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Establishment of *Azorhizobium caulinodans* in the rhizosphere of wetland rice (*Oryza sativa* L.)

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Abstract *Azorhizobium caulinodans* strongly colonized the rhizosphere of rice plants after incorporation of *Sesbania rostrata* in a field trial throughout the growing season and during the fallow period until 19 weeks after incorporation of *S. rostrata*. *A. caulinodans* became well established in the rhizosphere (7.17 log cfu g^{-1} dry rice root) and colonized subsequent *S. rostrata* test plants. Three traditional and three improved high-yielding rice varieties were inoculated with *A. caulinodans* under gnotobiotic conditions. In none of the combinations did acetylene reduction activity significantly increase. Ethylene production on colonized rice roots only started after the growth medium had been supplemented with an extra C source (0.1 to 0.25% Na-lactate). This indicates that the bacterial nitrogenase activity is limited by energy supply. Four possible inoculant-carriers (peat, coir dust, bagasse, rice straw) were compared for long-term survival of the bacterial strain. Independent of the storage temperature (26 °C or 4 °C), the survival of *A. caulinodans* in peat and coir dust was very high during a 12-month period (>8 log cfu g^{-1} dry carrier), whereas the bagasse and rice straw carriers showed a serious decline from 3 months onwards.

Key words *Azorhizobium caulinodans* · Rice · Inoculum survival · Inoculum carriers · *Sesbania rostrata*

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Introduction

Sesbania rostrata is considered to be an ideal green manure for lowland rice (Ladha et al. 1989b). The plant accumulates a substantial amount (up to 120 kg N ha^{-1}) of biologically fixed N_2 in a period of 7–9 weeks, when inoculated with *Azorhizobium caulinodans* (Ndoye et al. 1996). In Sri Lanka, farmers have not adopted green manuring with *S. rostrata*, although it was introduced in 1985. This indicates agronomic and/or socio-economic problems in its acceptance (Becker and Ladha 1996).

However, the possible association of *A. caulinodans* with rice roots (Ladha et al. 1989a) opens up new perspectives for biofertilization in paddy fields. *A. caulinodans* is the symbiont of an aquatic legume and thus adapted to submerged conditions. The strain fixes N_2 both as symbiont and as a free-living microorganism (de Bruijn 1989) and exhibits a high N_2–fixing potential. The nitrogenase activity of *A. caulinodans* as a free-living microorganism is around 1800 nmol of C_2H_2 produced per milligram of protein per hour at a partial O_2 pressure of 3 kPa (Dreyfus et al. 1988), as compared to only 800 nmol of C_2H_2 per milligram of protein per hour at a partial O_2 pressure in the range of 0.1–0.5 kPa with *Azospirillum* spp. (Hartmann and Zimmer 1994).

A. caulinodans is allochtonous in Sri Lanka, while a prerequisite for plant growth promotion is the survival of the introduced strain in the rice rhizosphere in competition with the native soil microflora (Glick and Bashan 1997). This study focused on the ability of the allochtonous bacterium, *A. caulinodans*, to colonize the rice rhizosphere. The establishment of *A. caulinodans* in the rice rhizosphere is described after incorporation of *S. rostrata*, in a field trial, followed by an incubation trial to mimic the post-harvest fallow period under agronomic conditions. Experiments under sterile conditions were set up to evaluate the influence of rice varieties and extra C supply on the acetylene reduction activity of the *A. caulinodans*-rice root association. Four inoculum carriers were tested for long-term survival of

