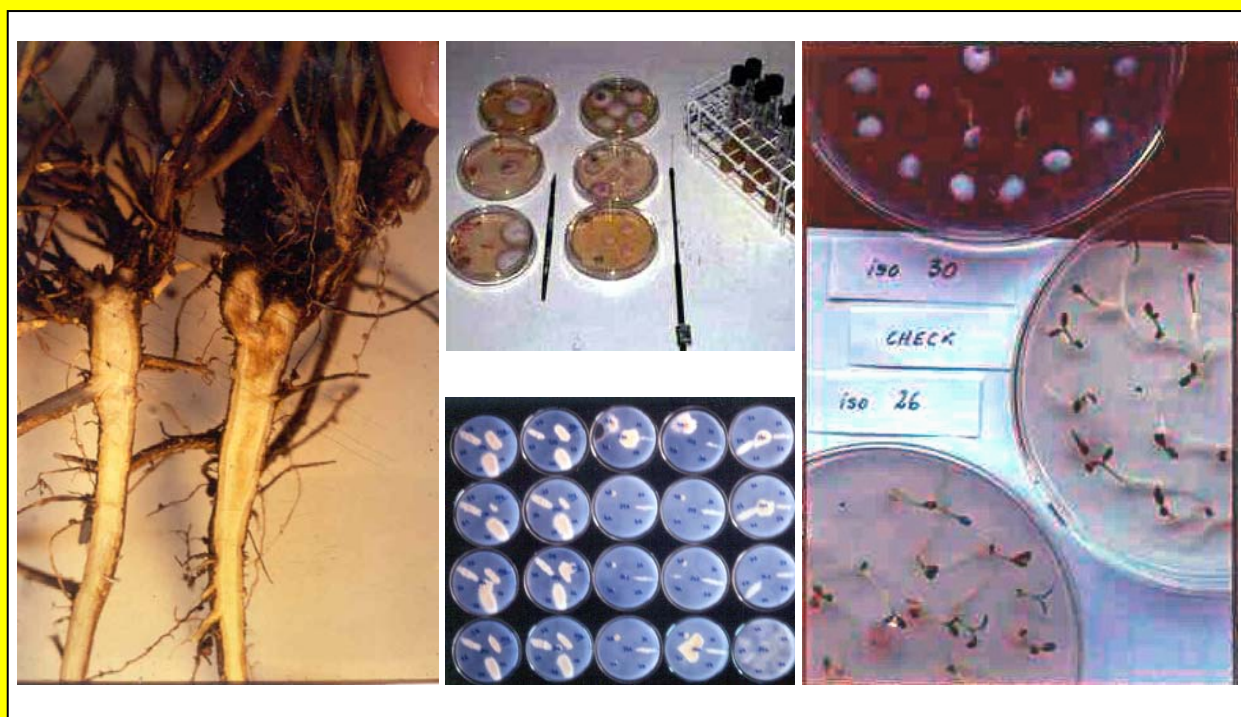


LOTUS NEWSLETTER 2005
Volume 35, Number 1



Editor: M. Rebuffo
INSTITUTO NACIONAL DE
INVESTIGACION AGROPECUARIA





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Front cover: The photographs on the front cover shows the symptoms of crown and root rot in a diseased birdsfoot trefoil plant (left), which was recorded during the epidemiological studies carried out in Uruguay by Altier and Kinkel (pp. 42-58). Middle-up: the most frequently and consistently isolated species from diseased crown and root tissues was *Fusarium oxysporum*. Middle-down: The lack of complementation among the isolates that composed the *F. oxysporum* population associated with birdsfoot trefoil indicated a large genetic diversity, as measured by vegetative compatibility. Right: A culture plate method was used by Altier and Groth (pp. 59-74) to characterize *F. oxysporum* isolates for aggressiveness to seeds and seedlings of birdsfoot trefoil: significant differences were observed among the isolates.

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Contents

Contents	i
Newsletter Announcements and Instructions	iv
Personalia	vi

Conference announcement

P.M. GRESSHOFF. Third International Conference on Legume Genomics and Genetics: From Genes to Crops	1
-----------------------------------------------------------------------------------------------------	---

Workshop abstracts

O. RUIZ (Chairperson). Interdisciplinary workshop on genetic, molecular and eco-physiological aspects of <i>Lotus</i> spp. and their symbionts. [Taller interdisciplinario sobre aspectos genéticos, moleculares y fisiocológicos del <i>Lotus</i> spp. y sus simbioses].	3
E. CAMADRO. Theoretical bases for the elaboration of a breeding program. <i>Lotus glaber</i> as an example.	7
M. REBUFFO. Plant breeding: <i>Lotus corniculatus</i> and <i>Lotus uliginosus</i> .	9
H. ACUÑA and A. CONCHA. Condensed tannin concentrations in <i>Lotus</i> spp.	11
A. CLÚA, M. BARRAGÁN, M.S. TACALITI, D. GIMÉNEZ and A.M. CASTRO. Assisted and traditional assessment of saline stress tolerance in <i>Lotus glaber</i> .	13
M. SISTERNA and G.A. LORI. Fungal diseases on <i>Lotus</i> spp in Argentina.	15
A. ALIPPI. Bacterial diseases of <i>Lotus</i> spp.	17
F. CASSÁN, V. LUNA and O.A. RUÍZ. Physiological studies of tolerance to saline stress in <i>Lotus glaber</i> and their correlation with the establishment and efficiency of the symbiotic association with <i>Mesorhizobium loti</i> .	19
C. LABANDERA and M. JAURENA. Rhizobiology research in <i>Lotus</i> species.	21
A.I. SANNAZZARO, A.B. MENÉNDEZ, E. ALBERTÓ and O.A. RUIZ. The arbuscular mycorrhizal fungi of <i>Lotus glaber</i> .	22
R. MENDOZA. Seasonal variation of arbuscular mycorrhizal fungi in temperate grasslands along a wide hydrologic gradient.	23
D. COGLIATTI, L. LETT, M. BARUFALDI, P. SEGURA and J. CARDOZO. Growth, Nitrogen and Phosphorus economy in two <i>Lotus glaber</i> Mill. Populations grown under contrasting P-availability.	24

