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DAKAR ORSTOM/CNRS/UNIVERSITY NITROGEN FIXATION GROUP

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1. STEM NODULATED LEGUMES

Since the discovery of stem nodulation of Sesbania rostrata in 1979, many laboratories throughout the world have initiated investigations on stem nodulated legumes because of their high N_2-fixing potential in the field and because they constitute unique experimental models for basic research both on Rhizobium and the host plants.

1.1. General characteristics of stem-nodulated legumes

B. Dreyfus, D. Alazard and E. Duhoux established that a characteristic of stem nodulated legumes is to bear predetermined nodulation sites. These sites always include a dormant root primordium whose base is infected by the rhizobia. The structure of the nodulation site varies greatly between two extreme types, forming a continuum between the most evolved types (Sesbania rostrata and Aeschynomene afraspera) and the least evolved ones (Aeschynomene elaphroxylon and Neptunia oleracea).

Rhizobia specific to stem-nodulated legumes differ in two respects from other known rhizobia: (1) their nitrogenase appears to be well protected against O_2, which could be an adaptation to their location in a photosynthetic nodule, (2) their ability to grow in the free-living state at the expense of N_2, which may confer upon these bacteria a selective saprophytic advantage.

In stem-nodulated legumes of the most evolved type such as Sesbania rostrata and Aeschynomene sp. nodulation sites are well above the soil and water level, which circumvents the problems of competition from indigenous rhizobia when the stems are inoculated.

Besides these characteristics, stem-nodulated legumes have the unusual capability of actively fixing N_2 even in the presence of high rates of combined nitrogen in the soil, which allows more nitrogen to be added to and less removed from the cropping systems.

In Sesbania rostrata, two types of strains have been isolated: stem-nodulating strains, called stem strains, capable of nodulating both stems and roots, and root-nodulating strains, called root strains, which nodulate only roots. Both types are fast growers. This root-stem specificity could be restricted to S. rostrata, since it has not been observed in other stem nodulated legumes up to now (B. Dreyfus).

1.2. In vitro studies of rhizobia from stem-nodulated legumes

1.2.1. Rhizobiophages and plasmids of Rhizobium isolated from Sesbania rostrata.

P. de Lajudie and D. Bogusz isolated two rhizobiophages RS_1 and RS_2 from ORS571, a typical stem strain of Rhizobium for Sesbania rostrata. The host range of both rhizobiophages was shown to be restricted to stem strain ORS571, all root strains being resistant. Phage RS_1 had an hexagonal head 63nm wide and a tail 87nm long and phage RS_2 and hexagonal head 60nm wide. These rhizobiophages could be used for typing Rhizobium strains or isolating Nod^- Rhizobium mutants on the basis of bacteriophage resistance.

Preliminary studies showed that the typical stem strain ORS571 contained a 5.6 Mdal plasmid and that a root strain of Sesbania rostrata contained a larger plasmid (100 Mdal).

1.2.2. Genetics of ORS571 (typical stem strain of Sesbania rostrata).

With the objective of localizing the genes of the symbiosis, a collaboration has been recently established between our lab and Pr. M. Van Montagu's group in Gent. By using transposon mutagenesis, 2000 mutants of ORS571 have been obtained. The screening of these mutants is under way simultaneously in Gent and Dakar.

The nif genes of ORS571 are studied at Pr. J.P. Aubert's laboratory in Paris.

1.2.3. In vitro N_2 fixation by Rhizobium strains from stem-nodulated legumes other than Sesbania rostrata

Only rhizobia from the most evolved types of stem nodulated legumes (e.g. Aeschynomene afraspera, A. sensitiva, A. schimperi) showed significant nitrogenase activity (200-300 nmoles $C_2H_4 h^{-1}$ mg protein^{-1}). Furthermore these strains were reported to grow at the expense of N_2. However the O_2 tension required for N_2-fixing growth was lower for these strains than for ORS571. The property to grow on N_2 could be a common feature among stem-nodulating rhizobia (D. Alazard; B. Dreyfus).

