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Consultant Final Report
IICA/EMBRAPA-PROCENSUL II

OPTICAL AND ELECTRON MICROSCOPY AND IMMUNOCYTOCHEMIST IN THE EXAMINATION OF TRANSGENIC EXPRESSION

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OPTICAL AND ELECTRON MICROSCOPY AND IMMUNOCYTOCHEMISTRY IN THE EXAMINATION OF TRANSGENIC EXPRESSION

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IICA/EMBRAPA-PROCENSUL II

Nicholas Harris

Brasília, janeiro de 1989

INSTITUTO INTERAMERICANO DE COOPERAÇÃO PARA A AGRICULTURA
EMPRESA BRASILEIRA DE PESQUISA AGROPECUARIA

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APRESENTAÇÃO

A reprodução e difusão dos Relatórios de Consultores, no âmbito restrito das Diretorias das Unidades do Sistema Nacional de Pesquisa Agropecuária, vinculado à EMBRAPA, tem como objetivo principal o de divulgar as atividades desenvolvidas pelos consultores e as opiniões e recomendações geradas sobre os problemas de interesse para a pesquisa agropecuária.

As atividades de consultoria são realizadas no âmbito do Projeto de Desenvolvimento da Pesquisa Agrtopecuária e Difusão de Tecnologia na Região Centro-Sul do Brasil - PROCENSUL II, financiado parcialmente pelo Banco Interamericano de Desenvolvimento - BID e a EMBRAPA conforme os contratos de Empréstimo 139/IC-BR e 760/SF-BR, assinados em 14 de março de 1985 entre o Governo Brasileiro e o BID.

As opiniões dos consultores são inteiramente pessoais e não refletem, necessariamente, o ponto de vista do IICA ou da EMBRAPA.

A coordenação dos Contratos IICA/EMBRAPA agradeceria receber comentários sobre estes relatórios.

Horacio H. Stagno
Coordenador Contratos IICA/EMBRAPA

INTER-AMERICAN INSTITUTE FOR COOPERATION ON AGRICULTURE
IICA/EMBRAPA CONTRACT

CONSULTANT FINAL REPORT

1. Consultant's full name: *Nicholas Harris*

2. Specialist in: *Eletronic microscopie and immonocitochemistry*

3. Title of IICA Project: *2.SB.3*

4. EMBRAPA Program for which consultancy is provided:

PROGRAMA : PROCENSUL II

SUB-PROGRAMA: 05-Recursos Genéticos/Controle Biológico

IICA Project Activity Code: *2.SB.3.05*	Administrative Code: *R 4884 B1B 03105*
Title of Activity of IICA Project corresponding to this consultancy	*Cooperation with EMBRAPA on research and applications of genetic resources, biotechnology and biologic control of plagues, diseases and weeds.*

CONSULTANT CONTRACT PERIOD	DUTY LOCATION (Center)
November 20th to December 16th., 1988	*CENARGEN*
CONTRACT EXTENTION PERIOD (If any)	DUTY LOCATION (Center)

5. Financial support: *PROCENSUL II*

6. ACTIVITIES UNDERTAKEN BY THE CONSULTANT AND RESULTS

6.1 RESEARCH DONE UNDER DIRECT RESPONSIBILITY OF THE CONSULTANT

Research activities developed	Results Achieved

During the period of the consultancy a number of research topics were examined, including:

6.1.1. Examination of samples of seed obtained from *Nicotiana* plants possibly transformed with the Brazil nut 2S gene: by optical and electron microscopy and immunocytochemistry

Background Examination of the 'when and where' of transgenic expression in plants is of great interest to both the academic and practical application and exploitation of the potential of this new bio-technology. To be successful, transgenic expression of particular proteins may require targeting to specific tissues or cell types; this requires both a knowledge of the interaction of the various promoters and targeting sequences, and assay of the temporal and spatial distribution of the transgenic proteins. The latter is determined by immunocytochemical techniques; an introduction to the background and general protocols has been given in a previous report to IICA (ref 2.SB.3.05 / R 4884 B1B 03105 from 11-21st April 1988).

