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International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

# Collaborative Research Programme

# TERMINAL EVALUATION REPORT

**UNIDO contract #** 92/036/CW

**ICGEB ref. #:** CRP/MEX91-05
(Project # GE/GLO/89/001)

**Project initiation:** July, 1992

**Project termination:** July, 1994

International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

# Collaborative Research Programme

## TERMINAL EVALUATION REPORT

Part 1

**Title of Project**

RELATION BETWEEN AMMONIUM ASSIMILATION AND NITROGEN FIXATION IN *Rhizobium* DURING SYMBIOSIS

Keywords: *Rhizobium*-legume symbiosis, symbiotic nitrogen fixation, ammonium assimilation.

**UNIDO contract #** 92/036/CW **ICGEB ref. #:** CRP/MEX91-05

**Project initiation:** JULY, 1992 **Project termination:** JULY, 1994

**Principal Investigator's name:** GEORGINA HERNANDEZ

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**Abstract:**

Bacteria of the genus *Rhizobium* have a symbiotic interaction with leguminous plants. A general dogma in this area is that the *Rhizobium* enzymes involved in ammonium assimilation are switched off during symbiotic nitrogen fixation, allowing fixed nitrogen in the form of ammonium to be excreted and assimilated by the plant. The evidence of participation of ammonium assimilation in the bacteroid during symbiosis is controversial. This project aims to solve this controversy.

The effect during symbiosis of a constitutive and stable expression of glutamate dehydrogenase (GDH) in *Rhizobium* was studied; the stable plasmid pAM1a, bearing the *gdhA E. coli* gene was constructed and conjugated into *Rhizobium*. The total inhibition of bean on alfalfa nodulation was observed when the minimum inoculum that gives optimal nodulation of *R. etli* CFN42/pAM1a was used. A drastic inhibition of the induction of the *nodA*-gene by bean root exudate and by naringenin was observed in the *R. etli* strain expressing GDH. This is explained by the repression of *nod*-gene expression by high ammonium concentration, which we determined for *R. etli*. An *ntrC*$^-$ mutant strain of *R. etli* bearing the pAM1a (GDH) plasmid do nodulate beans therefore, the inhibition of nodulation by nitrogen may be mediated by *ntrC* in *R. etli*. The inhibition of nodulation by the *R. etli* strain harboring the *gdhA* gene may be explained by a down regulation by nitrogen, mediated by *ntrC*, in the synthesis of the nodulation factors that are essential for the initial steps of the symbiosis. An instability of the plasmid harboring the *gdhA* gene was observed during symbiosis, indicating a strong selection against this plasmid.

A GOGAT$^-$ (Tn$^-$5 insertion) mutant strain of *R. etli* (DEM300) was obtained and characterized. It is a glutamate auxotroph and it shows a normal symbiotic phenotype. The substitution of the natural ammonium assimilation pathway of *Rhizobium* (GS-GOGAT) by the GDH-GS pathway was achieved by conjugating the stable pAM1a (GDH) plasmid into the *R. etli* DEM300 and into the *R. meliloti* AK330, GOGAT$^-$ mutant strains. The *E. coli gdhA* gene complements the glutamate auxotrophy of both strains. The expression of GDH in the GOGAT$^-$ *R. etli* mutant strain, also inhibits the bean symbiosis. We conclude that the capacity to assimilate ammonium by *Rhizobium* has to be precisely regulated for infection and/or nodulation to occur.

Part 2

## OBJECTIVES/METHODOLOGY
## (proposed at the time of the submission of the research proposal)

The project that we propose is aimed to understand what is the role of ammonium assimilation in the bacteroid during the symbiotic process of *Rhizobia* with a legume plant. We believe that there is still a controversy in this matter and the project tends to solve this controversy about an essential function of ammonium assimilation.

