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Nitrogen fixation in pure culture by rhizobia isolated from stem nodules of tropical *Aeschynomene* species

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1. SUMMARY

Asymbiotic nitrogenase activity was investigated in rhizobia strains isolated from stem and root nodules of several *Aeschynomene* species. All isolates from stem-nodulating species were able to develop nitrogenase activity ex planta in the presence or in the absence of combined nitrogen, whereas root isolates from *Aeschynomene* species related to the cowpea group of plants showed little or no activity. Nitrogenase activity in soft-agar and in liquid cultures displayed by strains ORS310 and ORS322, isolated from stem nodules of *A. indica* and *A. afraspera* respectively, was of the same order of magnitude as that found for *Azorhizobium caulinodans* ORS571 and ten times higher than for *Bradyrhizobium* strain CB756. Furthermore, like *A. caulinodans* ORS571, strains ORS310 and ORS322 were able to use atmospheric nitrogen as sole nitrogen source for growth.

2. INTRODUCTION

Asymbiotic nitrogenase activity in free-living cultures under appropriate conditions, has been demonstrated primarily in several strains belonging to the genus *Bradyrhhizobium, B. japonicum* [1-4] and cowpea *Bradyrhizobium* spp. [3,5], including the well characterized strains 32H1 [2,6–9] and CB756 [3,10,11]. Subsequently, a very limited number of *Rhizobium* strains has been shown to have some degree of acetylene-reducing ability ex planta: *Rhizobium* strain IHP100 isolated from *Cajanus cajan* [10], *Parasponia-Rhizobium* ANU 289 [12], and particular strains of *R. leguminosarum* [2,6,13], *R. meliloti* [14] and *R. trifolii* 0403 [15]. None of these strains was able to grow at the expense of molecular nitrogen, except *R. trifolii* 0403, which was shown after a treatment involving succinate to behave physiologically similar as nodule-derived bacteroids.

In free-living cultures of bradyrhizobia, nitrogen fixation always requires the presence of combined nitrogen and the fixed nitrogen is excreted into the culture medium [16]. Thus, in the free-living state as in the symbiotic conditions [17], ammonium assimilation appears to be disconnected from nitrogen fixation.

A notable exception among rhizobia isolates are the unusual strains from *Sesbania rostrata* stem nodules, now classified as a new genus *Azorhizobium caulinodans* [18,20]. These strains are characterized by a very high rate of free-living acetylene-reducing activity (up to 40 nmols C2H4/min/mg protein) and the ability to use the

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fixation products as sole nitrogen source for growth [18,19].

Isolation of a large number of strains from many tropical *Aeschynomene* species led me to classify these plants into three cross-inoculation groups [21]. This paper reports a study of asymbiotic nitrogen fixation, both in soft-agar and liquid cultures, of strains isolated from stem or root nodules of tropical *Aeschynomene* species belonging to the three cross-inoculation groups previously defined [21].

3. MATERIALS AND METHODS

3.1. Rhizobium strains

The 'cowpea' *Bradyrhizobium* strain CB756 was obtained from Dr. H.H. Keyser, USDA-ARS, Beltsville, MD, U.S.A., and *Azorhizobium caulinodans* strains ORS571 from Dr. B.L. Dreyfus.

Aeschynomene-Rhizobium isolates used in this study are listed in Table 1. Cultures were maintained and grown on yeast extract and mannitol (YM) medium [22]. Growth rate was determined in YM liquid medium at 30 °C. Acid or alkali production was determined by colour change of bromthymol blue (0.0025%) incorporated into the YM agar medium.

Nitrogen-fixing cultures were checked for purity by plating out single colonies and testing them for nodulation ability on appropriate host plant. Strains isolated from the resultant nodules exhibited the same cultural and physiological characteristics as original strains.

3.2. Media

The minimal growth medium (SA) contained (in mM): K_2HPO_4, 9.18; KH_2PO_4, 5.87; D-arabinose, 40; di-Na succinate, 40, $MgSO_4 \cdot 7\ H_2O$, 0.4; $CaCl_2 \cdot 2\ H_2O$, 0.27; NaCl, 0.85, and (in μM)

Table 1

Ex planta nitrogenase activity and host specificty of *Aeschynomene* rhizobia in soft-agar cultures after 7 days