Results Samples 1, 2 and 3 were provided from PGS, Belgium via Dr E Gander (EMBRAPA/CENARGEN) of which two were thought to be derived from *Nicotiana tabaccum* plants transformed with gene for the 2S Brazil nut high methionine protein, the third sample being from an untransformed control plant. The backgrounds of the seed samples were however not known nor was any definitive information available as to the extent or level of expression of the transgenic protein. Samples were however fixed for immunocytochemical examination, following protocols developed during a previous IICA supported visit to EMBRAPA/CENARGEN. After dehydration and embedding in a suitable acrylic resin, semi-thin sections were cut and the general histology examined by light microscopy. All samples exhibited a normal development of embryo tissues and endosperm storage reserves.

Antisera available for immunocytochemical examination of the seed sections had been shown to be specific for the 2S protein amongst a total extract of Brazil nut (by 2D gel) but it was not known whether there was any cross reactivity with any endogenous *Nicotiana* proteins. Nevertheless sections of the three samples were examined using LM immunocytochemical protocols with final visualisation by silver enhancement of a secondary antibody-gold colloid conjugate, and also by EM immunocytochemistry using secondary antibody-gold conjugates. Results obtained were not conclusive; all samples showed some seeds with a possible very low level of staining, thus indicating some non-specific labelling of endogenous *Nicotiana* proteins, but there was also some apparent variation within the samples. Without information on the background of the seed (e.g. as from a single seed ELISA study to determine the proportion of seeds with transgenic expression, and the relative level of transgenic expression over any non-specific background) it was not possible to form any conmclusions. Further information obtained later during the consultancy, from PGS Belgium, indicated that they had not been able to demonstate any transgenic protein expression in any of the samples provided, either by blotting or by immunocytochemistry carried out in Dr Engler's lab. Work on these samples was (cont

thus discontinued, although they had proved very useful for demonstrating both the problems that can arise during immunocytochemical localisation studies and also the various aproaches that can be used in attempts to overcome such problems (see 6.4.2.)

6.1.2. Examination of a series of samples of *Bacillus sphaericus* by electron microscopy: in association with Dr M Cabral and P Vilarinhos EMBRAPA/CENARGEN.

Background *Bacillus sphaericus*, like *B. thuringiensis*, produces protein crystals which, when degraded by the alkaline pH and proteases of the insect larval gut, generate toxins which kill the larvae. There is considerable scientific, practical and comercial interest in the production of such crystals and their application in biological control (ref eg review by Aronson et al. (1986) Microbiol Rev. 50: 1-24). Recently a new stain of *B. sphaericus* has been discovered at EMBRAPA/CENARGEN and a series of samples were examined to determine i) whether the new strain produces such crystals, and if so, ii) at what stage during the cell cycle, with particular emphasis on the timing in relation to sporulation. A preliminary ultrastructural study had already been carried out by E. Santana of EMBRAPA/CENARGEN

Results Seven samples were examined forming a time series of the development of organisms in culture. The samples represented 1) lag phase 2) beginning of log phase 3) mid of log phase 4) end of log phase / early sporulation 5) sporulation 6) lysis i, and 7) lysis ii. Samples were fixed in buffered aldehyde mixture and post fixed with osmium tetroxide prior to dehydration and embedding in Spurr resin. Sections revealed the ultrasture of the organism at the various stages of development, including the generation of both spores and crystalline inclusions within the cells from some samples. The work and results have been further detailed in a report to CENARGEN.

6.1.3. Examination of cultured Baculovirus, associated nuclear polyhedral bodies, and alkali-released virions from polyhedral bodies: in association with Dr M Pinheiro EMBRAPA/CENARGEN.

Background Among the interesting and important academic and practical applications associated with Baculovirus in their role in biological control, EMBRAPA/CENARGEN has already established a programme investigating the multiplication of Baculovirus in cultured insect cells. A preliminary ultrastructural study had already been undertaken by E. Santana to i) illustrate the development of virions and polyhedral bodies (PHB) during culture of the infected insect cells, ii) examine by negative staining isolated viruses, PHBs, and alkali/protease-released viruses (ARVs) from PHBs. Some practical problems associated with specimen preparation had been encountered with ii).