The experimental approach that we plan to follow in this project involves the following:

a) The effect during symbiosis of a constitutive and stable expression of glutamate dehydrogenase in the bacteroid. The plasmid pAB17 (Bravo *et al.* J. Bacteriol. 170, 1988) carries the *E. coli gdhA* gene. This gene will be cloned into integration vectors and integrated into the chromosome of *R. meliloti*. The symbiotic effect of the expression of GDH will be analyzed.

b) The determination of enzyme activities and of the presence glutamine synthetases in the bacteroid. The enzymatic activity of GSI and GSII in bacteroids of *R. meliloti* and *R. etli* during symbiosis will be measured. Using specific antibodies against one GS, the presence of each of the enzymes in the bacteroid during symbiosis will be detected by Western type gels.

c) The effect during symbiosis of a constitutive over expression of glutamine synthetase in the bacteroid. Each of the previously reported genes coding for GSII from *B. japonicum* (Carlson, *et al.* J. Bacteriol. 169, 1987). GSI from *R. meliloti* (De Bruijn *et al.* J. Bacteriol. 171, 1989) or the GSIII from *R. etli* will be fused to a constitutive promotor: either the *nptII* gene promotor or the cat gene promotor from *E. coli*. These have been shown to be expressed constitutively in *Rhizobium*. These gene fusions will be integrated into chromosome using integration vectors.

d) The effect of a total or drastic inhibition of glutamine synthetase using the antisense technology as a complementation to the previously obtained GS mutants of *Rhizobium*. Using the same genes, GSI, GSII and GSIII (described in c), antisense gene fusions will be constructed by fusing, either the whole coding region of a fragment in the opposite orientation of the message to a constitutive or inducible promotor. We will use the promotor of the *dctA* gene(discarboxylic acid transport) of *R. leguminosarum* which is induced by succinate and is expressed during symbiosis. The antisense gene fusions will be cloned into a wide host range vector and conjugated to *R. etli* and their effect in free life and in symbiosis will be tested. A GSIII antisense fusion will be constructed both with a constitutive and an inducible promotor and integrated into the chromosome of a double mutant GSI$^-$, GSII$^-$ of *R. meliloti* (de Bruijn *et al*, J. Bacteriol. 171, 1989). The total or very drastic inhibition of ammonium assimilation will be tested in *R. meliloti*.

e) The cloning of the glutamate synthase gene from *Rhizobium etli* and the study of its role during symbiosis. The clones of an *R. etli* CFN42 gene bank which complement for the glutamate auxotrophy will be tested for GOGAT activity. The cloned *glt* gene will be mapped and characterized. Also, *R. etli* GOGAT$^-$ mutant strains will be obtained and characterized both in free life and in their symbiosis with beans.

f) Determine if the ammonium assimilation is compatible with the presence of an active nitrogenase and with the expression of the different nitrogen fixation genes in the bacteroid. The possible inocmpatibility of ammonium assimilaiton either with a constitutive GDH or GS expression will be analyzed in terms of the nodulation, the activity of nitrogenase and the plant yield.

## RESULTS
## (compare against the set objectives)

The first objective of the project involved the study of the symbiotic effect of a constitutive and stable expression of GDH in *Rhizobium*. The original experimental approach proposed involved the integratrion into the *Rhizobium meliloti* chromosome of the *gdhA* gene from *E. coli*. After we sent the proposal and before this project was begun, the group of Dr. Helinski reported the construction of a plasmid which is stable in several bacteria, including *Rhizobium*, due to the presence of the *par locur* (Weinstein, *et al.* J. Bacteriol. 174, 1992). Since Dr. Helinski kindly provided us with this plasmid (pTR101) we decided, as a better alternative than that originally proposed, to clone the *gdhA* gene into this stable plasmid and then conjugate it to different species of *Rhizobium* and to study the effect of the constitutive and stable expression of GDH in the *R. etli* (previously called *R. phaseoli*)-bean and *R. meliloti*-alfalfa symbioses.