Strains	Host of isolation	Cross-inoculation group of plants	Generation time (h)	Ex planta N_2ase activity after 7 days (μmol C_2H_4/culture)	
				without glutamate	with glutamate
ORS 571	*Sesbania rostrata* (S [a])	–	5.5	11.0	20.0
CB 756	*Macrotyloma africanum* (R)	1	14.0	0.0	2.0
ORS 303	*Aeschynomene afraspera* (S)	2	5.5	8.5	4.5
ORS 308	*A. afraspera* (R)	2	5.0	10.5	5.0
ORS 312	*A. afraspera* (R)	2	4.8	9.00	18.5
ORS 322	*A. afraspera* (S)	2	4.8	8.0	20.0
ORS 326	*A. afraspera* (S)	2	5.7	5.5	8.0
ORS 337	*A. afraspera* (R)	2	5.2	5.7	11.0
ORS 306	*A. indica* (S)	3	7.5	4.0	7.0
ORS 310	*A. indica* (S)	3	6.5	9.5	18.0
ORS 318	*A. indica* subsp 1 (S)	3	7.8	5.0	4.1
ORS 319	*A. indica* subsp 1 (S)	3	7.5	8.0	16.0
ORS 320	*A. indica* (S)	3	4.5	5.0	15.5
ORS 328	*A. indica* subsp 2 (S)	3	5.2	8.5	14.0
ORS 330	*A. sensitiva* (S)	3	5.8	6.0	13.0
ORS 334	*A. tambacoundensis* (S)	3	4.2	7.5	10.0
ORS 301	*A. americana* (R)	1	6.0	0.0	0.0
ORS 302	*A. pundii* (S [b])	1	8.5	0.0	0.0
ORS 304	*A. elaphroxylon* (S [b])	1	5.0	7.0	12.0
ORS 305	*A. schimperi* (R)	1	7.5	0.0	0.2
ORS 309	*A. uniflora* (S)	1	8.0	0.0	0.0

Values are the mean of 4 replicates.

[a] S, strain isolated from stem nodule; R, strain isolated from root nodule.

[b] stem nodules were restricted to the lower and immerged part of the stem.

$FeCl_3 \cdot 6\ H_2O$, 15; $Na_2MoO_4 \cdot 2\ H_2O$, 40; $ZnSO_4 \cdot 7\ H_2O$, 0.76; $MnSO_4 \cdot H_2O$, 12; $CuSO_4 \cdot 5\ H_2O$, 0.32; biotin, 2.4; nicotinic acid, 10; Ca pantothenate, 2.6; agar Noble (Difco), 1%; pH adjusted to 6.8. The supplemented medium (SAG) was the minimal medium (SA) supplemented with glutamate 3 mM as nitrogen source.

3.3. Nitrogenase activity in soft-agar cultures

The procedure for setting up and growing the free-living cultures was as described by Bender et al. [10]. Cells pregrown in YM liquid medium at 30 °C were harvested, washed in distilled water and suspended in SA(G) medium. A total of 1 ml of the cell suspension (about 5×10^8 cells) was added to a test tube containing 4 ml of 1% SA(G) agar medium precooled at 45 °C. Sterile cotton plugs were pushed down to the middle of the tubes and the tubes were closed with sleeve-type rubber stoppers. Acetylene was injected to give a concentration in the gaseous phase of 5%. The cultures were incubated under air at 30 °C and gas samples were daily analyzed for ethylene production by gas chromatography [23].

3.4. Nitrogen-fixing liquid cultures

Experiments in liquid cultures were carried out in 570 ml serum flasks fitted with rubber sleeve stoppers. Cells prepared as above (about 5×10^8 bacteria) were grown in 15 ml SA medium on a rotary shaker operating at 150 rpm at 30 °C. Flasks were evacuated and flushed three times with pure N_2 and filled with N_2, 5% C_2H_2 and O_2 at the appropriate level. O_2 levels were determined at 12-h intervals and O_2 was added to maintain the desired concentration. The oxygen content was determined by gas chromatography with a thermal conductivity detector using a molecular sieve 5 Å column with helium as carrier gas. Gas samples removed from flasks were assayed for ethylene production.

3.5. Protein determination

Standard colorimetric assays generally used for determination of cell protein in agar cultures gave variable results due to interferences by culture products, arabinose and agar particularly. Therefore protein concentration was determined only in liquid cultures after harvesting cells by centrifugation at $5\,000 \times g$ for 10 min, using the Lowry method after alkaline digestion [24]. Bovine serum albumin was used as standard.

