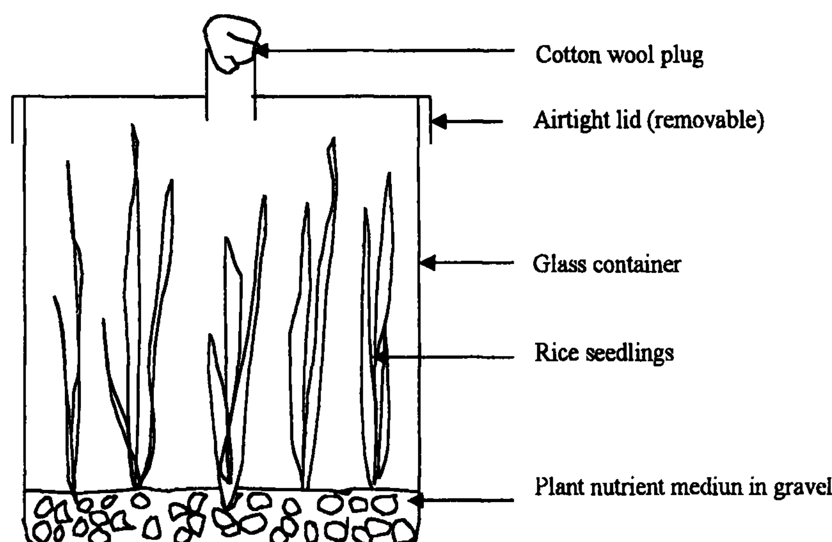

Figure 1. A single unit of an aseptic plant nursery

Pre-germination of seeds for aseptic nursery and *in vitro* experiments

Filled, mature paddy seeds were carefully de-husked manually without damaging the embryo or the endosperm and the seeds of similar size were selected. They were first left immersed in 70% ethyl alcohol for 3 minutes with occasional agitation. Ethyl alcohol was then drained off and the seeds were dipped in 0.1% $HgCl_2$ for 2 minutes. Finally they were washed thoroughly (8 washings) with sterile distilled water. About 10-12 surface sterilised seeds were transferred to Nutrient Agar (NA) plates under aseptic conditions, the platelets were incubated in the dark at 28°C. After 2-3 days seeds with uncontaminated radical initials were used for the experiments.

Preparation of inoculum

The bacterial strains were multiplied first in a 50 ml LB liquid medium (LB medium of Miller 1972 supplemented with of $CaCl_2$ and Mg SO_4, 2.5 m mol each) in an Erlenmeyer flask and incubated at 28°C for 24 h on a rotary shaker. This was subcultured in 500 ml of fresh sterile LB medium. The culture after 48 h of incubation was directly used as the inoculum. The density was determined by a plate count at the time of inoculation.

Acetylene reduction activity assay

The method reported by Barraquio *et al.* (1986) was used in this study, slightly modified for soil free roots. The rice plants from pots were gently uprooted and washed thoroughly with tap water to remove all the adhering soil particles. The upper portion of the plant was cut off leaving about 2 cm for mature plants and 0.5 cm for seedlings. A mature cut plant was placed in 400 ml of tap water in an incubation jar (1200 ml) and a seedling cut plant in 30 ml of water in a 100 ml

Erlenmeyer flask and sealed with a sterile rubber stopper. In the case of *in vitro* experiments, the cotton wool plug of the spermosphere was replaced with a sterile rubber stopper. Ten percent of the headspace air was replaced with C_2H_2 and the flask was incubated at 26°C in the dark. Gas samples were withdrawn after 24 h and analysed in a gas chromatograph (Shimadzu GC-9AM). The working temperatures of the GC were 80, 60 and 85°C for injection port, column (Porapak N) and FID detector, respectively.