The plasmid pAM1a, based in the stable vector pTR101 (Weinstein, *et al.* J. Bacteriol 174, 1992), containing the *E. coli gdhA* gene from pAB17 (Bravo *et al.* J. Bacteriol. 170, 1988) flanked by two transcription-translation terminators was constructed and conjugated to *R. etli* and *R. meliloti* wild type strains CFN42 and R41 respectively. Both strains bearing the pAM1a plasmid expressed a high GDH activity an a reduced GOGAT activity. *R. etli* CFN42/pAM1a synthesized and excreted high glutamate concentrations. The total inhibition of bean nodulation was observed when the minimum inoculum that gives optimal nodulation of *R. etli* CFN42/pAM1a was used. A drastic inhibition of the induction of the *nodA*-gene by bean root exudate and by naringenin was observed in the *R. etli* strain expressing GDH. The inhibition of nodulation by the R. *etli* strain harboring the *gdhA* gene may be explained by a reduction in the synthesis of the nodulation factors that are essential for the initial steps of the symbiosis.These data lead us to propose that the capacity to assimilate ammonium by *Rhizobium* has to be precisely regulated for infection and/or nodulation to occur. It appears that an ineffective symbiosis results when an increased amount of ammonium can be assimilated by the bacteria prior to symbiosis, and the bacteria must be nitrogen deprivated to be able to nodulate. We found that *R. etli nod*-gene induction is repressed by high extracellular ammonium concentrations, similarly as in *R. meliloti* (Puska & Kondorosi, Mol. Gen. Genet. 240, 1993) or *B. japonicum* (Wang & Stacey, Mol. Gen. Genet. 223, 1990). In addition the *R. etli* strain expressing GDH, represses the *nod*-gene expression at much lower ammonium concentrations. The observed nitrogen down-regulation of nodulation in *R. etli* could be mediated by NtrC, since an *ntrC*-mutant strain expressing GDH is able to nodulate beans normally.

When a higher concentration of *R. etli* or *R. meliloti* bearing the pAM1a (GDH) plasmid was used as inoculum for beans or alfalfa, respectively, nodulation was observed. But, the bacteria which formed the effective nodule have totally lost the GDH plasmid. So, despite the total stability of the pTR101 plasmid in *in-vitro* cultures or during symbiosis and also the total stability of the pAM1a plasmid in *in-vitro* cultures, there is a strong selection of the plant against the GDH plasmid. We observed that when the *R. meliloti*/pAM1a is incubated in media with alfalfa root exudate this plasmid is 80% lost after 6 weeks, and this is different to the lost of this plasmid in media without exudate (20% lost) or to the total stability of the pTR101 vector with or without exudates. The mechanisms involved in the selection of the plant (or root exudate) against the GDH stable plasmid remains to be investigated.

The next objectives of the project referred to study the symbiotic regulation of GS and to manipulate its expression in *Rhizobium*. We constructed a

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## Work plan and time schedule (originally envisaged)

a) Determine the effect during symbiosis of a constitutive and stable expression of GDH in the bacteroid. The *gdh*Agene will be cloned into integration vectors and integrated into the chromosome of *R. meliloti*. The GDH activity will be tested. The symbiotic effect of the GDH expression will be analyzed.

b) Determine the enzyme activity and the presence of GSI and GSII in the bacteroids of *R. phaseoli* and *R. meliloti*. This will be done using sensible immunological methods, that will allow us to conclude definitively if one of these enzymes is present in the bacteroids.

c) Determine what is the effect during symbiosis of the constitutive enhanced enzyme expression of GS in the bacteroides of *R. meliloti* during symbiosis. This will be obtained by integratring into *R. meliloti* chromosome a gene fusion of a GS gene and a constitutive promotor.

d) Determine the effect of a very drastic or a total GS inhibition during symbiosis of *R. meliloti* and *R. phaseoli* by expressing antisense-GS fusion with a constitutive or inducible promotor. The antisense fusions can be done using either, a constitutive or an inducible promotors, the latter would allow the inhibition of an enzyme in response to an specific inducer. The antisense-gene fusion will be integrated into the chromosome of *R. meliloti* mutant strain using integration vectors.

e) Cloning of the *glt* gene from a *R. etli* gene bank by complementation of the *E. coli* strain PA34 (glt$^-$). The GOGAT activity will be measured. The *glt* gene will be mapped and characterized. The subloning of the *glt* gene into the *Rhizobium* mutant (AK330) will be undertaken.

f) Based on the evidence that we have about the incompatibility of GDH activity and symbiotic nitrogen fixation could be that both a stable expression of GDH and GS are incompatible with symbiotic nitrogen fixation. If this is the case, with GDH, GS or both, we will determine if the nitrogenase is not working because the bacteroid lacks enough energy (used by GDH or GS) or because nitrogenase and the other nitrogen fixation genes are not expressed.