Results Variations in specimen preparation were tested and appropriate protocols for the negative staining of the viruses, polyhedral bodies and ARVs determined. These protocols have been detailed in a more extensive report of praticals details held by the appropriate EMBRAPA/CENARGEN staff. The work also indicated variations in the form of the ARVs dependent upon the method of their product from the PHBs from infected, cultured insect cells.

6.1.4. Examination of the structure of cocoyam corms and the localisation of specific gene transcription and translation products

Background As part of the intensive international studies into the control of plant gene expression with regard to possible exploitation in manipulation of transgenic expression, there is particular interest in the identification and isolation of sequences which confer tissue-specific or tissue-enhanced expression. In this context work by Drs LA Castro and M Carneiro at EMBRAPA/CENARGEN has identified sequences of potential significance to the control of gene (cont

expression during root/corm development. The temporal and spatial patterns of expresion of these genes need to be determined to assess their possible application in transgenic targeting to specific organs or tissues.

Three experiments have been initiated: i) an examination of the histology, cytology and ultrastructure of the developing corms, ii) an immunocytochemical investigation of the distribution of the specific protein of interest in the developing corms and iii) an ivestigation by hybridisation histochemistry of the distribution of the mRNA for the corm-enhanced protein of interest.

Results A series of developing corms of increasing size were fixed for histochemical, cytochemical, ulrastuctural and histochemical investigations. Tissue was fixed and, after prolonged dehydration and infiltration, embedded in wax and also in resin. Preliminary results were obtained showing histology of the developing corms. The pattern of localisation of storage reserves was found to be related to both the age of the developing corms and the form of the differentiating vascular system within the corms; preliminary ultrastructural studies indicated the subcellular location of the predominant storage reserves within the corm. Tissue was also prepared for the immunocytoogical and hybridisation histochemistry. Preliminary immunolocalisation studies were performed using an antiserum specific to the corm protein. This indicated some staining associated with the storage reserves although the level of staining was low; because of time restriction further experimentation was not possible during the consultancy although appropriate variations in protocols to improve 'signal' were discussed and the work should be carried out successfully. Unfortunately radiolabelling for generation of appropriate probes for the in situ hybridisation was not available during the consultancy but the methodology was described and discussed in detail (see 6.4.3). Full details of protocols employed during the consultancy, and the variations that are appropriate to their use with other differentiating tissues have been left in a report held by various EMBRAPA/CENARGEN staff. In particular attention was drawn to the variations sometimes required in specimen preparation, particularly fixation, to maximise the retention of histological and cytological detail but also allow for appropriate histochemical staining. A full range of publication references to the application of these techniques was also included.

6.2 SUPPORT TO RESEARCH UNDERTAKEN BY OTHER EMBRAPA RESEARCHERS

Research activities developed	Results achieved

During the period of the consultancy a number of research topics were discussed with other EMBRAPA workers and in some instances preliminary experimental work undertaken; such discussions included

6.2.1. Fixation techniques for the examination of cryo-preserved (mouse) embryos - with Dr R DeBem

As part of his doctorate studies in Paris, Dr DeBem had undertaken some ultrastructural investigations of normal and cryo-preserved embryos. This work was of high quality and indicated the potential significance of the Golgi-Endoplasmic reticulum-Lysosome system in the loss of embryo viability. A series of similar experiments, to be undertaken at EMBRAPA/CENARGEN were planned by Dr DeBem; some points of potential flexibility in the protocols which should aid the experimentation were pointed out. Attention was also drawn to the potential value of the zinc-iodine -osmium tetroxide post-fixation method which is particularly useful for studying the inter-relationships between the GERL and other parts of the endomembrane system. The detail of the protocol was discussed and appropriate references provided (eg Harris and Oparka (1983) Protoplasma 114: 93-102)

6.2.2. Examination of cyanophages by electron microscopy

Appropriate methods for the negative staining and examination of very small volumes of samples of cyanophages, which are currently under investigation at EMBRAPA/CENARGEN, were discussed and carried out.