Enumeration of *Azospirillum* density

Roots from gently uprooted plants were washed first with tap water to get rid of adhering soil particles and then with sterile distilled water. They were then cut into small pieces of about 2-3 cm in length. A 3 g sample from well-mixed root pieces was macerated with a sterile mortar and pestle and suspended in 30 ml of sterile distilled water. Soil from within the root mass was collected carefully before the roots were washed. One gram of a well-mixed sample was suspended in 100 ml of sterile distilled water. Ten fold dilution series were prepared from the supernatants of these suspensions. Four millilitres of nitrogen free malate semi-solid medium or N-free broth (Nfb) (Day and Doberreiner, 1976) was inoculated in triplicates with 100 µl from each dilution. After 2 days of growth in the dark, ARA was carried out for tubes which showed typical *Azospirillum* pellicles. Positive tubes for both pellicle and ARA from each dilution were counted and the bacterial population was estimated using standard MPN tables (Alexander, 1965).

Completely randomised design was used in both experiments and the means were compared using Duncan's Multiple Range Test for General Linear Model of SAS programme (1987).

RESULTS

The aseptic nursery developed was an effective tool for this kind of work. There was hardly any contamination in this set up compared to other techniques used to raise aseptic seedlings for inoculation experiments. This could be modified as required for other plant-microbe interaction studies as well.

Pot experiment

None of the inoculation treatments showed a significant increase in ARA. The ARA ranged between 14 and 30 n mol day^{-1}. Although soil was inoculated, it did not show a high activity than the ones with only native bacteria (un-autoclaved soil) at 30 and 80 DAT (Table 1). Although Ladha *et al.*, (1992) reported a higher ARA of native diazotrophs at heading stage of the plant compared to early growth stages, the differences in our experiment between 30 DAT and heading stage (80 DAT) were not significant.

Table 1

Acetylene reduction activity of soil free roots of different treatments

Treatment	C_2H_4 production (n mol plant^{-1} day^{-1})			
	30 DAT		80 DAT	
	Non-autoclaved soil	Autoclaved soil	Non-autoclaved soil	Autoclaved soil
T 0	19.75^a	14.74^a	24.02^a	17.24^a
T 1	14.14^a	17.19^a	22.20^a	15.09^a
T 2	15.47^a	18.85^a	14.16^a	28.45^a
T 3	18.85^a	14.24^a	23.26^a	18.02^a
Average	**17.05^A**	**16.26^A**	**20.86^A**	**19.70^A**

T0: Non inoculated; T1: 50 ml pot^{-1} of 1.2 x 10^9 CFU ml^{-1} bacteria 2 weeks after transplanting; T2: 50 ml pot^{-1} of 8 x 10^8 CFU ml^{-1} bacteria day before transplanting; T3: T1+T2. T2 and T3 plant roots were also dipped in a 8 x 10^8 CFU ml^{-1} density inoculum broth for 3 h just before transplanting. DAT: Days after transplanting. Data (means of 4 replicates) followed by the same lower case letter within a column or same upper case letter among averages at a particular sampling do not differ significantly according to the Duncan's Multiple Range Test at 5% probability level.

Plant growth in un-autoclaved soil was better compared to autoclaved soil (Table 2 and 3). Only the tiller number at 30 DAT was affected positively due to inoculation at 2 WAT in autoclaved soil but this was not prevalent at 80 DAT (Table 2). A similar reverting trend has been reported in *Zea mays* and *Lolium perenne* with *Azospirillum* spp. in a pot trial (Barea *et al.*, 1983).

Table 2

Tillering performance of plants as affected by different treatments

Treatment	Average number of tillers (plant^{-1})			
	30 DAT		80 DAT	
	Non-autoclaved soil	Autoclaved soil	Non-autoclaved soil	Autoclaved soil
T 0	3.25^a	2.00^b	8.00^a	7.67^a
T 1	3.33^a	2.67^a	9.25^a	7.00^a
T 2	2.75^a	2.00^b	8.67^a	7.00^a
T 3	3.33^a	2.00^b	8.25^a	7.00^a
Average	**3.14^A**	**2.15^B**	**8.57^A**	**7.15^B**

T0: Non inoculated; T1: 50 ml pot^{-1} of 1.2 x 10^9 CFU ml^{-1} bacteria 2 weeks after transplanting; T2: 50 ml pot^{-1} of 8 x 10^8 CFU ml^{-1} bacteria day before transplanting; T3: T1+T2. T2 and T3 plant roots were also dipped in a 8 x 10^8 CFU ml^{-1} density inoculum broth for 3 h just before transplanting. DAT: Days after transplanting. Data (means of 4 replicates) followed by the same lower case letter within a column or same upper case letter among averages at a particular sampling do not differ significantly according to the Duncan's Multiple Range Test at 5% probability level.