Time schedule

<u>First year</u>: Clonning of GDH gene and integration into the *R. meliloti* chromosome. Construction of GSI, GSII and GSIII gene fusions with constitutive promotors and antisense fusions. Determination of GS's activities and presence of enzymes in the bacteroid during symbiosis.

<u>Second year</u>: Study of the effect of atisense-GS gene fusions in free-living and symbiotic *Rhizobia*. Cloning of the GOGAT gene from *R. etli*. Study of the relation between ammonium assimilation and nitrogen fixation in the bacteroids of *R. meliloti*, expressing a constitutive activity of GDH or of GS.

gene fusion containing the coding region of the *B. japonicum* glnII gene and the *E. coli nptII* promoter which is a constitutive prpomotor for *Rhizobium*. We cloned this fusion in a stable plasmid for *Rhizobium* and try to complement *E. coli* or *R. meliloti* GS⁻ mutants with that plasmid. This was not possible, the GS from the gene fusion constructed did not expressed. We concluded that in the cloning procedure, important DNA sequences from the regulatory 5' end of the gene fusion, such as the SD sequence, had been distrupted and therefore the gene could not be properly transcribed. The glnII gene from *R. etli* was previously cloned in Dr. Mora's group in a 7.5 kb DNA fragment. An *R. meliloti* triple mutant strain (GSI⁻; GSII⁻; GSIII⁻) which is a glutamine auxotroph was obtained by Dr. J. Calderón. In this project we observed that the plasmid bearing the 7.5 kb DNA fragment from *R. etli* which contained the glnII gene did not complement the *R. meliloti* triple mutant GS⁻ strain and we propose that a DNA region of the plasmid appart from the *glnII* gene; prevents the expression, at a regulatory level, of this gene (this could be an artifact obtained during the cloning procedure). Therefore we subcloned from such plasmid a fragment containing the *glnII* gene which complements the triple mutant (GS) of *R. meliloti*.

Another objective of the work was to clone the *R. etli* structural gene(s) which code for GOGAT and to study the role of GOGAT in the *R. etli* bean symbiosis. We identified a clone from a *R. etli* gene bank which contains GOGAT the structural gene (*gltB*) in a 12 kb insert. This plasmid complements the glutamate auxotrophy of both an *E. coli* GDH⁻; GOGAT⁻ mutant strain and the *R. meliloti* AK330 (GOGAT⁻) mutant strain (Kondorosi *et al.* Mol. Gen. Genet. 151, 1977). Hybridization studies using *E. coli* specific gene probes indicate that the cloned DNA fragment of *R. etli* may contain, besides the structural GOGAT genes the regulatory *gltF* gene present in the *E. coli* operon. The cloned fragment was subcloned to a 8.5 kb fragment which still contains the genes of interest and this was used to obtain several MudlacZ insertions, which have been mapped. One of this insertions which interrupts the structural gene was subcloned into an integration vector for *Rhizobium* and experiment which will lead to the homogenotization of this gene fusion into the chromosome are being done. Strains with the MudlacZ insertion obtained will be used to study the regulation of GOGAT gene expression in *Rhizobium*. *R. etli* GOGAT⁻ mutant strains were obtained by random mutagenesis with Tn-5, selecting for glutamate auxotrophy. The mutant strain DEM300 was further characterized. It has a Tn-5 insertion in the 3' end of the *gltB* structural gene. It is a glutamate auxotroph and shows a normal symbiotic phenotype, similar to what has been reported for *R. meliloti* AK330 mutant strain.

The substitution of the natural ammonium assimilation pathway of *Rhizobium* (GS-GOGAT) by the GDH-GS pathway was achieved by conjugating the pAM1a plasmid (bearing *gdhA*) into the GOGAT⁻ mutant strains of *R. etli* (DEM300) and of *R. meliloti* (AK330). The *gdhA* gene is expressed and complements the glutamate auxotrophy of both strains. The expression of GDH in the *R. etli* GOGAT⁻ mutant strain inhibits nodulation of beans. The minimum *Rhizobium* inoculum that gives optimal nodulation was used. When a higher inoculum of *R. etli* DEM300/pAM1a or *R. meliloti* AK330/pAM1a was used to inoculate beans or alfalfa a normal symbiotic phenotype was observed but the nodule efficiency showed that the pAM1a plasmid was totally lost. Again, this data indicate a strong selection against the pAM1a (GDH) plasmid by the plant.