G.G. STRIKER, P. INSAUSTI and R.J.C. LEÓN. Comparative responses between <i>Lotus glaber</i> and <i>Paspalum dilatatum</i> to the flooding-grazing interaction.	25
A. ANDRES, B. ROSSO and O. SCHENEITER. Morpho-physiological characterization of <i>Lotus glaber</i> naturalized populations.	27
A.I. SANNAZZARO, E. ALBERTÓ, O.A. RUIZ and A.B. MENÉNDEZ. Influence of the arbuscular mycorrhizal fungus <i>Glomus intraradices</i> on the saline stress physiology of <i>Lotus glaber</i> .	29
R. PAZ, D.H. SANCHEZ, F. PIECKENSTAIN, S. MAIALE, A. SANNAZZARO, J. CRUZ CUEVAS, A. CHIESA, G. BONA and O.A. RUIZ. Molecular and biochemical approximation of polyamine roles in tolerance mechanisms to salt stress in <i>Lotus</i> spp.	31
P.A. SANSBERRO, F.D. ESPASANDIN, C.V. LUNA and L.A. MROGINSKI. Adventitious shoot regeneration in <i>Lotus glaber</i> Mill.	33
A.V. AVILA JACQUES and I. HERINGER. Fire on native pastures - effects on soil and vegetation.	35
M. BAILLERES. Is arriving the <i>Lotus glaber</i> time in the pampa deprimida del salado?	36
M. DALL´AGNOL and S.M. SCHEFFER-BASSO. Perspectives of utilization of native legumes in Rio Grande do Sul.	37
O.R. VIGNOLIO. <i>Lotus glaber</i> productivity changes under different management conditions.	39

Feature articles and reports

N.A. ALTIER and L.L. KINKEL. Epidemiological studies on crown and root rot of birdsfoot trefoil in Uruguay.	42
N.A. ALTIER and J.V. GROTH. Characterization of aggressiveness and vegetative compatibility diversity of <i>Fusarium oxysporum</i> associated with crown and root rot of birdsfoot trefoil.	59
J. REYNAUD and M. LUSSIGNOL. The Flavonoids of <i>Lotus corniculatus</i>	75
P. DÍAZ, O. BORSANI, A. MÁRQUEZ and J. MONZA. Nitrogen metabolism in relation to drought stress responses in cultivated and model <i>Lotus</i> species.	83
V.C. LEPEK and A.L. D´ANTUONO. Bacterial surface polysaccharides and their role in the rhizobia-legume association.	93
S.L. GREENE. U.S. Germplasm Collection of <i>Lotus</i> : Activities over the last decade.	106
C. PACIOS-BRAS. The symbiosis between <i>Lotus japonicus</i> and rhizobia: Function of nod factor structural variation. [Spanish version]	109

- R. MENDOZA, I. GARCÍA and V. ESCUDERO. Can the symbiosis between arbuscular mycorrhiza and *Lotus glaber* tolerate waterlogging in a saline-sodic soil? 115
- S. HERNÁNDEZ, M. REBUFFO, S. ARRIVILLAGA, M. JAURENA, C. LABANDERA, D. RISSO and J. CILIUTI. Evaluation of the genotype-environment interaction in the establishment of *Lotus uliginosus* (Schkuhr) with soil-cores. [Spanish version] 120

Research centers and projects

- P.M. GRESSHOFF. The Australian Research Council's Centre of Excellence for Integrative Legume Research 131
- J. SANJUÁN and M. REBUFFO. The LOTASSA proposal: the success of enthusiasm and tenacity. 134
- D. REAL, G.A. SANDRAL, J. WARDEN, M. REBUFFO, D.F. RISSO, J.F. AYRES, W.M. KELMAN and S. J. HUGHES. Breeding birdsfoot trefoil for Mediterranean-type environments in Southern Australia 136
- Lotus* activities: Background and present research 138
- Current list of *Lotus* researchers. [Database last updated August 30 2005](#) 143

Newsletter Announcements and Instructions

The Lotus Newsletter has been published annually since 1971 by Dr. W.Grant - Canada, Dr. R.McGraw and Dr. P.Beuselinck - USA, and it has been on the web site of INIA, Uruguay since 2003. It is intended as a worldwide communication link for all those who are interested in the research and development of *Lotus* species. Persons interested in trefoil improvement, genetics, molecular biology, microbiology, production, marketing, or utilization are invited to contribute to the *Lotus* Newsletter. Previous issues may be used as a guide. It is expected that the work reported will be developed further and formally published later in refereed journals. It is assumed that contributions in LN will not be cited unless no alternative reference is available.

The life of the Newsletter depends on the contributions. Therefore, many thanks to all contributors and to those that wrote the summary about their activities. In response to several contributors, that requested a more frequent edition of LN, two numbers of Volume 35 will be on the web site this year: Number 1 in August and Number 2 in December.

The web site is under transformation, since it will host two databases. The first database will be available by the end of year 2005 and it will consist of all researchers' information. The second database for the Literature will be available as a small database in 2005 and it will be built throughout year 2006 with the researchers' contributions. I would like to encourage everybody to make contributions to the list of bibliography for next year.

Acknowledgement

I would like to express my gratitude to Dr. Paul Beuselinck and Dr. William F. Grant for their permanent support and advice. We can provide electronic copies of all articles that are not available on the web, since Dr. Paul Beuselinck donated all his collection of LN issues. Dr. W. Grant made a great contribution last year with the list of cultivars of *Lotus* species of agronomic relevance. Although he will not be able to write new articles for LN, we will all share his permanent enthusiasm through the email.

INIA provided funding for my attendance to the XX International Grassland Congress, held in Dublin in June 2005, where I presented a poster about LN in the session "The role of the International Grassland Congress and Grassland Societies in technology interaction and influencing policy: a discussion session". All papers related to trefoil will be summarized for Volume 35 Number 2, and I invite all who wish to contribute with their view of the Congress.

The priority of the Newsletter was the update of the recipient list, and I am pleased to inform that the registration increased to 185 researchers in August 2005. Thanks to all for helping me with this task.

What to contribute?

Send us the kind of information you would like to see in LN.

- Contributions should be current, scholarly, and their inclusion well-justified on the grounds of new information.
- Results of recently concluded experiments, recent additions to germplasm collections, information on new or tentative cultivars with descriptions. We want to include an adequate cultivar description, including disease reactions and origin if possible.
- Notes on acreage, production, varieties, diseases, etc., especially if they represent changing or

- unusual situations.
- Genome maps and information on probe-availability and sequences, and populations synthesized for specific traits being mapped. Glossy black and white prints of maps should be included, if possible. Partial maps can also be submitted.
 - Short reports of workshops, conferences, symposia, field days, meetings, tours, surveys, network activities, and recently launched or concluded project.
 - Details of recent publications, with full bibliographic information and short reviews.
 - Personal news (new appointments, awards, promotions, change of address, etc.)

How to format contributions

- Include the full address with telephone, fax, and e-mail numbers of all authors.
- Keep the items brief – remember, LN is a newsletter and not a primary journal.
- Give the correct Latin name of every crop, pest, or pathogen at the first mention.
- If possible, table should fit within the normal typewritten area of a standard upright page (not a ‘landscape’ page). You may include figures and photographs (black and white or color). Please send disk-files (with all the data) whenever you submit line figures and maps.
- Supply the essential information: round off the data-values to just one place of decimal whenever appropriate, choose suitable units to keep the values small (e.g. use tons instead of kg).
- All lists of references should have been seen in the original by the author and year. Provide all the details such as author/s, year, title of the article, full title of the journal, volume, issue, and page numbers (for journal articles), and place of publication and publishers (for books and conference proceedings) for every reference. Incomplete references will not be accepted.
- The language of the Newsletter is English, but we encourage researchers to submit their articles in other languages as well; the translation will be linked within the LN web site. Authors should closely follow the style of the reports in this issue. Contributions that deviate markedly from this style will be returned for revision, and could miss the publication date. If necessary, we will edit communications so as to preserve a uniform style throughout the Newsletter. This may shorten some contributions, but particular care will be taken to ensure that the editing will not change the meaning and scientific content of the article. Wherever we consider that substantial editing is required, we will send a draft copy of the edited version to the contributor for approval before printing.
- Contact the Editor for detailed guidelines on how to format text.