Table 3

Plant biomass production as affected by different treatments

Treatment	Plant dry weight (g plant^{-1})			
	30 DAT		80 DAT	
	Non-autoclaved soil	Autoclaved soil	Non-autoclaved soil	Autoclaved soil
T 0	0.62^{a}	0.45^{a}	14.17^{a}	11.75^{a}
T 1	0.57^{a}	0.49^{a}	14.64^{a}	12.03^{a}
T 2	0.57^{a}	0.37^{a}	16.61^{a}	11.57^{a}
T 3	0.57^{a}	0.35^{a}	15.01^{a}	10.60^{a}
Average	**0.58^{A}**	**0.41^{B}**	**14.95^{A}**	**11.53^{B}**

T0: Non inoculated; T1: 50 ml pot^{-1} of 1.2 x 10^9 CFU ml^{-1} bacteria 2 weeks after transplanting; T2: 50 ml pot^{-1} of 8 x 10^8 CFU ml^{-1} bacteria day before transplanting; T3: T1+T2. T2 and T3 plant roots were also dipped in a 8 x 10^8 CFU ml^{-1} density inoculum broth for 3 h just before transplanting. DAT: Days after transplanting. Data (means of 4 replicates) followed by the same lower case letter within a column or same upper case letter among averages at a particular sampling do not differ significantly according to the Duncan's Multiple Range Test at 5% probability level.

None of the plant parameters showed any positive effect due to inoculation in un-autoclaved soil indicating the poor competitive ability of the introduced strain with the native population. Although different inoculation treatments have affected the plant N yield negatively at 30 DAT in both autoclaved and un-autoclaved soil, it has recovered with time (Table 4). The average total N yield of plants in the autoclaved soil 80 DAT was higher compared to unautoclaved soil (Table 4), indicating that the dead biomass has contributed to the plants only at later stages of its growth.

Table 4

Nitrogen assimilation by plants under different treatments

Treatment	N yield (mg plant^{-1})			
	30 DAT		80 DAT	
	Non-autoclaved soil	Autoclaved soil	Non-autoclaved soil	Autoclaved soil
T 0	16.82^{a}	15.61^{a}	102.37^{a}	131.89^{a}
T 1	8.68^{b}	11.80^{ab}	113.03^{a}	141.10^{a}
T 2	14.81^{ab}	14.15^{ab}	132.64^{a}	129.03^{a}
T 3	13.83^{ab}	10.04^{b}	111.15^{a}	129.83^{a}
Average	**13.86^{A}**	**12.99^{A}**	**113.01^{B}**	**133.59^{A}**

T0: Non inoculated; T1: 50 ml pot^{-1} of 1.2 x 10^9 CFU ml^{-1} bacteria 2 weeks after transplanting; T2: 50 ml pot^{-1} of 8 x 10^8 CFU ml^{-1} bacteria day before transplanting; T3: T1+T2. T2 and T3 plant roots were also dipped in a 8 x 10^8 CFU ml^{-1} density inoculum broth for 3 h just before transplanting. DAT: Days after transplanting. Data (means of 4 replicates) followed by the same lower case letter within a column or same upper case letter among averages at a particular sampling do not differ significantly according to the Duncan's Multiple Range Test at 5% probability level.

The total *Azospirilum* population density reduced drastically from 10^7 to 10^4 CFU ml^{-1} at 30 DAT and 10^3 CFU ml^{-1} or below at 80 DAT (Table 5). Similar results have been reported in a field study, where an inoculated bacterial population (a mixture of 4 bacteria) on rice roots decreased with time during the rice growth period (Ali *et al.*, 1998). Even repeated inoculations did not sustain a high density in our study indicating the involvement of one or more limiting factors. Inoculation at nursery and transplanting stages has sustained a higher number of bacterial cells compared to single inoculation at 2 WAT, during the early 30 days (Table 5). However, this did not have any positive effect on the host plant possibly due to the strain not establishing a stable association with the host plant although it was surviving. The reason for this could be lack of sufficient C substrates.