6.2.3. Fixation techniques for the examination of insect eggs - with Dr E Fonz

The phase partition method of specimen fixation was explained and samples of insect egg were duly processed. Because of the extended nature of resin infiltration required for such samples the specimens were not examined during the consultancy but potential variations and adaptaions of the method were explained should the initial protocol require further modification.

6.2.4. Methods for examination of fungally-infected insects to determine distribution of hyphae and conidia - with Dr M Tigano

Samples of fungally-infected insects had been prepared by E. Santana of EMBRAPA/CENARGEN. The samples showed good retention of fungal structure etc but they had not provided the required information on the relationship between the fungal hyphae, fruiting bodies and insect exoskeleton. This was merely due to the fact that the sectioning had not been fortunate enough to cut in an appropriate part of the samples. Methods to minimise the time input required to obtain sections through an appropriate plane were explained; these involve a combination of semi-thin sectioning and conventional thin sectioning, but may still require a substantial amount of sectioning before 'striking lucky' (alternatively the appropriate section may be obtained very quickly!)

6.3 TRAINING ACTIVITIES DEVELOPED BY THE CONSULTANT

Date	Training subject matter	Type of event*	Number of beneficiaries	
			From EMBRAPA	From other institutions

The training provided is described more appropriately in 6.4.

* Short courses, seminars, conferences, etc.

6.4 IN-SERVICE TRAINING PROVIDED BY THE CONSULTANT

In-service training subject matter	Names of counterparts

6.4.1. Techniques for negative staining of samples for ultrastructural analysis

Increasing use is being made of the negative staining technique for examination of, for example, baculovirus, cyanophage and bacteria samples being produced at EMBRAPA/CENARGEN. Some excellent results had already been obtained by E Santana but there were, on some occassions, various problems resulting in problems in image interpretation. The causes of such problems were discussed, the advantages of taking a varied approach towards specific aspects of the preparation protocols were considered, and a series of samples were also prepared. This training was appropriate to work being carried out under 6.1.2, 6.1.3 and 6.2.2 above.

6.4.2. Immunocytochemistry at histological, cytological and ultrastructural levels

Following on from work initiated out under an earlier IICA contract (ref 2.SB.3.05 / R 4884 BIB 03105 from 11-21st April 1988) further instruction in the detailed application of immunocytochemical techniques to studies of plant development and

differentiation were given to E Santana and M Loureiro (EMBRAPA/CENARGEN). Particular attention was drawn to the flexibility required when preparing samples, and to the need to consider the size and nature of the tissues rather than just follow published protocols too rigourously.

6.4.3. Hybridisation histochemistry at histological and cytological levels.

With E. Santana and Marcelo Loureiro: the various criteria to be coinsidered when undertaking in situ hybridisation studies were discussed at length, and in particular the potential variations in protocol timings required for different types of tissues and experimental approaches. Tissue was prepared, and the importance of testing different fixation methods stressed. Unfortunately we were unable to prepare suitable radioactively labelled probes during the consultancy but this work is to carried out in the follow-up work at EMBRAPA/CENARGEN.

6.4.4. Variations in standard histological and electron microscopy protocols to accommodate 'unusual' samples

Eliana Santana undertakes the processing and ultrastructural examination of a wide range of biological samples at EMBRAPA/CENARGEN without the formal supervision of an academic with any extensive background in such work. Under these circumstances she has provided a service of high quality. Numerous discussion were held throughout the consultancy in which the variations in application of different EM methods were considered; in particular with regard to the matching of general outline procedures to the specific requirments of a particular tissue or problem as opposed to a strict following of a protocol which had been applied to different tissues or problems.

6.5 ACTIVITIES IN SUPPORT OF RESEARCH STRATEGY AND PLANNING

Research subject matter	Research program to which subject matter is concerned

6.5.1. Examination of material for possible transgenic expression

Various discussions were held with EMBRAPA/CENARGEN staff about the ananlysis of transgenic material by a range of immunological methods. Background references and specific protocols, for eg single seed ELISA assay, have been provided and are detailed in the technical report of the consultancy, copies of whichare held be yvarious members-of EMBRAPA/CENARGEN

6.5.2. Development of programme for establishing in situ hybridisation methodology

In association with 6.4.3, extensive discussions about the value and specific application of the in situ hybridisation method (particularly in association with parallel immunocytochemical studies) were held. Development and application of such work is planned (see section 9.)