A. caulinodans, in order to introduce high cell numbers in the immediate surroundings of the germinating seeds.

Materials and methods

Field/incubation trial

A field trial was conducted in the Wet Zone of Sri Lanka during the winter rain season 1991/1992. The survival of *A. caulinodans*, after incorporation of *S. rostrata*, was compared with the occurrence of *Azospirillum* spp. during and after the rice-growing season. Two treatments were arranged in a completely randomized block design with four replicates each: (1) incorporation of inoculated *S. rostrata*; and (2) control plots without *S. rostrata* incorporation. Each replicate plot was 12 m^2. The soil characteristics were: Dystropept (Soil Survey Staff 1975); pH (KCl) 5.0; total N 1.8 g kg^{-1}; organic C 18 g kg^{-1}; Olsen P 0.01 g kg^{-1}; CEC 11 $cmol_c$ kg^{-1}; Ca 2.07 cmol kg^{-1}; Mg 0.54 cmol kg^{-1}; Na 0.26 cmol kg^{-1}; K 0.15 cmol kg^{-1}. Scarified *S. rostrata* seeds were immersed in a suspension of washed *A. caulinodans* cells and broadcasted in the field (25 kg seeds ha^{-1}). A second inoculation was carried out 3 weeks after sowing. *A. caulinodans* cells (500 ml m^{-2}, 10^7 cfu ml^{-1}) were spread over the seedlings with a lever-operated knapsack sprayer. The chopped *S. rostrata* plants (8 cm) were incorporated 9 weeks after sowing (3400 kg dry matter ha^{-1}). Two-week-old rice seedlings (variety Bg 300, Table 1) were transplanted 2 days after incorporation of *S. rostrata*. No extra fertilizers were added.

After the rice harvest, 11 weeks after transplanting (WAT), three soil samples (75 cm^3 each) were collected from each replicate of the different treatments. The soil samples were combined in a single composite sample per replicate. The soil was dried at 45 °C to 6% water content, thoroughly mixed and sieved (<2 mm). Soil (500 g) was then incubated in sterilized jars.

Soil and plant samples were taken at regular intervals during the experimental period, until rice harvest (11 WAT) and until 19 WAT for the incubated soil samples.

Inoculation experiments under bacteriological controlled conditions

The three traditional local rice varieties and the three improved high yielding (HY) varieties chosen for the inoculation experiments, were all widely used by the farmers. Some agronomic characteristics of these varieties are mentioned in Table 1. The Rice Research Stations of Batalagoda, Bombuwella and Labuduwa, Sri Lanka, provided the varieties. Rice seeds were surface-sterilized and allowed to germinate following the procedure of Ladha et al. (1989a). Three-day-old sterile seedlings were transferred into cotton-plug test tubes (250×20 mm). Tubes were filled with 15 ml of N-free, semi-liquid (0.4% agar) nutrient medium for rice (Yoshida et al. 1976). In a second set with the variety Ld 181–5 only, the effect of an extra C source on the nitrogenase activity of the inoculated strain on the rice roots was evaluated. The medium was supplemented with 0, 0.025, 0.05, 0.1 or 0.25% Na-lactate.

In both experiments, all treatments were carried out in 10 replicate tubes. The inoculation of the seedlings was done at transplanting, with 1 ml washed *A. caulinodans* cells (10^8 cfu ml^{-1}). Fourteen days after inoculation the nitrogenase activity of the bacteria on the rice roots was measured. The rice seedlings were harvested, rinsed with sterile water and plant parameters were measured. Subsequently the seedling roots were shaken in 9 ml of sterile PBS, (0.1 M, 0.75% NaCl, pH 7.0) for 1 h on a rotary shaker (200 rev. min^{-1}). Plating decimal dilution series of the bacterial suspension on yeast extract bacteriological plates (YEB, Geelen et al. 1995) tested the survival of the inoculum.

Inoculum carrier evaluation

Four locally available carriers were selected for potential use.

1. Peat is traditionally used as an inoculum carrier (Somasegaran and Hoben 1994).
2. Rice straw can be collected from the field, instead of being burned.
3. Bagasse is a by-product of the sugar cane industry.
4. Coir dust is a solid organic waste material obtained after the extraction of coconut fibers from the coconut husks.