1.3. Establishment and fonctionning of the symbiosis

1.3.1. Morphogenesis of the nodules

Studies on the initial stages in the morphogenesis of the N_2-fixing stem nodules of Sesbania rostrata have been simultaneously carried out in three laboratories working in collaboration: the ORSTOM Laboratory in Dakar (B. Dreyfus), the Department of Microbiology, University of Minnesota (B. Tsien and E.L. Schmidt), and the Cytology laboratory of the University of Dakar (E. Duhoux). Similar investigations are under way to determine the ontogenesis of nodules on stem nodulated legumes other than Sesbania rostrata, especially different species of Aeschynomene (D. Alazard; E. Duhoux).

1.3.2. Leghemoglobin and nodulins

Using the chromatographic procedure, D. Bogusz fractionated root nodule leghemoglobin into three components, and stem nodule leghemoglobin into four components. One of the components was found to be specific to stem nodules, the other ones were common to both root and stem nodules. Root and stem nodule leghemoglobins of Sesbania rostrata bound nicotinate as already reported for soybean leghemoglobin. Antibody against stem nodule leghemoglobin did not cross-react with nodule extract from soybean and alfalfa.

Using the immunoferritin technique D. Bogusz and E. Duhoux found that leghemoglobin was present only in the plant cell cytoplasm of root and stem nodules of Sesbania rostrata. However the presence of a very low amount of leghemoglobin inside the membrane envelope cannot be totally excluded.

Further investigations will be devoted to the identification and the study of Sesbania nodulins.

1.4. Studies on the host plant

1.4.1. The triple potential of the nodulation site of Sesbania rostrata.

The nodulation sites of S. rostrata are formed continuously throughout the growth of the stem and remain sensitive to Rhizobium infection during the whole life of the plant. In the absence of Rhizobium infection, the dormancy of the root primordia can be broken by immersing the stem in water, which induces adventitious roots, or by placing an internodal stem cutting into a nutrient medium (M. M. Barreto).

In the latter case, the upper primordium of the cutting develops into a shoot bud, indicating the triple potential of the nodulation site.

The growth of root primordia was also shown to be influenced by different organs of the plant, especially roots and buds, but the mecanisms involved have not yet been elucidated.

1.4.2. Flowering characteristics of Sesbania rostrata

E. Duhoux plans to initiate the study of the morphology and organogenesis of the flowers of Sesbania rostrata and elucidate the process of pollinisation and fecondation. Related results should facilitate the development of methods for sexual hybridization.

1.4.3. Tissue cultures

The ultimate objectives of the investigations based on the use of tissue cultures are(1) to obtain an experimental model facilitating the analysis of the relations between the host plant and the endophyte,(2) to transfer the nodulating ability from stem-nodulating to non-stem-nodulating plants,(3) to devise methods breaking the dormancy of root primordia existing on the stem of some important legumes (e.g. Asian cultivars of soybean).

In a preliminary phase B. Dreyfus obtained calluses from hypocotyls of S. rostrata and succeeded in the induction of complete plants from the calluses (December 1983). The next objectives are (1) to regenerate plants from calluses originating from nodules, attempting to detect nodulines or noduline-like substances in the regenerated plants,(2) to isolate and cultivate protoplasts of different species of Sesbania (only S. rostrata is stem nodulating), thus preparing the next step, i.e. the fusion of a non-nodulating Sesbania protoplast with a S. rostrata protoplast.

1.5. Tolerance of N_2-fixing activity of Sesbania rostrata to combined nitrogen

The threshold of tolerance of N_2-fixing activity of Sesbania rostrata to combined nitrogen has not yet been accurately determined. However preliminary experiments indicate that N_2 fixation (ARA) by root nodules was inhibited when NO_3NH_4 concentration in the hydroponic culture was higher than 0.5mM whereas stem nodules still exhibited a significant ARA when NO_3NH_4 concentration was 1-3mM.