Table 5

Inoculum population densities of root and rhizosphere soil

Treatment	*Azospirillum* inoculum density (CFU g^{-1} substrate)			*Azospirillum* density 30 DAT ($x10^3$ CFU g^{-1})				*Azospirillum* density 80 DAT ($x10^3$ CFU g^{-1})			
	Nursery $6.7x10^7$	At trans. $2.9x10^7$	14 DAT $1.05x10^7$	Non-sterilised soil		Sterilised soil		Non-sterilised soil		Sterilised soil	
				root	soil	root	soil	root	soil	root	soil
T 0	-	-	-	$<10^3$	$<10^3$	2.8	$<10^3$	$<10^3$	$<10^3$	$<10^3$	$<10^3$
T 1	-	-	+	1.4	$<10^3$	28	$<10^3$	$<10^3$	$<10^3$	1.7	$<10^3$
T 2	+	+	-	2.1	14	2	240	$<10^3$	2.1	$<10^3$	2.4
T 3	+	+	+	1.4	14	91	17	$<10^3$	0.21	$<10^3$	2.0

T0: Non inoculated; T1: 50 ml pot-1 of 1.2 x 109 CFU ml-1 bacteria 2 weeks after transplanting; T2: 50 ml pot-1 of 8 x 108 CFU ml-1 bacteria day before transplanting; T3: T1+T2. T2 and T3 plant roots were also dipped in a 8 x 108 CFU ml^{-1} density inoculum broth for 3 h just before transplanting. DAT: Days after transplanting. CFU: colony forming units.

In vitro experiment

Plants responded to external N, but not to malate under these conditions (Table 6). Therefore the effect of malate, if any on the plant in any plant-bacterial association tested should be, through the bacteria. Acetylene reduction activity was not detected in any association. However, inoculated strains survived at sampling time. All three bacterial strains have affected plant growth (Table 6). This was reflected from significant increases in plant biomass. The positive effect was found in N-free medium and the effect of the strain *A. irakense* KBC1 was similar to that of N in N supplemented medium. This effect was also higher than that of its IAA excreting mutant. Hence, involvement of IAA in affecting plant growth by the mutant could not have occurred. Malate did not show any particular effect on any of the bacterial strains tested in the absence of N. The effect of malate was negative on *A. irakense* KBC1 in affecting plant growth in N supplemented medium (Table 6). Although the effect of malate was positive on *A. faecalis* strain, it did not trigger N_2 fixation despite it being a suitable carbon substrate for *Azospirillum* (Khammas *et al.*, 1989).

Table 6
Response of rice variety BW 267-3 to inoculation, nitrogen and malate, 30 days after transplanting.

Inoculation	Nitrogen (29.2 mg tube^{-1})	Malate (0.542 mg tube^{-1})	Plant dry weight (mg plant^{-1})
Non-inoculated	-	-	26.8^{f}
	-	+	29.3^{ef}
	+	-	54.8^{ab}
	+	+	59.7^{a}
Azospirillum irakense KBC1	-	-	58.8^{ab}
	-	+	55.5^{ab}
	+	-	40.0^{de}
	+	+	26.0^{f}
A. irakense KBC1 (P 0.2)	-	-	41.5^{cd}
	-	+	49.5^{abcd}
	+	-	46.0^{bcd}
	+	+	48.0^{abcd}
Alcaligenes faecalis	-	-	49.0^{abcd}
	-	+	53.8^{abc}
	+	-	46.3^{bcd}
	+	+	60.5^{a}

Data (Means of 6 replicates) followed by the same letter do not differ significantly according to the Duncan's Multiple Range Test at 5% probability level. '+': presence, '-': absence

DISCUSSION

It is not clear why the ARA of native and introduced diazotrophs was low compared to that found in a separate study (data not shown) where it was 330 n mol day^{-1} without inoculation. Except for the absence of N fertilizer all the other conditions were same in the present study. Also under *in vitro* conditions the strain has promoted the growth of this rice variety apparently via BDF as discussed elsewhere.