Standard procedures for carrier preparation were followed (Somasegaran and Hoben 1994). Each bag, containing 10 g of carrier material, was aseptically injected with *A. caulinodans* broth (10^9 cfu ml^{-1}). The broth:carrier ratios (v:w) were chosen according to the water-holding capacity of each substrate [broth:peat (5:10), broth:straw (12:10), broth:coir dust (20:10) and broth:bagasse (25:10)]. Two subtreatments simulated realistic conditions of storage: room temperature (26 °C) and refrigerated (4 °C). In addition, a set of broth cultures was diluted with glycerol (75:25) for storage at –20 °C. Destructive sampling was done in four replicate bags per treatment. Inoculum density was checked 1, 3, 6, 12 months after inoculation (MAI) by plating decimal dilution series in PBS of 1 g stored material on YEB medium, supplemented with 200 μg ampicillin ml^{-1}.

Table 1 Agronomical characteristics of the rice varieties used for the field trial and the microorganism–host preference screening under sterile test tube conditions

	Maturity duration[a]	Yield potential[b]	Fertilizer response	Iron toxicity tolerance	Pest/disease resistance	Pericarp colour
Field trial						
Bg 300	3.0	5.5	High	Susceptible	Moderate	White
Test tubes						
Traditional local variety						
Devererderri	5.0	2.2	Lodging	n.r.[c]	Moderate	Red
Rathu heenati	4.5	n.r.	Low	n.r.	Moderate	White
Herath banda	3.0	2.5	Lodging	Tolerant	Low	White
High yielding variety						
Bg 94–1	3.5	7.0	High	Moderate	High	White
Bw 267–3	3.5	8.0	Moderate	Tolerant	High	White
Ld 181–5	3.5	5.0	Moderate	Tolerant	Moderate	Red

[a] Maturity duration: months
[b] Yield potential: 10^3 kg ha^{-1}
[c] No records

Azorhizobium culture and inoculum

The *A. caulinodans* strain ORS571 was obtained from the BCCM-LMG culture collection, Gent, Belgium. This wild type strain is intrinsically resistant to 200 μg ampicillin ml^{-1}. The strain was grown in YEB broth, pH 6.8, at 26 °C for 3 days on a rotary shaker (150 rev. min^{-1}). The medium was supplemented with ampicillin (200 μg ml^{-1}) when used for isolation of *A. caulinodans* under non-sterile conditions. *A. caulinodans* inoculum was prepared in YEB broth. The cells of fully grown cultures were centrifuged, washed twice with, and resuspended in, sterile water.

Enumeration of total microbial numbers, *Azospirillum* spp. and *A. caulinodans* in plant and soil samples

At each sampling in the field trial, three rice plants per plot were carefully uprooted and rinsed under running tap water. Similarly, three root-free soil cores (75 cm^3 each) per plot were collected and thoroughly mixed. A subsample of 10 g roots or soil slurry (on a fresh weight basis) was used for bacterial counts. The samples were placed in a bottle containing 90 ml sterile physiological water. Subsequently the samples were shaken for 1 h on a rotary shaker (200 rev. min^{-1}). Soil bacteria (aerobic and facultative anaerobic) and *Azospirillum* spp. were counted by inoculating decimal dilution series of the bacterial suspension on tryptic soy agar (0.3%) and in semi-liquid N-free malate medium (Döbereiner 1995), respectively. The plates and test tubes were incubated at 32 °C and observations were made after 72 h. The survival of *A. caulinodans* on rice roots and in soil was checked with MPN counting by the plant infection method according to Ladha et al. (1989a). Stem nodule appearance was assessed 3–4 weeks after inoculation.

Acetylene reduction activity assay

Acetylene reduction activity (ARA) assays were done on rice seedlings, grown under sterile conditions, 14 days after inoculation. The cotton wool stopper of the test tubes was replaced by a sterile rubber stopper. The whole rice plant was exposed to C_2H_2 at a partial pressure of 10 kPa. All samples were further treated as described earlier (Barraquio et al. 1986). The samples were incubated at 26 °C in the dark. Gas samples were collected after 24 h and analysed in a gas chromatograph (Shimadzu GC-9AM). The GC working temperatures were 80, 60 and 85 °C for injection, column (Poropak N) and FID detector, respectively.