1.6. Field experiments (G. Rinaudo)

1.6.1. A microplot experiment showed that the effect of Sesbania rostrata as green manure on the rice yield was as marked in soils with high clay content as in sandy soils where the trials has been conducted up to now. In both soil types the yield was increased 2.5-fold.

1.6.2. N_2 fixation was the highest under the climatic conditions prevailing in August and September in Dakar, which confirms that high temperatures (ca.30°C) are the most favorable.

1.6.3. The methodology of the use of Sesbania rostrata as green manure in paddy fields has been progressively improved.

2. ACTINORHIZAL SYMBIOSES (mainly Casuarina-Frankia symbiosis)

There are two major reasons for studying these symbioses:

a) Plants (which are mostly perennial ones) associated with Frankia can thrive in very poor soils, where they give high yield of biomass or actively contribute to the improvement of these soils to the benefit of agriculture (rotational agriculture) or associated non-fixing trees (e.g. pines, eucalypts).

b) Frankia strains exhibit a high variability suggesting that this organism could be manipulated when the necessary genetic tools have been obtained. Furthermore the fact that eight widely different families can be associated with Frankia suggests that the transfer of the nodulating ability to plants other than the presently known actinorhizal species would be very worthwhile.

2.1. Biology of Frankia in vitro

2.1.1. Isolation

H.G. Diem has developed an isolation technique for Frankia from Casuarina, probably the most difficult endophyte to grow in culture (because of the very low number of nodular structures able to give colonies).

2.1.2. N metabolism of Frankia in vitro

At the same time as Tjepkema et al, D. Gauthier established that some strains of Frankia are able to exhibit a significant acetylene reduction activity in vitro and can grow with N_2 as the sole nitrogen source.

Using strain ORS020602, D. Gauthier showed that the biosynthesis of nitrogenase was maximum when pO_2 in the atmosphere above the culture was 10kPa whereas the nitrogenase activity was apparently not affected by O_2 tension. The question arose as to whether ammonium originating from nitrogenase activity was assimilated through the GS-GOGAT pathway in Frankia as it is in other nitrogen-fixing prokaryots. A first attempt to answer this question was to follow the derepression of nitrogenase biosynthesis in Frankia in the presence of ammonium with or without MSX (methionine sulfoximine), known to inhibit GS and GOGAT. When MSX was added, nitrogenase could be synthetized even in the presence of ammonium, which supports the hypothesis that GS could play a role in the regulation of nitrogenase biosynthesis in Frankia. Preliminary results confirmed the presence of GS, GOGAT, and alanine dehydrogenase in Frankia growing in nitrogen-free medium. N_2 fixation occurred only when vesicles were present in the culture. In some media vesicles could appear without expressing any nitrogenase activity. Such a situation is reminiscent of that of preheterocysts of cyanobacteria.

Diem showed Frankia from Casuarina exhibited four types of structures: hyphae, vesicles, typical sporangia and sporangia-like structures (SLS). SLS appear to play a major role as regeneration structures.

Future investigations on the biology of Frankia in vitro will be developed in three directions:

a - Collecting strains of Frankia from Casuarinaceae. Efforts shall be made to obtain strains compatible with Allocasuarina and Gymnostoma species. Characterization of the strains will be initiated in a attempt to establish a taxonomic classification of the Casuarina strains in relation to the Rhamnale cross-inoculation group.

b - Improving the culture of Frankia by stimulating the production of SLS. Methods will devised to prepare new forms of Frankia inoculant suitable for long conservation, transportation and large scale utilization.

- The genetics of Frankia is in its infancy. We strongly feel the necessity of initiating studies in that field with the following objectives:
  . obtaining the required genetic tools;
  . attempting to locate the genes responsible for the specificity in Frankia strains;
  . attempting to improve the yield of SLS.
  This program will be initiated as soon as a geneticist joins our group.

2.2. Functionning of the symbiosis

Strains of Frankia from Casuarina isolated up to now exhibit a very strict specificity, which probably explains the failure of introduction of Allocasuarina outside their natural distribution area.