The reason for no response in plants due to inoculation and reduction of bacterial density in the pot experiment could be insufficiency of specific C substrates for these diazotrophs. The plant may not be exuding sufficient amounts of C substrates to support a high bacterial density and N_2-fixation. Glick (1994) has stated that although it may seem the diazotrophs in general have a competitive advantage over non-diazotrophic bacterial strains in N poor soils, the presumed larger amount of energy required for N_2-fixation may put the diazotroph at a disadvantage. It has been reported that only some bacteria have the capability of inducing exudation. If the introduced bacteria were unable to induce exudation or they did not find a specific substrate in soil they would not have thrived and would not have been in a position to support the plant (Heulin *et al.*, 1987). Repeated

inoculation with high densities thus may be a process that adds another competitor to the available soil C pool. Also the changes occurred in the soil by autoclaving would have altered the activities and survival of the introduced strain. Bacteria in associative systems are constituents of a group of microorganisms including non-fixers. In such groups cross-feeding including supply of growth factors between fixers and non-fixers may be important (Vose, 1983). Here it may be the ratio of introduced to indigenous microbes that is important and not the strain that dominates. Thus introducing a high density of diazotrophs alone does not seem effective under our experimental conditions in improving the rice root-bacterial association.

The death of microbial biomass due to autoclaving and its subsequent decomposition and mineralisation would increase the soil N pool and this has benefited the plants. However, the introduced strain has not benefited from this killed biomass and apparently was at a disadvantage due to heat sterilising of the soil. Although heating during autoclaving the soil would have caused a flush of substrates from killed microorganisms and those become available for the introduced strain, other changes caused by heating would have been unfavourable for the introduced strain. Loss of organic C in mass scale starts at temperatures between 100°C and 200°C due to distillation of the volatile constituents of the organic matter (Kang and Sajjapongse, 1980). Modifications brought about by heating also include structural changes in humic and fulvic acids and an increase in aromatic structures, which has been proposed to increase the resistance of organic matter to microbial attack (Knicker *et al.*, 1996).

Lack of plant response to inoculation in non-sterilised soil has been reported previously. Chalk (1990) quoting several others reported that inoculation of non-sterilised soils with *Pseudomonas* spp. and *A. lipoferum* failed to show any effect on plants. However, in studies with Indica-type rice using homologous strain of *Klebsiella oxytoca* a significant contribution to the plant was demonstrated in heat-sterilised soil but not in non-sterilised soil. However, in our study despite being a non-homologous strain the KBC1 had affected the tiller number due to inoculation 2 WAT, at early stage of the plant growth in autoclaved soil. Despite the conducive non-competitive environment for colonisation, no positive effect on plants was observed in the autoclaved soil at later stages in response to inoculation. This could be attributed to the poor competitive ability of the introduced strain with the subsequent colonizers in establishing a stable association with the plant roots. According to the protein profile comparison of reisolated bacteria with that of the KBC1 strain at sampling times (data not shown), diazotrophs other than the inoculated strain were found indicating a subsequent microbial succession.

The plant growth improvement in the *in vitro* experiment could be attributed to associative BDF. The reason for not detecting ARA could be because nitrogen fixation at sampling had been repressed apparently if the N supply was adequate. When a plant can express its whole potential the effect of inoculation is less pronounced and can even be hidden (Fages, 1994). Other possible mechanisms of plant growth promotion, particularly enhanced N acquisition by the strains as

mentioned by Vose (1983) could not have taken place at least in N-free treatments. Siderophore action has been discussed by Glick (1994) but it was also not possible as this was a monoxenic system. Lack of nitrogenase activity may be also due to the low concentration of malate added (0.07%). In a similar study with the rice variety LD 181-5 inoculated with *Azorhizobium caulinodans*, low concentrations of Na-lactate (0.025% and 0.05%) did not affect ARA but 0.1% and 0.25% concentrations increased ARA six fold over that in the control though the rice seedling growth was negatively affected (Van Nieuwenhove *et al.*, 2000).

Under our experimental conditions repeated inoculation with high densities of a non-homologous strain even under non-competitive soil conditions was not useful. Supplementing the inoculum with a suitable C substrate under low levels of N might be beneficial and should be tested under field conditions.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Sri Lankan Government and the Belgian Administration for Development Coorporation (BADC) for their financial assistance to the BNF project at the IFS, Kandy and members of the BNF project for their support.

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