Statistical analysis

All data were subjected to statistical analysis. The ANOVA procedure of the SAS program was used for General Linear Model Analysis to determine the main and interaction effects. Tukey's studentised range test was used for the comparison of means.

Results and discussion

Survival of *A. caulinodans* after incorporation of inoculated *S. rostrata* during the rice cropping season and the post-harvest fallow period

Despite being introduced in Sri Lanka in 1985, *S. rostrata* has not yet been used as green manure in the area selected for the experiment. Therefore, *A. caulinodans* was completely absent in the soil before the start of the experiment (Table 2). The *Azorhizobium* counts in the control treatment during the experiment – small as they were ($<10^2$ cfu g^{-1}) – are due to contamination through irrigation water. Total microbial counts and *Azospirillum* numbers were not significantly different between the control and the treatment with incorporated *S. rostrata.* Therefore, results shown in Table 2 only represent the microbial counts of soil and rhizosphere samples after incorporation of *S. rostrata*. Total microbial numbers and the populations of *A. caulinodans* and *Azospirillum* spp. were significantly higher in the rhizosphere than those in the bulk soil throughout the growth season. It seems likely that the total counts, in the rhizosphere as well as in the soil samples, are underestimated. The method used to estimate total bacterial numbers only considers the aerobic and facultative anaerobic microflora, disregarding the anaerobic microflora. The population of *A. caulinodans*, although significantly lower, is only a factor 10 lower than the total microbial numbers (Table 2).

The first sampling after transplanting the rice seedlings (4 WAT) shows significantly increased rhizosphere populations (Table 2). The growing rice plant

Table 2 Development of microbial colonization (log cfu g^{-1} soil slurry or dry root) in soil and rhizosphere samples during the rice growing season after incorporation of *Sesbania rostrata* under field conditions. Data followed by the same letter within a column do not differ significantly by Tukey's studentised range test ($P=0.05$)

	Soil			Rhizosphere		
	Azorhizobium	*Azospirillum*	Total count	*Azorhizobium*	*Azospirillum*	Total count
Rice season[a]						
0	0.00 d	2.15 c	6.34 c	–	–	–
4	5.23 c	2.62 b	7.70 a	6.38 c	3.63 b	7.86 a
7	6.97 a	3.00 ab	7.45 ab	7.96 a	4.54 a	8.24 a
11	6.11 b	3.10 a	7.18 b	7.17 b	3.89 b	7.90 a
Avg[b]	5.67	2.72	7.17	7.17	4.02	8.00
Fallow period[a]						
12	5.22 c	–	6.45 c	–	–	–
19	6.58 a	–	6.49 c	–	–	–

[a] Weeks after transplanting
[b] Average of microbial counts over the rice growing season

provides O_2 and growth substances for the microflora, native as well as inoculated. The colonization of the rice roots was highest at the heading stage (7 WAT); this corroborates the findings of Boddey (1987) and Ladha et al. (1988). Accumulation of reduction products results in a more reducing rhizosphere, especially after panicle initiation, as reflected in the decline of the microbial population in the later growth stage (11 WAT) (Kimura et al. 1979; Sethunathan et al. 1983). After harvest (12 WAT), the *A. caulinodans* population decreased to 5.22 log cfu g^{-1}, but recovered gradually to 6.58 log cfu g^{-1} at 19 WAT. The availability of extra C sources from decaying rice roots and stubble and the aerobic incubation conditions resulted in a remarkable survival of *A. caulinodans* throughout the fallow period. Sufficient numbers of *A. caulinodans* survived at the end of the fallow to induce spontaneous nodulation on the stems of *S. rostrata* test plants inoculated with soil extracts.