The last objective of the project was to determine if the expression of ammonium assimilation enzymes in the bacteroid is compatible with symbiotic

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nitrogen fixation. For this purpose we propose to construct chimeric genes with the coding region of GDH or GS fused to a *Rhizobium* nitrogen fixation promotor. We choose a *P. etli nifH* promotor which has been characterized in Dr. Mora's group, it is highly expressed in the bacteroid at the beginning of nitrogen fixation This part of the project was done in collaboration with Dr. Gary Roberts from University of Wisconsin, in a visit to his lab from one of us, where we learned from his experience in doing bacterial gene fusions. We obtained the *nifHprom-gdhA* gene fusion and cloned it in the stable vector (pTR101) for *Rhizobium*. We will study the symbiotic phenotype of different *Rhizobium* species bearing this plasmid.

**Work plan and time schedule**
**(actual)**

Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services, training).

1. The effect of the stable and constitutive expression of GDH in *Rhizobium*.

| | First Year | Second Year |
|---|---|---|
| Cloning of the *E. coli gdha* gene into a stable vector for *Rhizobium*: pAM1a plasmid | X | |
| Study of the free-life and symbiotic phenotypes of the *R. etli* strain expressing GDH | X | |
| Study of the *R. etli nod*-gene induction in response to nitrogen. | | X |
| Study of the role of *ntrC* in the nitrogen regulation of nodulation in *R. etli*. | | X |
| Study of the free life and symbiotic phenotypes of *R. meliloti* expressing GDH study of the instability of the plasmid with GDH in the presence of plant exudates. | | X |

2. The role of GOGAT in the *R.etli*-bean symbiosis

| | | |
|---|---|---|
| Cloning and partial characterization of the GOGAT(s) gene(s) of *R. etli*. | X | |
| Obtention of *R. etli* DEM300, GOGAT$^-$ mutant strain (Tn-5 insertion in *gltB*) | | X |
| Study of the free-life and symbiotic phenotype of *R. etli* DEM300. | | X |

3. The substitution of the *Rhizobium* GS-GOGAT ammonium assimilation pathway.

| | | |
|---|---|---|
| Conjugation of pAM1a (GDH) plasmid into *R. meliloti* and *R. etli* GOGAT mutant strains. | | X |
| Study of the free-life and symbiotic phenotypes of *R. meliloti* and *R. etli* expressing the GDH-GS pathway. | | X |

4. Is the expression of ammonium assimilation enzymes in the bacteroid compatible with nitrogen fixation?

| | First year | Second Year |
|---|---|---|
| Construction of a chimeric gene with the *nifHc* promotor from *R. etli* and *gdhA* from *E. coli*. | | X |
| Cloning of such chimeric genes into stable vector for *Rhizobium* and conjugation into *R. etli* and *R. meliloti*. | | X |

5. The modification of GS expression and the study of its regulation during symbiosis.

| | First Year | Second Year |
|---|---|---|
| Subcloning of the *glnII* gene of *R. etli* and its expression in *R. meliloti*. | | X |
| Intention to construct constitutive promotors-*gln* gene fusion. | X | |

## NETWORKING

THIS PROJECT WAS NOT DONE IN COLLABORATION WITH ANOTHER AFFILIATED CENTER.

## PUBLICATIONS


- Palacios, R., Mora, J. and Newton, W. NEW HORIZONS IN NITROGEN FIXATION. Kluwe Academic Publishers. Dordrecht, The Netherlands.

- Mendoza, A., Leija, A., Hernández, G. and Mora, J. THE ENHANCEMENT OF AMMONIUM ASSIMILATION IN *Rhizobium etli* PREVENTS NODULATION OF *Phaseolus vulgaris*. Submitted to Molecular Plant-Microbe Interactions, 1994.

**Work plan and time schedule (actual)**

Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services, training)

The results of this project have been presented on three international meetings: Mendoza, A., Hernández, G. and Mora, J. THE EFFECT OF THE EXPRESSION OF AMMONIUM ASSIMILATION ENZYMES IN *Rhizobium* DURING SYMBIOSIS. VII PAABS Congress. (Panamerican Biochemical Society). Ixtapa, México. September 27-October 2, 1992.