By contrast, Frankia from Rhamnales (especially Colletia that we have recently studied) appear to belong to a continuum ranging from non-infective Casuarina strains to typical Rhamnale strains.

2.3. Research on the host plant

The investigations are to be initiated at the beginning of 1984 in two main directions.

2.3.1. Propagative multiplication in vitro

There is a strong requirement for mastering this technique:
- to obtain indentical individuals for nursery or field experiments;
- to multiply the "plus" trees screened for their growth and nodulation ability.

2.3.2. Regeneration of Casuarina from calluses

The objective will be mainly to obtain plants which are resistant to high levels of salt and still fix N_2 actively. This work will be carried out at the ORSTOM Station and at the University of Dakar (E. Duhoux).

2.4. Assessment of N_2 fixation in the field

Ironically, 90% (or more) of people working on N_2 fixation are not concerned with the amount of N_2 that is actually fixed in

the field. As a matter of fact measuring N_2 fixation in the field is a difficult venture. Two types of problems are encountered. The first ones are related to the methodology, the second ones are specific to perennial plants (large volume of soil explored by roots; recycling of elements through litter fall).

By comparing four methods of assessment (the difference method, the usual isotope dilution technique, the A value method, the fractionation method), we arrived at the conclusion that, in conditions simulating those prevailing in the field (microplot studies), the difference method and the usual isotope method overevaluated (by ca.30%) the actual N_2 fixation if the A value method was considered as the reference method. If analyses are carefully performed the fractionation method gives data that are probably similar to those obtained with the isotope dilution methods.

In a field experiment carried out in Senegal in 1982-1983 using a pure strain culture (ORS021001) as an inoculant we found that fixation expressed as g N_2 $tree^{-1}$ was negligible till the trees were 4.5 months old, but it was 2.31 ± 1.47 (confidence interval) during the interval of time between 4.5-11 months, indicating that N_2 fixation dramatically increased with plant age.

Large variations in the N_2-fixing activity of individual trees occurred. These variations can be attributed neither to the endophyte (trees were inoculated with a pure strain of Frankia and contamination by external strains was unlikely) nor to the soil heterogeneity (the soil was carefully homogenized before filling the microplots). The observed variations were probably related to the host plant genotype. Exploiting such a variability would probably be a fruitful approach to increase N_2 fixation of Casuarina stands.

Future field studies will be directed:

3.1. to establishing the time course of N_2 fixation from 0 to 3 year old Casuarina, using the A value and the difference method. This study should allow the calibration of the difference method, which is a necessity since ^{15}N studies cannot be used as a routin technique in large field studies.

3.2. to evaluating the need for double inoculation with Frankia and mycorrhizal fungi (ecto- or endo-). Large scale experiments will be set up in Senegal and Egypt (in collaboration with the U. of Alexandria)

3. N_2-FIXING LEGUMINOUS TREES (NFT)

This program, characterized by its applied character, will be partially sponsored by the National Academy of Sciences (USA). It is carried out in collaboration with ISRA/CNRF.

Phase 1. Determining suitable NFT for reforestation of sahelian and soudanian areas of Western Africa. Preliminary studies are directed to finding out the most actively N_2-fixing species among the following trees which are already known to have a satisfactory growth rate:

Prosopis juliflora, Erytrophlaeum guineense, Prosopis africana Acacia holosericea, Albizia ferruginea, Cordyla pinnata, Parkia biglobosa, Daniella ogea.

For each of the listed species, the investigations will be as follows:

- collection of Rhizobium strains and evaluation of their effectivity;
- study of the host spectrum of the strains;
- nursery experiments for assessing the N_2-fixing potential and the need for inoculation with Rhizobium and endomycorrhizal fungi.

Phase 2. For the most promising species, evaluation of the growth and development characteristics, particularly assessment of the N_2-fixing capabilities.