Material may be submitted at any time during the year. Deadline for Volume 35 Number 2 will be 30th November 2005. Please send all contributions and request for inclusion in the recipients list to:

Monica Rebuffo, Lotus Newsletter Editor, c/o INIA La Estancuela, 70000 Colonia, Uruguay
Email lnl@inia.org.uy Fax +598-574-8012

Personalia

Welcome to the new subscribers of the *Lotus* Newsletter: Aïssa Abdelguerfi – Algeria; Adriana Alippi, Analía Sannazzaro, Marina Sisterna and Pedro Alfonso Sansberro – Argentina; Steve Hughes – Australia; Edgar Cárdenas – Colombia; Douglas Martin and Andy Pollard – Falklands Islands; Seishiro Aoki – Japan; Katharina Pawlowski – Sweden; Heathclife Riday and Matt Sanderson – USA; Athole Marshall, Jilliam Perry and Christina Marley – United Kingdom.

Several *Lotus* researchers have been recently reassigned or no longer work on the subject. Dr Lene Krusell moved from Max Planck Institute of Molecular Plant Physiology – Germany to University of Aarhus – Denmark. Dr. Jiri Stiller – USA, has switched the fields and currently work on sugarcane. Dr. Mark McCaslin – USA, is president of Forage Genetics International. Dr. Andreas Roussis – Denmark, is presently working at the Center for Human and Clinical Genetics, Leiden University Medical Center in The Netherlands. Dr. Ken Vogel – USA, no longer works with *Lotus* since his research focus on sorghum and forage grass species. Dr. Mónica Tourn – Argentina, works with tropical pastures at the present time.

The following personal information has been updated during the current year: Ana Arambarri, José De Batista and Miguel Cahuépe – Argentina; John Ayres – Australia; William F. Grant – Canada; Lene Heegaard Madsen, Christina Cvitanich, Niels Sandal and Lene Krusell – Denmark; Emanolis Flementakis – Greece; Ariel Asuaga – Uruguay;

Third International Conference on Legume Genomics and Genetics: From Genes to Crops

[PETER M. GRESSHOFF*](#)

ARC Centre of Excellence for Integrative Legume Research, The University of Queensland, St. Lucia, Brisbane, QLD 4072, Australia

<http://www.iclgg3.org/>

**Corresponding author*

The major aim of research is to discover the connection between structure and function. This biennial conference focuses on the genetics and genomics of one of the major plant groups, namely the legumes. Legumes are major crop plants for the benefit of human food, animal feed, vegetable oil and nutraceutical production. Their benefits are recognized around the world. Legumes also serve as a model plant, providing useful information for the improvement of other crops such as tomato, sunflower, cotton, corn and rice. The biochemistry of legumes is distinct from that of other plant groups and many unique molecules, often used in biomedical applications, are found among legume metabolites. Isoflavones and plant sterols are just two major examples.

The ICLGG-3 will be held in Brisbane, Australia and is hosted by the ARC Centre of Excellence for Integrative Legume Research. The meeting will bring together world experts, reporting their findings in both crops as well as model legumes. The model legume *Lotus japonicus* and *Medicago truncatula* have led the recent push towards understanding the genomic structure of legumes. By April 2006 a significant part of their genomes will be available, allowing comparative genomic approaches. Concurrently the rate of gene discovery in complex developmental processes, such as lateral branching, embryogenesis, flower development, and nodulation has increased to a rapid rate, providing first-time insights into the mechanistic events underlying plant development. Many of the discoveries were impossible in the existing model plant rice and Arabidopsis, because of their growth habit and biological inabilities to develop, for example, nodules.

The central theme will be: From genes to crops. Thus reductionist and fundamental science will be reported together with 'real world' applications and progress. The format of the meeting will include plenary lectures of broader interest and overview, short research reports, and integrative discussion sessions. Experts from related fields of yeas, *Arabidopsis* or human genomics will provide possibilities to discuss future directions of the legume genomics field. Experts from the bio-pharmaceutical and nutritional sciences, representing end users will be invited. Posters will provide a venue of large scale data presentation and a forum for discussion.

Brisbane Convention & Exhibition Centre
Queensland, Australia



3RD INTERNATIONAL CONFERENCE ON
Legume Genomics and Genetics
9-13 APRIL 2006

Mark your diary NOW!

The Conference
The ABC Centre of Excellence for Integrative Legume Research is pleased to announce the 3rd International Conference on Legume Genomics and Genetics. The event is expected to attract over 350 delegates and will offer a stimulating plenary program featuring the world's leaders in legume research, a diverse poster program and enjoyable social activities. The theme for ICGG3 is Genes to Crops. The Conference will provide an ideal opportunity to discover learn and share knowledge & experience.

www.icgg3.org

G E N E S T O C R O P S

Destination
Brisbane is Australia's largest city in the subtropics, enjoying an enviable climate of hot, bright summers and warm, mild winters that makes any time of the year a good time to visit. A modern city dotted with parks and lying beneath clear blue skies makes Brisbane a refreshing change from most major capitals. The Conference is being held at the Brisbane Convention & Exhibition Centre, located at South Bank, Brisbane. The Centre has been purpose built for large and diverse conferences. It is situated in the cultural and entertainment precinct, located directly opposite the Brisbane Central Business District on the south bank of the beautiful Brisbane River.

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Program Topics

- Comparative Genomics, Gene Structure and Evolution
- Agricultural Applications
- Developmental Mechanisms - Signalling
- Developmental Mechanisms - Gene Networks
- Interactions
- Defining Gene Function
- Gene Regulation
- Frontiers of Legume Research
- Natural Biology of Legumes

Important Dates

Registration Brochure Available	July 2005
Poster Abstract Submission Deadline	October 2005
Earlybird Registration Deadline	February 2006

For further information or to register your interest, visit www.icgg3.org

**Interdisciplinary workshop on genetic, molecular and
eco-physiological aspects of *Lotus* spp. and their symbionts.
[Taller interdisciplinario sobre aspectos genéticos, moleculares y
fisiocológicos del *Lotus* spp. y sus simbioses].**

Chairperson: [OSCAR A. RUIZ*](#)

Held at: Instituto de Investigaciones Biotecnológicas - Instituto Tecnológico de Chascomús.
IIB-INTECh. (UNSAM-CONICET). Chascomús. Provincia de Buenos Aires. Argentina.
9 –10 September 2004

* Corresponding author

Program

Day 9 September:

Plant breeding workshop

Discussion Leader: Ing. Agr. MSc, RAÚL RODRÍGUEZ (Unidad Integrada Balcarce)

Planteo teórico sobre cómo se elabora un plan de mejoramiento genético, tomando al *Lotus* como base [Theoretical bases for the elaboration of a breeding program, with *Lotus* as an example.] Dra. ELSA CAMADRO, Unidad Integrada Balcarce, CONICET, Argentina

Mejoramiento genético: *Lotus corniculatus* y *Lotus uliginosus*. [Plant breeding: *Lotus corniculatus* and *Lotus uliginosus*.] Ing.Agr. M.Phil. MÓNICA REBUFFO, INIA, Estación Experimental “La Estanzuela”, Colonia, Uruguay.