Mendoza, A., Leija, A., Hernández, G. and Mora, J. THE EXPRESSION OF *E. coli* GLUTAMINE DEHYDROGENASE (GDH) IN *Rhizobium phaseoli* INHIBITIS NODULATION. 9TH International Congress on Nitrogen Fixation. Cancún, México. December 6-12, 1992.

Mendoza, A., Reyes, J., Castillo, A., Leija, A., Hernández, G. and Mora, J. THE SUBSTITUTION OF THE AMMONIIUM ASSIMILATION PATHWAY (GS-GOGAT) BY THE GDH-GS PATHWAY IN *Rhizobium meliloti* AND *Rhizobium etli*. 1st EUROPEAN NITROGEN FIXATION CONFERENCE. Szeged, Hungary. August 29-September 2, 1994.

There has been one publication, in a book, about this project:

Mendoza, A., Leija, A., Hernández, G. and Mora, J.
THE EXPRESSION OF *E. coli* GLUTAMINE DEHYDROGENASE (GDH) IN *Rhizobium phaseoli* INHIBITS NODULATION. In: "New Horizons in Nitrogen Fixation". R. Palacios, J. Mora and W. Newton (eds.). Kluwer Academic Publishers. Dordrecht, The Netherlands. p. 244, 1993.

In addition, the manuscript entitled "The Enhancement of Ammonium Assimilation in *Rhizobium etli* prevents nodulation of *Phaseolus vulgaris*" by A. Mendoza, A. Leija, G. Hernández and J. Mora has been resubmitted (after doing the corrections suggested by the editor) to Molecular Plant-Microbe Interactions.

Several scientists that participated in the project have been trained in different labs or have asisted to international scientific meetings:

- Dr. Alberto Mendoza, assistant professor, visited the lab of Dr. Gary Roberts, University of Wisconsin, during one month and was trained in molecular cloning techniques to perform chimeric gene constructs using nitrogen fixation gene promotors and genes for ammonium assimilation enzyme.

- M.S.Mario Ramírez, a graduate student that participates in this work, received training in México city (Escuela Nacional de Ciencias Biológicas) and was funded by this project.

- Dr. Jaime Mora, professor, assisted to the ASM Conference and Interactive Behavior of Bacteria, Woodshole, MA, (USA) on March, 1993, funded by this project.

- Dr. Georgina Hernández, associate professor, assisted to the Gordon Conference on Plant Cell & Tissue Culture in Wolfeboro, NH (USA) on June, 1993 and to the 4th International Congress on Plant Molecular Biology, in Amsterdam, (The Netherlands), in June, 1994 funded by this project.

Part 4

## STATEMENT OF EXPENDITURES

| To be filled by ICGEB | | To be filled by the Affiliated Centre | |
|---|---|---|---|
| **Budgets as per original proposal** | | **Summary of expenditures** * | |
| 1) Capital equipment | US$ ..... | 1) Capital equipment | US$ 10975 |
| 2) consumables | US$ ..... | 2) consumables | US$ 24567 |
| 3) training | US$ ..... | 3) training | US$ 14458 |
| 4) literature | US$ ..... | 4) literature | US$ ..... |
| 5) miscellaneous | US$ ..... | 5) miscellaneous | US$ ..... |
| **TOTAL GRANT** | **US$.....** | **TOTAL** | **US$** 50000 |

**Please itemize the following budget categories (if applicable)**

Capital equipment

1 Microcentrifuge
1 Vortex
1 Freezer
1 Vacum pump
2 Computers (Macintosh Quadra 605)

Training (provide names, duration of training, host laboratory)

- Mario Ramírez (student) 2 years. National University of México, México city.
- Alberto Mendoza (investigator) 1 month. University of Wisconsin, USA, Dr. G. Roberts.
- Jaime Mora (investigator) 1 week. "ASM Conference on Multicellular and Interactive Behavior of Bacteria". Woodshole, MA. USA.
- Georgina Hernández (investigator) 1 week. Gordon Conference on Plant Cell & Tissue Culture, Wolbeforo, WH, USA. 1 week. International Congress on Plant Molecular Biology. Amsterdam, The Netherlands.

Literature

* Please do not send invoices, receipts etc.; these should be kept by the Affiliated Centre for future reference and sent to ICGEB upon request.