The rhizosphere effect (R:S, microorganisms in the rhizosphere vs microorganisms in the non-rhizosphere), averaged over the growth season, was 4.29 for the total microflora (aerobe and facultative anaerobe) and 11.82 for *A. caulinodans*. These values are of the same magnitude as the values for lowland rice plants reported earlier (Jalaluddin 1975), ranging from 1.25 to 16.20. The ratios are low when compared with the figures given by Bazin et al. (1990) for upland crops. Kimura et al. (1979) attributes this to the reducing environment in the submerged soils. The significantly higher rhizosphere effect of *A. caulinodans* compared to the total rhizosphere effect suggests an enrichment of the inoculated bacteria in the rice rhizosphere, as was also reported by Ladha et al. (1989a). In our experiment, *Azorhizobium* proved to be highly competitive with the native soil microflora to colonize the roots and the rhizosphere of rice.

Colonization of different rice varieties by *A. caulinodans* under gnotobiotic conditions

The system, *A. caulinodans*/rice root association, was scaled down to a sterile model under controlled conditions. Six rice varieties were selected for a microorganism-host preference test under sterile conditions. The germinating rice seeds were directly inoculated with the bacterial strain, without intervention of its natural host, *S. rostrata*. Owing to this direct inoculation, the interfering effects of mineralization of a large amount (3400 kg dry matter ha^{-1}) of incorporated green manure on the nitrogenase activity could be excluded.

There was no significant difference between the six varieties either in survival of the inoculum (cell density) or in nitrogenase activities (ARA) (Table 3). The ARA between the rice varieties ranged between 86 and 93 nmol 24 h^{-1} per seedling. This lack in difference be-

Table 3 Effect of rice varieties and increasing concentrations of Na-lactate on plant parameters (seedling length, dry weight), survival of the inoculum (cell density) and nitrogenase activity (ARA). Data followed by the same letter within a column do not differ significantly by Tukey's studentised range test ($P=0.05$)

	Treatment[a]	Length (cm)	Dry weight (mg/plant)	Cell density (cfu/g or /ml)	ARA (nmol/24 h)
Rice variety					
Devererderri	I	20.2 a	32 a	1.0E+10	88 c
	NI	22.0 a	35 a	–	89 c
Rathu heenati	I	23.0 a	34 a	8.0E+11	87 c
	NI	23.1 a	36 a	–	87 c
Herath banda	I	21.4 a	32 a	8.2E+11	89 c
	NI	21.4 a	30 a	–	88 c
Bg 94-1	I	18.9 b	21 b	8.0E+11	92 c
	NI	17.6 b	21 b	–	90 c
Bw 267-3	I	17.6 b	20 b	3.0E+11	88 c
	NI	17.8 b	19 b	–	86 c
Ld 181-5	I	18.3 b	17 b	2.0E+11	89 c
	NI	18.7 b	18 b	–	93 c
Na-lactate concentration (%)[b]					
0.000	S	21.0 a	22 b	2.0E+11	90 c
	NS	–	–	2.4E+10	92 c
0.025	S	17.5 b	18 b	8.0E+10	155 c
	NS	–	–	6.5E+10	145 c
0.050	S	15.0 c	16 c	1.6E+11	235 c
	NS	–	–	2.0E+11	142 c
0.100	S	15.5 c	16 c	2.0E+11	899 b
	NS	–	–	7.0E+10	160 c
0.250	S	14.0 c	16 c	1.8E+11	1177 a
	NS	–	–	2.0E+10	192 c

[a] Treatments: *I* inoculation, *NI* no inoculation, *S* with rice seedling, *NS* without rice seedling
[b] Rice variety Ld 181–5 was used, all treatments *A. caulinodans* inoculated

tween rice varieties after inoculation was also stated by Watanabe et al. (1979) for *Azospirillum* inoculation.