Evaluación de las concentraciones de taninos en una población de *Lotus glaber* naturalizada. [Evaluation of tannin concentrations in a population of naturalized *Lotus glaber*.] Ing.Agr. MSc. PhD HERNÁN ACUÑA, INIA, Estación Experimental Quilamapu, Chile.

Evaluación de una población seleccionada de *Lotus glaber* frente al estrés salino. [Evaluation of a selected population of *Lotus glaber* under saline stress.] Dra. ANA CASTRO, Facultad de Ciencias Agrarias, Universidad Nacional de La Plata, Argentina.

Phytopathology Workshop

Discussion Leader: Ing.Agr. ADRIANA M. ALIPPI (CIC - Centro de Investigaciones de Fitopatología, Fac. de Cs. Agrarias y Ftales, UNLP).

Enfermedades de origen fúngico. [Fungal diseases.] *Ing.Agr. GLADYS A. LORI and Ing.Agr. MARINA SISTERNA, CIC - Centro de Investigaciones de Fitopatología, Facultad de Ciencias Agrarias y Forestales, UNLP, Argentina.*

Enfermedades de origen bacteriano. [Bacterial diseases.] *Ing.Agr. ADRIANA ALIPPI, CIC - Centro de Investigaciones de Fitopatología, Facultad de Ciencias Agrarias y Forestales, UNLP, Argentina.*

Microbiology Workshop

Discussion Leader: Dr. JUAN SANJUAN (CSIC – Estación Experimental del Zaidin, Departamento de Microbiología del Suelo y Sistemas Simbióticos, Granada, Spain).

Evaluaciones de biodiversidad y algunas consideraciones tecnológicas acerca de la producción de un inoculante eficiente para el *Lotus glaber*. [Evaluation of biodiversity and some technical considerations on the production of efficient inoculant for *Lotus glaber*.] *Dr. FABRICIO CASSÁN, Universidad Nacional de Río Cuarto e IIB-INTECh (UNSAM-CONICET), Argentina.*

Actividades de Investigación del Departamento de Microbiología de Suelos en Rhizobiología en especies del Género *Lotus*. [Research activities of the Department of Soil Microbiology on Rhizobiology in species of the Genus *Lotus*.] *Licenciado MARTÍN JAURENA, Laboratorio de Microbiología de Suelos, Ministerio de Agricultura, Ganadería y Pesca, Uruguay.*

Biodiversidad de micorrizas asociadas al *Lotus glaber* en la Pampa Deprimida del Salado. [Biodiversity of mycorrhiza associated to *Lotus glaber* in the Pampa Deprimida del Salado.] *Licenciada ANALÍA SANNAZZARO. IIB-INTECh (UNSAM-CONICET), Argentina.*

Asociación *Lotus*-Micorrizas en pastizales afectados por gradiente hidrológico y salino. [Association *Lotus*-Mycorrhiza in grasslands affected by hydrologic and saline gradient.] *Dr. RODOLFO MENDOZA, CEFYBO-CONICET, Argentina.*

Day 10 September:

Physiology Workshop

Respuestas comparativas entre *Lotus glaber* y *Paspalum dilatatum* a la interacción pastoreo-inundación. [Comparative responses between *Lotus glaber* and *Paspalum dilatatum* to the flooding-grazing interaction.] Dr. GUSTAVO STRIKER, Dr. PEDRO INSAUSTI and Dr. ROLANDO LEÓN, IFEVA, Facultad de Agronomía de la Universidad de Buenos Aires (UBA), Argentina.

Caracterización morfo-fisiológica de poblaciones naturalizadas de *Lotus glaber*. [Morpho-physiological characterization of *Lotus glaber* naturalized populations.] Ing.Agr. MSc. PhD. ADRIANA ANDRES, Ing.Agr. MSc. BEATRIZ ROSSO, Ing.Agr. MSc. OMAR SCHENEITER, INTA-Pergamino, Argentina.

Economía del nitrógeno y del fósforo en dos poblaciones de *Lotus glaber* crecidas bajo condiciones contrastantes de fósforo. [Nitrogen and Phosphorus economy in two populations of *Lotus glaber* Mill. grown under contrasting conditions of Phosphorus.] Dr. DANIEL COGLIATTI, Ing. LINA LETT, Ing. MÓNICA BARUFALDI, Ing. PAOLA SEGURA and Dr. JORGE CARDOZO. Facultad de Agronomía de Azul, Universidad del Centro de la Provincia de Buenos Aires y Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

Influencia de las micorrizas sobre la fisiología del estrés salino en *Lotus glaber*. [Influence of mycorrhiza on the saline stress physiology of *Lotus glaber*.] Dr ANA MENÉNDEZ, Facultad de Ciencias Exactas de la UBA e IIB-INTECh (UNSAM-CONICET), Argentina.

Biotechnology Workshop

Una aproximación bioquímica y molecular del rol de las poliaminas en la tolerancia al estrés salino del *Lotus glaber*. [Molecular and biochemical approximation of polyamine roles in tolerance mechanisms to salt stress in *Lotus glaber*.] Licenciada ROSALÍA PAZ, IIB-INTECh (UNSAM-CONICET), Argentina.

Cultivo *in vitro* de *Lotus glaber*. [In vitro culture of *Lotus glaber* Mill.] Dr PEDRO SANBERRO, IBONE, Facultad de Ciencias Agrarias de la Universidad Nacional del Nordeste (UNNE), Argentina.

Ecology, Management and Production Workshop

Queima de pastagens naturais - efeitos sobre o solo e a vegetação. [Fire on native pastures - effects on soil and vegetation.] Dr. AINO VICTOR AVILA JACQUES, Professor Titular Aposentado da Universidade Federal do Rio Grande do Sul – UFRGS, Brazil.

Lotus glaber, su nuevo rol en los sistemas ganaderos de la Cuenca del Salado. [*Lotus glaber*, its new role in the grazing systems of “Cuenca del Salado”.] Ing. Agr. MATÍAS BAILLERES, Estación Experimental de Manantiales, Ministerio de Asuntos Agrarios de la Provincia de Buenos Aires, Argentina.