4. RESULTS AND DISCUSSION

4.1. Nitrogenase activity in soft-agar cultures

Nineteen strains isolated from *Aeschynomene* species belonging to the three cross-inoculation groups were examined. Strains ORS571 and CB756 were included as controls. Table 1 reports the generation time and the nitrogenase activity determined in the soft-agar medium. The nineteen strains had a generation time ranging from 4.2 to 8.5 h in YM medium and can be considered as fast- or intermediate-growing rhizobia. However, all produced alkali in YM medium, which is a feature usually encountered with slow-growing rhizobia. This was in agreement with previous reports suggesting that *Aeschynomene* rhizobia were members of an intermediate type of *Rhizobium* sharing physiological and nutritional characteristics with both fast and slow growers [25,26].

Nitrogenase activity was determined on cultures grown on soft-agar. Soft-agar allows the creation of an oxygen gradient, compatible with growth of microaerophilic bacteria. The growth pattern was different depending on the presence or the absence of glutamate in the medium. In nitrogen-free medium, growth was observed as a layer several millimeters below the surface medium. *Aeschynomene* strains appeared to be more oxygen sensitive than strain ORS571 as evidenced by the relative depth at which they grew, the layer formed by *Aeschynomene* strains being deeper (data not shown). When supplemented with glutamate, all the cultures showed growth at and near the agar surface.

All strains isolated from *Aeschynomene afraspera* (group 2) and those from plants belonging to the *A. indica* cross-inoculation group (group 3) grew well under the conditions described above and displayed nitrogenase activity ranging from 50 to 100% of that determined with ORS571 (Table 1). Except for strain ORS304 isolated from

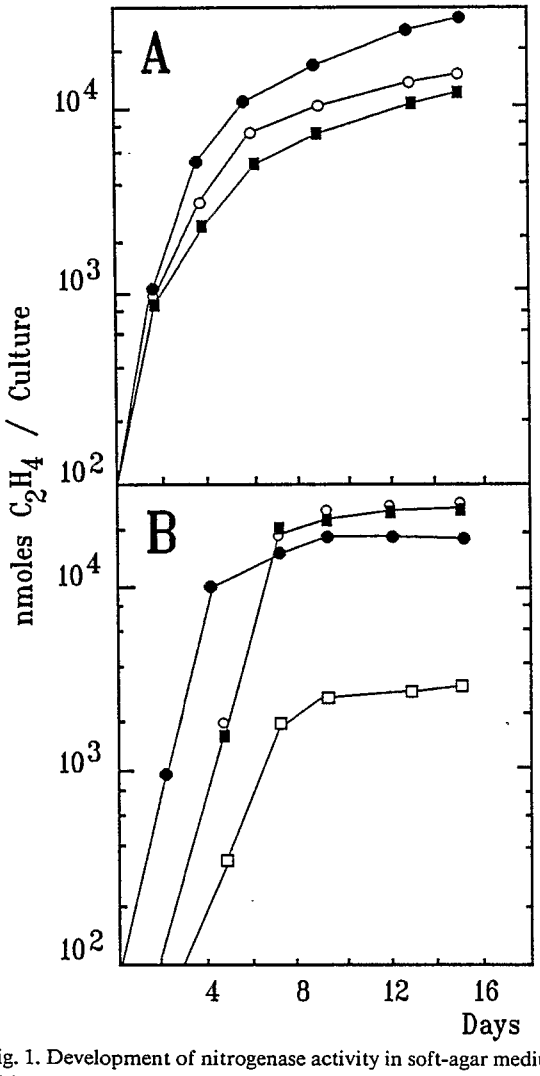

Fig. 1. Development of nitrogenase activity in soft-agar medium without (A) and with (B) 3 mM Na glutamate by *Azorhizobium caulinodans* ORS571 (●), *Aeschynomene* rhizobium strains ORS322 (○) and ORS310 (■), and cowpea *Bradyrhizobium* strain CB756 (□). Oxygen tension in the gas phase: 3% for strain ORS571; 0.5% for strains ORS310 and ORS322.

crown nodules of *A. elaphroxylon*, isolates from group 1 plants (including CB756) grew only when glutamate was present and display very little or no nitrogenase activity (Table 1).

Kinetics of ethylene production is shown in Fig. 1 for strains ORS310 and ORS322, as compared to kinetics obtained with ORS571 and CB756. The two *Aeschynomene* strains were chosen as representative of inoculation groups 3 and 2 respectively and because they displayed a high nitrogenase activity. In the absence of glutamate, the kinetics observed with ORS310 and ORS322 was almost identical to that observed with ORS571 (Fig. 1A), and as expected no activity was detectable with CB756 (not shown). In the presence of glutamate, a lag of two days before apparition of nitrogenase activity was observed with the two *Aeschynomene* strains (Fig. 1B). The lag was 7 days in the case of CB756. In addition, the maximal nitrogenase activity of CB756 was ten times lower than that of the other strains (Fig. 1B). This finding was in agreement with previous reports [10].