6.5.3. Methodology and specific protocols for future histological, cytological and ultrastructural work

A range of projects were discussed with various EMBRAPA/CENARGEN staff with reference to the specific requirements and variations of appropriate methods of specimen preparation and examination (some detailed in sections 6.1 and 6.2 above

6.6 ACTIVITIES IN SUPPORT OF RESEARCH STRATEGY AND PLANNING
CENTERS LINKS WITH ABROAD

Subject matter on which links were recommended	Persons, centers and universities recomended for contact

6.7 PUBLICATIONS AND REPORTS UNDERTAKEN WITH THE CONSULTAT'S PARTICIPATION

Author(s)*	Title of publication or Report and other bibliografic identification

A series of publications and reports will be derived from the work undertaken during the consultancy, including the work on cocoyam, baculovirus and B. sphaericus. Work undertaken during the previous consultancy has now been the subject of conference proceedings, reports and some publications which are in the final stages of preparation prior to submission to journals.

* Personal, institutional, etc.

6.8 SUPPORT PROVIDED TO EMBRAPA RESEARCHERS IN THESIS AND DISSERTATION WORK

Name of the student	Thesis subject matter and sinthesis of advice

8. CONSULTANT'S SUGGESTIONS AND TECHNICAL OR INSTITUTIONAL RECOMMENDATIONS FOR THE IMPROVEMENT OF THE RESEARCH SERVICE

A general scientific background to some of the major research programmes being undertaken at EMBRAPA/CENARGEN, and involving the histological, histochemical and ultrastructural studies, has been given in an earlier report to IICA, and others are outlined above in 6.1.
Similarly, some recommendations concerning the establishment of staff and management procedures for the EM unit have also been made previously.

During this consultancy it was apparent the the EM-associated technician (Eliana Santana) was beginning toprovide the research groups with a good service with regard to their requirements for backup ultrastructural information. She has proved keen and willing to learn and adapt new procedures, and is presently limited by the lack of an appropriate supervisor with an extensive background in histological and cytological work; some help and advice is however available from UnB and the two laboratories seem to have a useful inter-relationship. It might be appropriate for EMBRAPA to consider the possibility of sending Eliana for a period of further training in the application of histological, histochemical and ultrastructural techniques to one or more laboratories where advanced techniques appropriate to those required by the research programmes at EMBRAPA are currently being undertaken. The initiation of such a training programme might be more appropriate to the specific needs of the EMBRAPA/CENARGENresearch groups than for example the training of staff who are currently employed only on short term contracts and are possibly likely to leave. Alternatively the appointment of a senior member of staff, or the continuation of visits by appropriate consultants would be other methods by which her developing expertise could be encouraged and promoted.

9. AGREEMENTS OR COMMITTMENTS ESTABLISHED WITH EMBRAPA RESEARCHERS IN-SERVICE OF THE FUTURE DEVELOPMENT OF RESEARCH IN THE CONSULTANT'S FIELD OF SPECIALIZATION

Following some general discussions about the potential desirability of continued collaborations between the consultant's research group and various EMBRAPA groups, two specific programmes were determined for future collaborations, including the possibilities of exchange of staff and joint research funding applications.

10. CONSULTANT'S COMMENTS ON CIRCUMSTANCES WHICH AFFECTED THE CONSULTANCY WORK

It is again a great pleasure to acknowledge the enthusiasm, keen interest and help and assistance provided by the scientific staff and workers at EMBRAPA/CENARGEN. It may be apparent from the above that a wide and extensive programme of work was undertaken during the consultancy and some interesting and (hopefully) valuable results obtained; such a programme would not have been possible without the keen participation of all of those involved. It is perhaps a little unfair to pick out specific names but I should record the valuable help of Eliana Santana, the considerable enthusiasm of a very able student Marcello Loureiro, and the scientifically valuable interactions with Drs Cabral, Carneiro, Pinheiro and Paulo Vilarinhos. Last but by no means least, it is true to say that without the considerable help and efforts of Dr MJA Sampaio I have no doubt that the visit would have either not taken place at all or, at best, been far less successful than it was.