The plant biomass production (dry weight) was significantly higher for the traditional varieties in comparison with the improved HY varieties. This is in agreement with their plant physiological characteristics (Weerakoon, IIMI, SCOR Project, Galenbindunuwewa, Sri Lanka, personal communication). Traditional varieties, which are the long-duration genotypes, were, however, not better hosts for the bacteria than the short-duration HY genotypes under these experimental conditions This is in contrast to the findings of Ladha et al. (1988).

Triggering nitrogenase activity under increasing energy supply

The lack of ARA in the rice roots was puzzling despite the successful survival of the bacterium under gnotobiotic conditions. The relation between the degree of bacterial colonization of the rice roots and nitrogenase activity remained obscure. To investigate the hypothesis that a limiting supply of carbohydrates prevented measurable nitrogenase activity on the colonized roots, one rice variety (Ld 181–5) was selected for a further test in which stepwise increasing amounts of available C were added to the rice-growing medium. Variety Ld 181–5 was selected because its seedlings were best adapted to the artificial test-tube conditions and showed healthy growth even 14 days after inoculation.

Data in Table 3 represent the results obtained with the Ld 181–5 seedlings exposed to increasing Na-lactate concentrations. Na-lactate was not effective in increasing the ARA at concentrations of 0.025% and 0.05%, but at concentrations of 0.1 and 0.25% the ARA increased several-fold over that in the control (899 vs 160 nmol C_2H_4 24 h^{-1}). However, rice seedling growth was negatively affected, but the inoculum (cell density) survived well with increasing Na-lactate concentrations.

The growth conditions of the rice seedlings in the test tubes were apparently not conducive for C_2H_2 reduction. In this regard, Heulin et al. (1987) reported that C was the most important limiting factor for N_2 fixation on rice seedlings grown under similar conditions. Our experiment with the rice variety Ld 181–5 also showed a high C_2H_4 production after supplementation with an extra C source. Not only the available C source, but also the prevailing O_2 tension at the root surface is a limiting factor for significant nitrogenase activity (Vanderleyden et al. 1995). Our results support this claim. The marked difference of C_2H_2 reduction activity between the test tubes with and without rice seedling shows that *A. caulinodans* is not able to fix N_2 as a free-living bacterium under anaerobic conditions. Aerenchymatous tissue in rice plants delivers the required O_2 and atmospheric N_2 to the microorganism (Sethunathan et al. 1983).

Long-term survival of *A. caulinodans* in four inoculum carriers

Since emerging seedling roots are scarcely colonized by microorganisms, it is possible to establish a population of a selected strain in this niche via inoculation (Höflich et al. 1995). Introduction of sufficient cell numbers in the immediate surrounding of the germinating seed can be done through the use of high-quality inoculants. The development of a reliable inoculation technology determines whether *A. caulinodans* inoculant will be easily adopted in future agricultural production (Okon and Labandera-Gonzalez 1994). Long-term survival of inocula in locally available carrier materials will reduce the cost of production.

In all materials, except straw dust, *A. caulinodans* showed a significantly increased growth 1 MAI at room temperature (26 °C), as a result of the favourable conditions allowing proliferation even within the substrata (Fig. 1A). This 'maturing of the inoculum' cannot take place at a storage temperature of 4 °C. This is clearly shown in Fig. 1B, where all bacterial counts were signif-