4.2. Nitrogenase activity in cells grown in nitrogen-free liquid medium

Maximum nitrogenase activity for *Aeschynomene* strains cultured in nitrogen-free medium was obtained with 0.5% oxygen in the gas phase at the initial cell density used. In contrast, strain ORS571 showed the highest level of acetylene reduction in 3% oxygen in the gas phase [18].

The kinetics of nitrogenase activity in strains ORS571, ORS310 and ORS322 grown in shaken liquid cultures is shown in Fig. 2. Patterns of nitrogenase development were quite similar in the three strains tested and the level of activity obtained was about 5×10^5 nmols of C2H4 produced per flask after 4 days at their respective optimum oxygen tension. This level was a lot higher than recorded previously for any rhizobial culture.

Growth of strains ORS310 and ORS322 increased from about 10 μg protein/ml up to 100 μg after 6 days as compared to 20 μg protein in the control under air (Table 2). Then, except for the small amount of combined nitrogen in the vitamins provided, asymbiotic nitrogen fixation was able to sustain growth. Thus, in addition to the capacity to reduce acetylene in culture at a very high level, strains ORS310 and ORS322 have in common with strain ORS571 the ability to use the products of fixation as a sole nitrogen source for growth.

Kaminski et al. [27] identified recently, in *Azorhizobium caulinodans* ORS571, a new gene

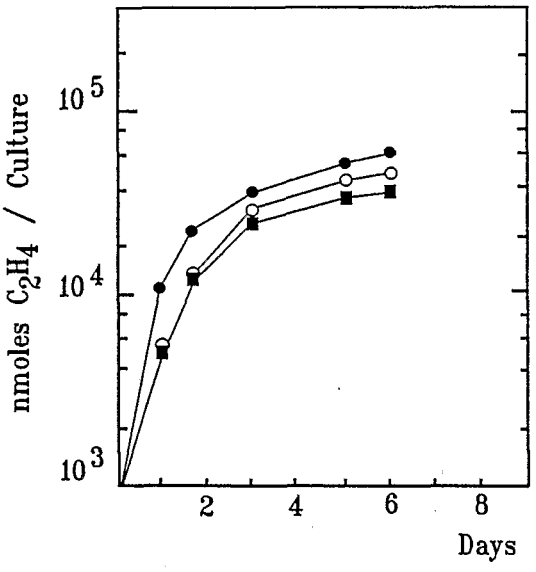

Fig. 2. Development of nitrogenase activity in nitrogen-free liquid medium by *Azorhizobium caulinodans* ORS571 (●), *Aeschynomene* rhizobium strains ORS322 (○) and ORS310 (■).

(*nif*O) involved in nitrogen fixation in the free-living state. By hybridization experiments, they found a strong homology between a *nif*O probe and the total DNA from *Rhizobium* strain ORS322, but neither with *Bradyrhizobium japonicum* nor with typical *Rhizobium* species.

Aeschynomene rhizobia and *Azorhizobium* strains have in common the property to nodulate stems of aquatic legumes. Thus, the unique property to grow on atmospheric dinitrogen seemed correlated to the stem-nodulating ability.

The ability of *Aeschynomene* rhizobia to grow in the free-living state at the expense of molecular nitrogen under low oxygen concentration implies a coupling between nitrogen fixation and nitrogen assimilation whereas these processes are decoupled when rhizobia are symbiotically associated with leguminous plants. A better knowledge of the synthesis and regulation of nitrogenase and ammonia-assimiling enzymes ex planta deserives further study.

Table 2

Nitrogenase activity and bacterial growth after 6 days in nitrogen-free liquid medium

Strains	Nitrogenase activity μmol C_2H_4/mg protein	Growth μg protein/ml
ORS 571	29.7	130
ORS 310	26.6	95
ORS 322	28.5	105

Values are the means of four replicates. The initial cell protein content was from 8 to 10 μg/ml. Controls under air yielded protein contents of 25, 20 and 18 μg protein/ml for strains ORS571, ORS310 and ORS322 respectively. No nitrogenase activity could be detected under air.

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ERRATUM

Delete from Fig.1 and transfer to Fig. 2: "Oxygen tension in the gas phase: 3% for strain ORS571; 0.5% for strains ORS310 and ORS322."