Date: Signature

Programa II. Geração e Transferência de Tecnologia

O Programa de Geração e Transferência de Tecnologia é a resposta do IICA a dois aspectos fundamentais: (i) o reconhecimento, por parte dos países e da comunidade técnico-financeira internacional, da importância da tecnologia para o desenvolvimento produtivo do setor agropecuário; (ii) a convicção generalizada de que, para aproveitar plenamente o potencial da ciência e da tecnologia, é necessário que existam infra-estruturas institucionais capazes de desenvolver as respostas tecnológicas adequadas às condições específicas de cada país, bem como um lineamento de políticas que promova e possibilite que tais infra-estruturas sejam incorporadas aos processos produtivos.

Nesse contexto, o Programa II visa a promover e apoiar as ações dos Estados membros destinadas a aprimorar a configuração de suas políticas tecnológicas, fortalecer a organização e administração de seus sistemas de geração e transferência de tecnologia e facilitar a transferência tecnológica internacional. Desse modo será possível fazer melhor aproveitamento de todos os recursos disponíveis e uma contribuição mais eficiente e efetiva para a solução dos problemas tecnológicos da produção agropecuária, num âmbito de igualdade na distribuição dos benefícios e de conservação dos recursos naturais.

INSTITUTO INTERAMERICANO DE COOPERAÇÃO PARA A AGRICULTURA

O Instituto Interamericano de Cooperação para a Agricultura (IICA) é o organismo especializado em agricultura do Sistema Interamericano. Suas origens datam de 7 outubro de 1942, quando o Conselho Diretor da União Pan-Americana aprovou a criação do Instituto Interamericano de Ciências Agrícolas.

Fundado como uma instituição de pesquisa agronômica e de ensino, de pós-graduação para os trópicos, o IICA, respondendo às mudanças e novas necessidades do Hemisfério, converteu-se progressivamente em um organismo de cooperação técnica e fortalecimento institucional no campo da agropecuária. Essas transformações foram reconhecidas oficialmente com a ratificação, em 8 de dezembro de 1980, de uma nova convenção, que estabeleceu como fins do IICA estimular, promover e apoiar os laços de cooperação entre seus 31 Estados membros para a obtenção do desenvolvimento agrícola e do bem-estar rural.

Com um mandato amplo e flexível e com uma estrutura que permite a participação direta dos Estados membros na Junta Interamericana de Agricultura e em seu Comitê Executivo, o IICA conta com ampla presença geográfica em todos os países membros para responder a suas necessidades de cooperação técnica.

As contribuições dos Estados membros e as relações que o IICA mantém com 12 Países Observadores, e com vários organismos internacionais, lhe permitem canalizar importantes recursos humanos e financeiros em prol do desenvolvimento agrícola do Hemisfério.