Perspectivas de Utilização de Leguminosas Nativas en RGS. [Perspectives of utilization of native legumes in Rio Grande do Sul.] Dr MIGUEL DALL’AGNOL Professor da UFRGS, Faculdade de Agronomia, Dep. de Plantas Forrageiras e Agrometeorologia, Brazil.

Cambio de la productividad del *Lotus* spp. bajo distintos tipos de manejo. [Changes in the productivity of *Lotus* spp. under different management conditions.] Dr. OSVALDO VIGNOLIO, Universidad Nacional de Mar del Plata, Unidad Integrada Balcarce, Argentina.

Theoretical bases for the elaboration of a breeding program. *Lotus glaber* as an example.

[ELSA CAMADRO*](#)

EEA Balcarce, INTA-FCA, UNMdP. CONICET. C.C. 276, 7620 Balcarce, Bs. As.,
Argentina

* Corresponding author

Before starting a breeding program, problems or aspects that need to be improved in the species of interest have to be identified and listed. Among others, the following are apparently of concern in *Lotus glaber*: slow seed germination, seedling weakness, lack of persistence, susceptibility to stresses (abiotic: soil salinity and flooding, and biotic: insects) and winter and early spring forage production. Next, it is necessary to consider: which problems can be solved by cultural and/or chemical practices, what is the effectiveness of these practices and their cost in relation to the expected results, which could be solved only by breeding and, if the solution to a problem can be approached in both ways, which of them is the most cost-effective. If the breeding approach seems convenient from the economic or environmental point of view, it is necessary, before making a decision, to know (or determine if not known) the type of genetic control of the trait(s) of interest: (a) nuclear (mono- or oligogenic with none or little environmental influences on the expression, or oligo- or polygenic, with the expression highly influenced by the environment) or (b) cytoplasmic. Nuclear genes have biparental inheritance and follow Mendel's laws, thus predictions can be made regarding expected genotypes/phenotypes and proportions in controlled crosses, in contrast with cytoplasmic genes that can have either uni- or biparental inheritance and do not follow Mendel's laws. Also, it has to be considered that a given trait can be under control of nuclear or cytoplasmic genes, or under control of cytoplasmic genes that interact with nuclear genes (i.e. chlorophyll content or male sterility) and that an enzyme can be composed of various polypeptides, some of them codified by nuclear genes and the others by cytoplasmic genes (i.e. the enzyme RUBISCO).

If breeding is deemed appropriate, it has to be decided what type of cultivar will be developed (improved population, synthetic variety, hybrid, etc.). For doing this, it has to be considered: (a) that *Lotus glaber* is a species herbaceous, perennial, diploid and self-incompatible, that reproduces sexually and has related diploid and polyploid species that can be manipulated in vitro and are amenable to transgenesis, and (b) the complexity of the process, the minimum required time, the likelihood of maintenance of the genetic identity of the cultivar (given the feasibility of natural hybridizations and re-seeding), the available infrastructure and equipments, and costs in relation to benefits. The first step to start a breeding program is to gather a collection of germplasm (commercial cultivars, advanced selections, natural populations), and to study the heritable variation within and between genetic materials. If no heritable variation is found for the trait(s) of interest, the

breeder can recur to: (a) interspecific hybridization (a collection of species and genotypes has to be gathered and controlled crosses performed; if hybridization barriers are detected, strategies have to be applied or developed to circumvent them, i.e., ploidy level manipulations), (b) *in vitro* manipulations to select pre-existing somaclonal variation or generate it, (c) induced mutagenesis with chemical or physical agents, (d) transgenesis for monogenic traits. It is important to analyze the advantages and disadvantages of each approach taking into account effectiveness and costs. For *in vitro* manipulations, the correlation with the *in vivo* behavior has to be established. Breeding has been considered an art and a science. But modern breeding has to be carried out on solid scientific bases, making use of sophisticated tools only if the cost-benefit relations warrant it.

Plant breeding: *Lotus corniculatus* and *Lotus uliginosus*.

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Beef and dairy production are based on grazing systems in Uruguay. Natural grasslands dominated by grasses represent over 70% of the total grazing area (about 13 millions hectares), while agricultural areas are cultivated with legume pastures in rotation with cereal crops (1.2 millions hectares). *Lotus corniculatus* (birdsfoot trefoil), either pure or in mixtures, is the legume most extensively utilized, whereas the relevance of *Lotus uliginosus* (big trefoil) to improve forage production and quality of natural grasslands has sharply increased in recent years.

The main restriction for forage productivity in Uruguay is the lack of persistence of the legumes. Birdsfoot trefoil breeding program began in 1988. The objectives are the improvement of persistence and forage production, maintenance of seasonal forage distribution and seed production. San Gabriel is the most widely utilized cultivar in the country. Altier (1997) clearly pointed at the root rot complex (*Fusarium oxysporum*, *F. solani*, *Colletotrichum*, etc) as the main reason for the lack of persistency of this cultivar. The progress in persistence has been sustained throughout 4 cycles of recurrent selection performed under field conditions (Rebuffo and Altier, 1997). Dead plants in the third year dropped from 88% in Cycle 0 to 53% in Cycle 4. Breeding determined the increment in the tolerance to *F. oxysporum* (Altier *et al.*, 2000), in addition to the increment in crown size and in the proportion of plants with lateral roots. Cultivar INIA Draco, the outcome of Cycle 2, produces 15% more forage than San Gabriel (range from 5% up to 61 % increment in years with high incidence of root rot diseases). Additional strategies to improve persistency have been added to the objectives of the breeding program, such as the introgression of rhizome into the adapted germplasm.

The main restriction of big trefoil late flowering materials is the low seed production, due to the frequent water deficit during summer. The program has been working with diploid and tetraploid materials looking for the improvement of seed production. The breeding program started in 1983, with the introduction of a large collection of diploid accessions. The aim was to identify germplasm suitable for forage and seed production in the country. An early flowering experimental line is under evaluation at the present time. Its high seed production could build up the soil seed bank, and therefore facilitate reseeding in natural grasslands. The recent appearance of severe damages caused by *Uromyces* (rust) added another objective for this species (Ciliuti *et al.*, 2003). At the present time we are evaluating rust resistant lines for introgression into outstanding forage and seed production lines.

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Condensed tannin concentrations in *Lotus* spp.

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The most important species of genus *Lotus* used in Chile are *L. corniculatus* (Lc), *L. glaber* (Lg) and *L. uliginosus* (Lu). These species, like other forage legumes, contain appreciable amounts of condensed tannins (CT) which are important compounds for reducing incidence of pasture bloat, produced by the formation of the stable foam originated in the soluble plant protein released in the rumen, preventing the normal expulsion of the gases (Tanner *et al.*, 1995). Condensed tannins are also recognized as a factor that affects the palatability and digestibility of feeds, reducing intake and nutrient absorption (Barry, 1984). Low levels of CT in plants may improve utilization of herbage protein by ruminants without impairing feed intake and digestibility (Wang, 1996).