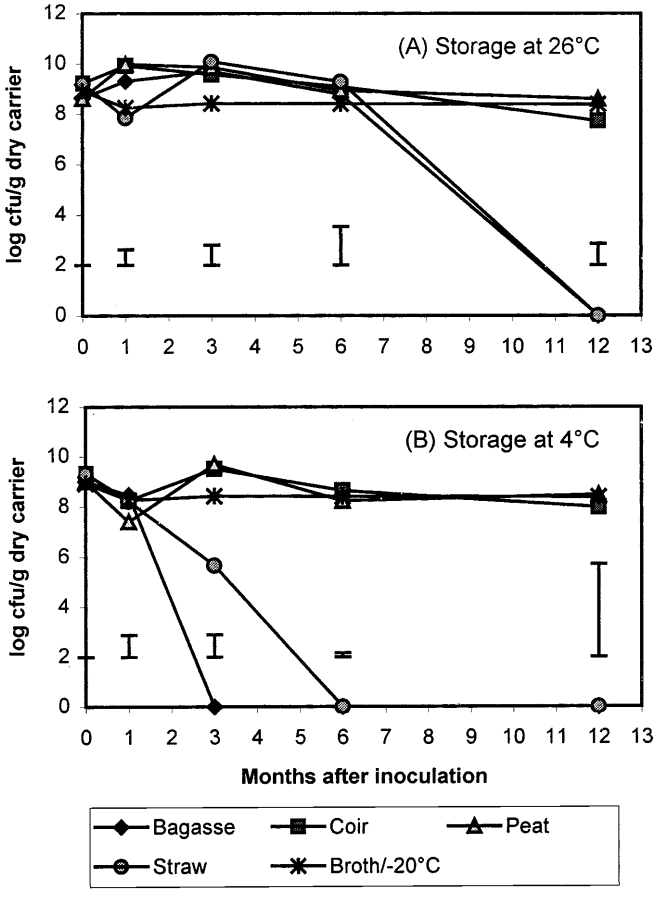

Fig. 1 Long term survival of *Azorhizobium caulinodans* (log cfu g^{-1} dry carrier) on four sampling dates, in four different carriers (coir dust, peat, bagasse, straw dust). Stored at 26 °C (**A**), at 4 °C (**B**) and in broth stored at –20 °C. *Error bars* indicate the minimum significant difference (5%) for comparing treatments on each sampling date

icantly lower ($P=0.05$) 1 MAI than at the initial stage. *A. caulinodans* survived in high numbers, in all carriers during at least 6 months at room temperature (Fig. 1A). Bagasse and straw samples showed a serious decline in the survival of *A. caulinodans* from 3 MAI onwards, when the samples were stored at 4 °C (Fig. 1B). Twelve MAI, the survival of *A. caulinodans* in all bagasse and straw samples was very low or nil. The quality of the other treatments decreased slightly but not below the level of the broth-glycerol samples stored at –20 °C. The latter samples remained very stable during the experimental period, after an initial decrease from 8.92 to 8.25 log cfu ml^{-1} medium. From the results it is clear that the storage of the samples at 26 °C is significantly better ($P=0.05$) than storage at 4 °C with an average survival (over 12 months) of the inoculum of 8.22 vs 6.51 log cfu g^{-1} dry substrate respectively. The high standards for inoculants, recommended by Okon and Labandera-Gonzalez (1994), containing cell numbers of the order of 1×10^9 to 1×10^{10} cfu g^{-1} or ml^{-1} were, however, not reached.

The high survival of *A. caulinodans* in coir dust can be explained by its intrinsic capacity to retain moisture. Moisture loss in combination with high temperatures was mentioned by Roughley et al. (1995) as the main factors affecting the numbers of rhizobia in inoculants. The low availability of nutrients in the coir is compensated for by the low degree of contamination in comparison with bagasse. While peat shows a good result, it is economically and environmentally not acceptable to be exploited on a large scale in Sri Lanka, where the main peat lands are located within nature reserve areas.

A. caulinodans proved a proficient colonizer of rice roots, persistent in the field and abundant on rice roots under sterile and field conditions. Since the rice roots are surrounded by an O_2–rich atmosphere through their aerenchyma (Justin and Armstrong 1987), semi-aerobic microorganisms, like *A. caulinodans*, have better chances to proliferate in the rice root vicinity and compete better for the nutrients exuded by the roots than anaerobic ones. However, a direct substantial contribution to the rice yield remains to be demonstrated. The present investigations seem to indicate that the energy requirements of *A. caulinodans* are not met in lowland paddy fields. Our experiments on the establishment of a new and viable microsymbiont could so far not prove that *A. caulinodans* is a more effective associative microorganism for rice than the indigenous population.

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