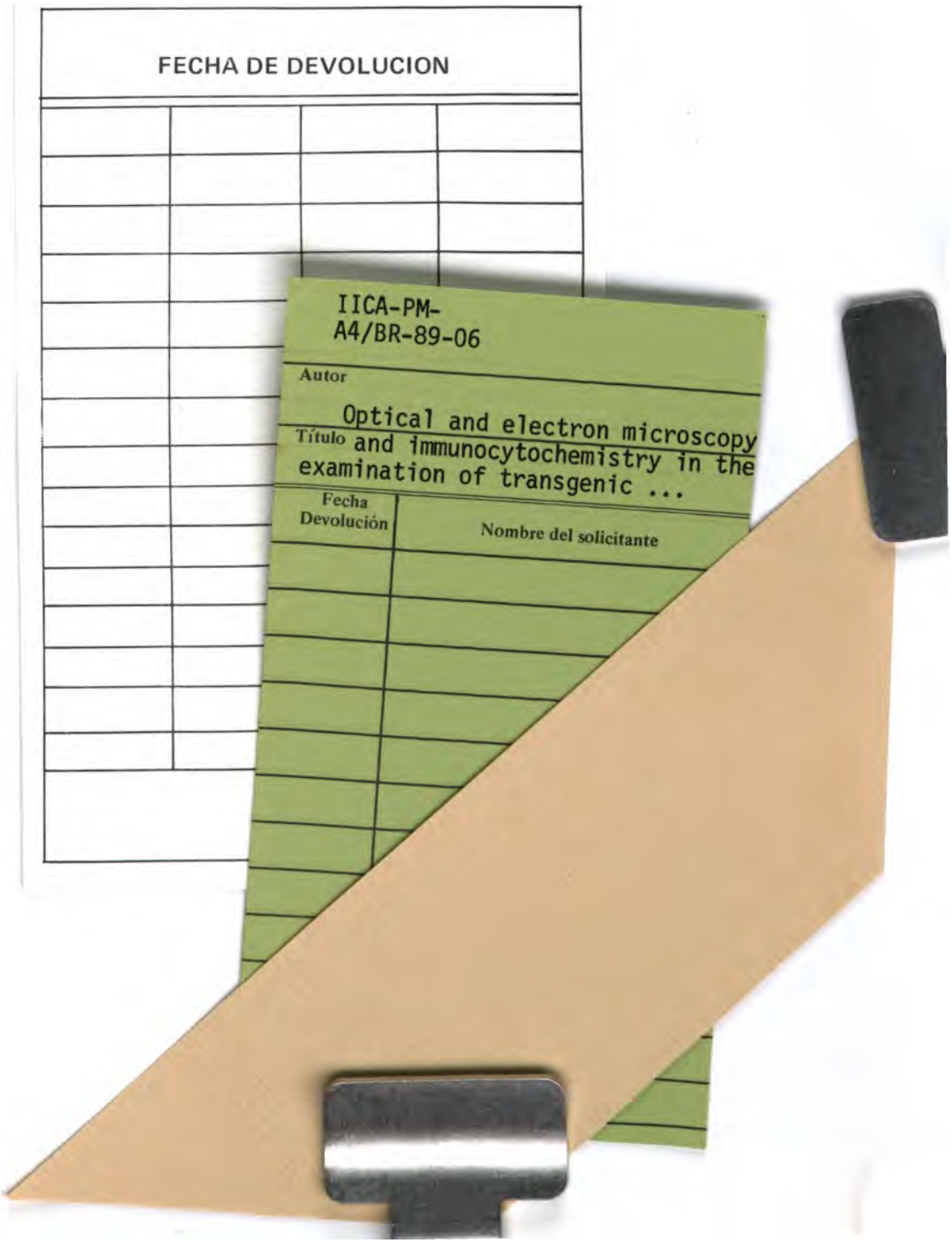
O Plano de Médio Prazo 1987-1991, documento normativo que assinala as prioridades do Instituto, enfatiza ações voltadas para a reativação do setor agropecuário como elemento central do crescimento econômico. Em vista disso, o Instituto atribui especial importância ao apoio e promoção de ações tendentes à modernização tecnológica do campo e ao fortalecimento dos processos de integração regional e sub-regional.

Para alcançar tais objetivos o IICA concentra suas atividades em cinco áreas fundamentais, a saber: Análise e Planejamento da Política Agrária; Geração e Transferência de Tecnologia; Organização e Administração para o Desenvolvimento Rural; Comercialização e Agroindústria, e Saúde Animal e Sanidade Vegetal.

Essas áreas de ação expressam, simultaneamente, as necessidades e prioridades determinadas pelos próprios Estados membros e o âmbito de trabalho em que o IICA concentra seus esforços e sua capacidade técnica, tanto sob o ponto de vista de seus recursos humanos e financeiros, como de sua relação com outros organismos internacionais.

Esta publicação foi reproduzida na Gráfica do Escritório do IICA no Brasil, em Brasília, em janeiro de 1989, numa tiragem de 100 exemplares.

Responsáveis pela reprodução: Jadir José dos Santos e Murillo Sodré da Silva.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