The objective of this study was to determine CT concentrations in Lc cultivar (13) and in Lg (11) and Lu (22) accessions collected in Chile between 32° - 38° S and 36° - 45° S, respectively, by Butanol – HCl method, and to detect variability attributable to genetics.

The experiments were established in autumn 1998 at Cato, volcanic medium textured soil: Cabrero, sandy soil; Chillán, clay soil under irrigated conditions; San Ignacio and Vilcún, both localities of volcanic soil without irrigation. There was one experiment per species at each location in a two random block design. Terril *et al.* (1992) procedure was used to separate total CT in forage into extractable, protein-bound and fibre-bound fractions. The samples for analysis were taken in December 1999 (2d cut), 40 – 45 days after the first cutting, when plants were completely blossomed.

The results show, in general, significant differences ($P < 0.05$) between Lc cultivar or Lg and Lu accessions, in fractions and in total CT concentrations, in all different environments. *Lotus corniculatus* range from 1.9 to 5.6, 0.8 to 3.1 and 0.9 to 2.1 % of DM for extractable, protein-bound and fibre-bound CT, respectively. *Lotus uliginosus* DM % of CT range from 3.2 to 6.5, 1.3 to 3.9 and 0.9 to 2.9 for extractable, protein-bound and fibre-bound fractions, respectively. The above values are in the ranges reported in the literature, but the concentrations of CT in Lg are notoriously high (around 4.5 % of total CT in DM) considering that some authors reported absence of CT in Lg leaves, presence in stems and abundance only in roots, measured with vanillin-HCl reaction (Strittmatter *et al.*, 1994). It is concluded that the studied materials show enough variability to be used in future breeding programs to develop cultivars of these species which contain appropriate amounts of CT to avoid pasture bloat without negative effects on digestibility or herbage intake.

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Assisted and traditional assessment of saline stress tolerance in *Lotus glaber*.

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Narrowleaf birdsfoot trefoil is a naturalized grass in the Salad River Basin, which is a region with variable type of soils, being the Natralboles and Natracualfs the most frequent (Salazar *et al.* 1980). These soils induce plant salinity stress. High salinity in soils is a common abiotic distress, producing deprived water supply, affecting plant growth and productivity by osmotic stress and/ or ion toxicity. The most typical plant symptom of salinity injury is a retarded growth due to inhibition of cell elongation (Nieman, 1965). Largely differences have been found between and within species in the degree of adaptation to water stress. It is important to investigate the metabolic changes involved in plant adaptive strategies to water stress and their physiological basis could be useful for breeding and management purposes. Consequently, the aims of this work are to study: (i) the Narrowleaf birdsfoot trefoil intrapopulation variation in osmotic adjustment capability; (ii) the morphological and metabolic changes in response to water stress caused for salinity, and (iii) the assessment of biochemical and molecular markers associated with tolerance to saline stress.

The birdsfoot trefoil plants were collected from different populations of the Salado river basin, living in contrasting environments. Shoot explants of 5 cm long, were sectioned from plants to obtain clones, under natural conditions of light, temperature, and humidity. The explants grew in trays containing Hoagland's solution (Hoagland & Arnon 1950). After 20 days half plantlets of every genotype were transferred to another tray containing 200mM of ClNa. The rest of the plantlets remained in Hoagland solution as controls. It was recorded the survival, fresh and dry weights in aerial and root parts, root volume, foliar area, non-structural carbohydrates, protein contents, proline and isozymes. Samples were collected after 15 days of treatment. A sample of 0.5 gr of fresh weight was collected in one-week plantlets to analyze DNA by RAPDs in a bulk screening method.

The most tolerant genotypes subjected to saline stress showed the highest survival and their foliar area, fresh and dry weights and root volumes were not significantly different from control plants. On the other hand, susceptible genotypes showed losses of 60-100% in the above mentioned parameters. Similarly, reduced, non-reduced and total carbohydrates were similar in control and stressed plants of tolerant genotypes. The susceptible genotypes showed a significant increase of the carbohydrate contents when subjected to saline stress, comparing with their controls. The protein contents decreased in every genotype under stress. Tolerant genotypes * showed a high significant increase of proline under stress

compared to their checks. Peroxidase system discriminated the tolerant genotypes from susceptible ones under stress. Three RAPDs were identified only in tolerant genotypes.

The traditional and assisted selection permitted the identification of tolerance to saline stress in *Lotus glaber*.

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Fungal diseases on *Lotus* spp in Argentina.

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Diseases can limit persistence of *Lotus* spp. in production systems. Several pathogens are involved in a “disease complex”. Nevertheless, little is known about diseases and their impacts, both in the world and in Argentina. Fungal pathogens are the most prevalent organisms and according to the plant tissue they can affect, diseases are classified as follows (the references correspond to the diseases cited for Argentina):

Crown and root diseases:

They are considered chronic diseases, causing plant death and losses of 60 to 80 %. Typical symptoms are rot and wilt. The main Genus is *Fusarium* (*F. solani*, *F. oxysporum*, *F. verticillioides* and *F. equiseti*). (Teyssandier, 1976a; Dal Bello and Sisterna, 1992; Madia de Chaluat, 1994; Monterroso *et al.*, 1998; Juan *et al.*, 2000).

Stem and foliar diseases:

In contrast to crown and root diseases, foliar ones do not cause directly the plant death. They contribute to the progressive weakness of the plant through the effects on the basic metabolic processes. The reported pathogens are: *Phomopsis loti* (blight) (Teyssandier, 1976b; *Colletotrichum destructivum* (anthracnose) (Wolcan and Dal Bello, 1988; Monterroso *et al.*, 1998); *Stemphylium loti* and *S. spp.* (leaf spot) (Dal Bello and Wolcan, 1989; Monterroso *et al.*, 1998; Juan *et al.*, 2000); an unidentified rust (Dal Bello, 1986); *Uromyces loti* (rust) (Monterroso *et al.*, 1998; Juan *et al.*, 2000) and *Botrytis cinerea* (Monterroso *et al.*, 1998; Juan *et al.*, 2000).

Seed diseases:

They cause decrease in germination and damping-off. Fungi genera present in the seed are: *Alternaria*, *Aspergillus*, *Bipolaris*, *Botrytis*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Epicoccum*, *Fusarium*, *Leptosphaerulina*, *Penicillium*, *Phoma*, *Phomopsis*, *Stemphylium* and *Verticillum*. (Teyssandier, 1976a, b; Wolcan and Dal Bello, 1988; Dal Bello and Sisterna, 1992; Madia de Chaluat, 1994).

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Bacterial diseases of *Lotus* spp.

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There are no reports about diseases of bacterial origin affecting species of *Lotus* in Argentina. On a world-wide basis, only *Pseudomonas viridiflava* and *Clavibacter michiganensis* subsp. *insidiosus* have been reported affecting birdsfoot trefoil (*Lotus corniculatus*; Bradbury, 1986).