Up to now the main effort was directed to studying Acacia holosericea which seems to be a promiscuous host, but an active N_2-fixing species (precise data will be available when the ^{15}N analysis under way are completed). Because of its marked sensitivity to P, Acacia holosericea responds to mycorrhizal infection (G. mosseae) only in very P deficient soils. Thus P fertilization combined with mycorrhizal inoculation may depress the growth of Acacia holosericea. Field experiments showed a satisfactory but transient response to Rhizobium inoculation. Inoculation with Glomus mosseae did not affect the growth, however it resulted in the improvement of the stand homogeneity and increased the survival of transplanted trees.

N_2-fixation by Acacia cyanophylla

This Acacia plays an important role in Northern Africa, where it is used for reclamation of sandy soils, production of wood, charcoal and forage.

In collaboration with the University of Dakar (E. Duhoux) and Institut National de Recherches Forestières de Tunisie (IDRC partially sponsering the project) we have just initiated a research

program with the following objectives:
- selecting the host plant exhibiting the best N_2-fixing potential;
- obtaining, by tissue culture, plants that are tolerant to high levels of salt in the soil;
- developing an *in vitro* method of vegetative propagation of *A. cyanophylla*;
- checking in the field the results obtained *in vitro*.

4. THE AZOLLA-ANABAENA SYMBIOSIS

4.1. Genetic studies on Anabaena

The first objective of the investigations was to compare the plasmid content and the Nase structure genes of symbiotic *Anabaena* extracted from five species of *Azolla* (*A. pinnata*, *A. caroliniana*, *A. filiculoides*, *A. mexicana*, *A. microphylla*) to the related characteristics of non-symbiotic *Anabaena* in order to identify the main genetic traits involved in the symbiosis. Results obtained to date are as follows:

4.1.1. Comparison of the plasmid content of symbiotic and non-symbiotic strains of Anabaena (C. Franche)

Strains of *Anabaena* extracted from *Azolla caroliniana*, *A. filiculoides* and *A. mexicana* contained one 25 Mdal plasmid whereas no plasmid was found in *Anabaena* extracted from *Azolla pinnata* or *A. microphylla*. By contrast non-symbiotic tropical strains of *Anabaena* had a variable number of plasmids (1 to 5, generally 3) with molecular weights ranging from 1.5 to 120 Mdal. Until now no specific function could be attributed to these plasmids.

4.1.2. Homology between the nitrogenase structure genes of Anabaena variabilis strain PCC7120 (non symbiotic Anabaena) and total DNA extracted from four symbiotic Anabaena azollae (C. Franche and G. Stanier).

Nif HDK genes of symbiotic and non-symbiotic *Anabaena* were clearly homologous. For both groups of *Anabaena* the arrangement of *nif* HDK genes was quite similar; *nif* K was not adjacent to *nif* H and *nif* D. Preliminary results suggest that, like the non-symbiotic strain of *Anabaena* PCC7120, the symbiotic strains of *Anabaena* have several copies of *nif* H gene.

The restriction map of A. azollae fragments homologous to the nif HDK genes of PCC7120 was similar for the different strains of A. azollae, suggesting that only one relatively primitive strain of A. azollae exists.

4.2. Microplot studies (P. Reynaud)

Factors affecting the growth and N_2 fixation of Azolla pinnata var. africana were studied. An experiment was devised to study N_2 fixation and the fate of N_2-fixed, using the isotopic dilution method.

5. NON SYMBIOTIC BLUE-GREEN ALGAE (P. Reynaud)

The statistical analysis of data from 82 biotopes showed that the following factors which most affected the distribution of N_2-fixing blue-green algae (BGA) were: pH (positive effect), plant cover (positive effect), soil water content (negative effect), abundance of non N_2-fixing BGA (positive or negative effect).

Microplot experiments at the ORSTOM Bel Air station indicated that algalization of rice by BGA was successful only when performed at the middle of the growth cycle, a consequence of the sensitivity of BGA to excessive light and competition with other algal strains.