Main symptoms caused by *P. viridiflava* on trefoil are wilting of affected plants and reduction of root growth. The tops were wilted and the crowns appeared yellowish gray (Lukezic *et al.*, 1983).

P. viridiflava also produces other diseases in several plants. It has been reported to cause basal stem rot of tomato, fruit rot on tomato, discolored pith on chrysanthemum, bacterial wilt of sweet onion, root and crown rot of alfalfa, bacterial canker of poinsettia, blossom blight of kiwifruit, necrosis of melon, blite, eggplant, basil, bean, cabbage, cauliflower, dill, grape, lettuce, lupine, parsnip, passion fruit, pea, poppy, pumpkin, rape, and other hosts (Gitaitis *et al.*, 1998; Alippi *et al.*, 2003). It has also been reported as a secondary invader and epiphyte. In Argentina *P. viridiflava* has been associated with symptoms of pith necrosis of tomato and pepper (Alippi *et al.*, 2003) and leaf necrosis of basil (Alippi *et al.*, 1999), being a potential pathogen to species of *Lotus* due to its ubiquitous nature and transmission through contaminated seed.

Bacterial wilt caused by *C. m.* subsp. *insidiosus* has been reported affecting *Medicago sativa*, *Lotus* spp., *Melilotus* spp., and *Trifolium* spp. (Bradbury, 1986). The disease occurs throughout most of the alfalfa growing areas of the world (Graham *et al.*, 1980). Infected plants are scattered throughout the stand and are easily detected by their yellow-green color and stunted growth. Mild symptoms consist of leaf mottling with slight cupping or upward curling of the leaflets and some reduction in plant height. Severely infected plants are stunted and yellow-green, with many spindly stems and small, distorted leaflets. Diseased plants are usually most evident in the regrowth after clipping with appearance of witches' broom symptoms. Cross sections of taproots show first a yellowish brown discoloration of the outer vascular tissue, and subsequently the entire stele discolors. When the bark is peeled away, the stele is yellowish brown, in contrast with the white of healthy plants. Pockets of infection sometimes appear on the inner surface of the bark (Graham *et al.*, 1980). The pathogen can survive in plant material in the soil, hay and seed for several years. It can be spread plant to plant via rain, irrigation or contaminated implement. Long distance spread is

due to contaminated seed and hay. Bacteria usually infect plants through wounds in the root and crown that can be caused by winter injury, nematodes, or mechanical injury.

Dwarfing symptoms of alfalfa, probably caused by *Xylella fastidiosa* has been reported in USA (Graham *et al*, 1980), and similar symptoms were described on *L. corniculatus* in Uruguay (Altier, 1997), but the identity of the causal agent was not confirmed. Due to the wide range of hosts reported for *X. fastidiosa* there is high probability that the bacterium can also affects *Lotus spp.* From this limited information about bacterial diseases, it is obvious that much remain to be done to elucidate interactions between bacterial diseases and development of *Lotus spp.*

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Physiological studies of tolerance to saline stress in *Lotus glaber* and their correlation with the establishment and efficiency of the symbiotic association with *Mesorhizobium loti*.

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Soil salinity represents the main cause of abiotic stress in cultivable plants around the world. At least 34.000.000 Has. are subjected to excess of water and mineral salts in Argentina, These factors determine extreme ecological conditions causing a drastic reduction in their productivity. Salt stress has two components: osmotic stress, caused by a relative increase of solute concentration and the consequent decreased in water availability in the soil, and ionic stress, caused by the modification of the K^+/Na^+ relationship and by concentrations of Na^+ and Cl^- that are harmful for plant tissues. Plants strategies to overcome these conditions are directed to the activation of multiple metabolic pathways in order to: facilitate the acquisition and retention of water, protection of cell functionality, modifications of growth patterns and maintenance of general homeostasis. These biochemical and physiological modifications include changes in mechanisms such as: (a) phytohormones balance, (b) osmoprotectans synthesis, (c) specialized proteins synthesis for active species of oxygen (AOS) scavenging, (d) toxic ions mobilization and compartmentation.

The rapid increase in world population makes it necessary to find alternative ways to improve agricultural productivity and make the available resources be used more efficiently. One of the strategies consists on obtaining plants tolerant to different stresses in order to introduce them in unfavourable lands. The Argentinean region called Pampa Deprimida del Salado concentrates near 70% of the Argentinean livestock activity, and their productivity is intimately linked to its edaphic characteristics: nutritionally poor soils with high concentrations of minerals salts, high pH, and high fluctuations of the useful water determined by seasonal cycles of drought and flood. In this way, the search for tolerant plant populations, as well as the understanding of the physiological basis of salinity tolerance in Pampa Deprimida ecosystem species, can be considered as a high-priority topic to be studied. Preliminary observations in the IIB-INTECh indicate the existence of high salinity tolerant genotypes in different populations of *Lotus glaber* (Vignolio *et al.*, 1994). The soil conditions in the Pampa Deprimida are a natural obstacle for the efficient installation of pastures based in traditional leguminous as alfalfa or clover. In this sense, the possibility of obtaining improved populations of *Lotus glaber* represents an important productive alternative for those ecosystems and makes this species an excellent experimental model. In relation to its symbiotic associations, previous work in the IIB-INTECh indicates that for a

significant nodulation in an established population of *Lotus glaber* it is necessary to use several *Mesorhizobium loti* strains (Fulchieri *et al.*, 2001). Studies to verify if the physiological conditions of the tolerant plant affect the infection process, colonization and effective biological N fixation have not been addressed yet, nor has the question if the fixation efficiency is due to a minimum number of nodulating strains in the soil.

Our hypothesis suggests that salinity tolerance in *Lotus glaber* would depend, at least in part, on the plant capacity to associate with different native *Mesorhizobium loti* strains. The physiological conditions of the plant in this moment would be decisive for the effective bacterial colonization and later biological N fixation. To corroborate this hypothesis we propose the following general objectives: (a) to elucidate physiological and biochemical salinity tolerance mechanisms in *Lotus glaber* populations and their nodulation through association with *Mesorhizobium loti*; (b) to identify efficient symbiotic associations among tolerant populations of *Lotus glaber* and *Mesorhizobium loti* strains that could facilitate their successful installation under adverse edaphic conditions.

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Rhizobiology research in *Lotus* species.

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The Uruguayan natural grasslands are the nutritional base of livestock production and occupy more than 70 % of the surface of the country. These grasslands are very stable, but they present limitations of quantity and quality of forage inside and between years. The improvements of grasslands are been done by sod seeding of forage legumes inoculated with specific strains of *Rhizobium sp.* and phosphate fertilizers. This technology was validated in commercial farms in the eastern and the center regions of the country. These improvements are realized principally with species of the genera *Lotus* (*L. subbiflorus* cv. "Rincón ", *L. uliginosus* cv. "Makú ", *L. corniculatus* cv. "San Gabriel "). However the situation is not the same in the basaltic region in the north part of the country, for which new species of forage legumes are been evaluated, since traditional *Lotus* species are not adapted due to restrictions related to edaphically factors (mix of superficial and deep soils) and climatic conditions (high temperature in summer and freezes in winter). Main lines of rhizobiological research in species of *Lotus* are:

- Rhizobia strain selection for species of agronomic interest.
- Evaluation of compatibility between rhizobia strains and *Lotus* species
- Analysis of biodiversity patterns of strains able to nodulate *Lotus corniculatus*.

In the first case the introduction and isolation of new rhizobia strains allows the evaluation in symbiosis of the agronomic potentiality of *Lotus* species of current and potential use. This activity begins with a preliminary testing of symbiotic efficiency of strains and isolations in growth chambers under controlled conditions. Superior strains pass to greenhouse evaluation in "soil cores" to imitate field conditions. In this stage there is an evaluation of *Lotus* and grassland dry matter production. The strains of better behavior in greenhouse go to field evaluation in plots in several localities.

Final selection is based in field results. Selected strains are evaluated for genetic stability and are used to study interspecific compatibility, and ability to growth in the industry conditions for inoculant production.

We are also performed biodiversity studies of the native and introduced rhizobia populations in soils and in the roots system associated with the persistence of commercial *Lotus* species. The principal outcomes of this research relates with:

- I) the knowledge of the characteristics of the native and introduced rhizobia strains,
- II) the isolation and conservation of about 150 isolations able to nodulated *Lotus* species,
- III) Inoculant development and use in farm conditions.

The arbuscular mycorrhizal fungi of *Lotus glaber*.

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We intended to study the composition and structure of arbuscular mycorrhizal (AM) fungal community associated with *Lotus glaber* in sodic soils of the Salado River basin.

Roots were cleared, stained with Trypan blue and the amount of intraradical mycorrhizal structures estimated by the slide method. Spores were identified after isolation by wet sieving and decanting. The chemical analysis of rhizospheric soil was performed.

Spores of eighteen different AM species were detected. *Glomus geosporum*, the most frequently isolated AM fungus, was also the dominant one. Shannon-Wiener diversity index varied between 0.65 and 1.65.

Morphological types of AM fungi associated with *L. glaber* were also studied. At least eight colonization patterns (IP) of AM fungi in roots of *L. glaber* were observed. *Arum*- and *Paris*-types of infection were found in the same plant species. This result supports the idea that the morphology of AM is not solely under plant control, but is also influenced by fungal identity. One IP presumably corresponding to *G. intraradices* and a second one possibly assignable to *G. tenue* were the most commonly found.

Additionally, DNA from the root samples was isolated and intergenic DNA sequences from the AM fungi were amplified through a nested PCR with taxon-specific primers. Problems in cloning and sequencing caused that only two fragments have been successfully sequenced. Sequencing of these clones and the comparisons with the data in the Genbank revealed that the amplified fragments possess a high homology percentage with sequences from *G. intraradices*

Our findings reinforce previous suggestions that *G. geosporum* and *G. intraradices* are well adapted to sodic-saline conditions and would play a role in the resistance of *L. glaber* to these soils.

Seasonal variation of arbuscular mycorrhizal fungi in temperate grasslands along a wide hydrologic gradient.

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We studied seasonal variation in population attributes of AM fungi over two years in four sites of temperate grasslands of the Argentinean Flooding Pampas. The sites represent a wide range in soil conditions, hydrologic gradient, and floristic composition. *Lotus glaber*, a perennial herbaceous legume naturalised in the Flooding Pampas, was dominant at the four plant community sites. Its roots were highly colonised by AM fungi. Temporal variations in spore density, spore type, AM root colonisation, floristic composition and soil chemical characteristics occurred in each site and were different among sites. The duration of flooding had no effect on spore density but depress AM root colonisation. Eleven different types of spores were recognized and four were identified. Two species dominated at the four sites: *Glomus fasciculatum* and *Glomus intraradices*. Spore density was highest in summer (dry season) and lowest in winter (wet season) with intermediate values in autumn and spring. Colonisation of *L. glaber* roots was highest in summer or spring and lowest in winter or autumn. The relative density of *G. fasciculatum* and *G. intraradices* versus *Glomus* sp. and *Acaulospora* sp. had distinctive seasonal peaks. These seasonal peaks occurred at the four sites, suggesting differences among AM fungus species with respect to the seasonality of sporulation. Spore density and AM root colonisation when measured at one time were poorly related to each other. However, spore density was significantly correlated with root colonisation three months prior suggesting that high colonisation in one season precedes high sporulation in the next season.

Growth, Nitrogen and Phosphorus economy in two *Lotus glaber* Mill. Populations grown under contrasting P-availability.

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With the aim of comparing the growth and economy of phosphorus (P) and nitrogen (N) of two *Lotus glaber* populations differing in their ploidy level, an experiment was performed under two soil P-availabilities. Plants were grown in the open air from mid January to April 2003 in soil filled pots kept under field capacity. The experimental design was a 2x2 factorial consisting of two *Lotus glaber* populations, a colchicine-induced autotetraploid (Barufaldi et al., 2000) and diploid cv. Chaja, provided by KWS Argentina S.A., and two P-fertilization doses (0 and 100 ppm of P as triple super phosphate). The original extractable P concentration was 4 ppm (Bray and Kurtz N°1). At transplanting time, all germinated seeds were inoculated with 10⁸ cells per plant of the commercial inoculant strain LL32 produced by Laboratorios Biagro S.A. Argentina. Twenty plants were harvested at 46, 74 and 102 days after germination and on each occasion growth parameters, P and N concentration were determined. In addition to the greater growth of all fertilized plants, the two populations showed similar growth rate (GR) and relative growth rate (RGR) at each P-availability. Variability of dry biomass was greater for tetraploid than for haploid plants. In spite of similar means at each harvest, the tetraploid population included the plants with the highest individual dry biomass. Leaf area of diploid plants at the end of the experiment was larger than that of tetraploids, due to a higher biomass partitioning to leaves. Phosphorus concentration was similar between populations and greater in P-fertilized plants. Differences were not found either for P-absorption and P-utilization efficiencies or P-partitioning between shoots and roots. As was expected, P-fertilization increased P-uptake and reduced P-utilization efficiencies, but partitioning was not affected in either population. In a similar manner to the results obtained with phosphorus, neither N-concentration nor N-use efficiencies were different between populations. Differences in N-content observed between P-availabilities were partially attributed to the higher number of nodules observed in P-fertilized plants. Under P-fertilization higher nodule number and nodule dry weight were observed in the diploid population.

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Comparative responses between *Lotus glaber* and *Paspalum dilatatum* to the flooding-grazing interaction.

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Flooding and grazing are the two most important factors of the disturbance regime that affects the natural grasslands of the Flooding Pampa. There is enough evidence that indicates that two functional groups of these grasslands, native gramineous and exotic dicotyledonous differ in the tolerance of the above-mentioned disturbances (Insausti *et al.*, 1999). *Paspalum dilatatum* and *Lotus glaber*, representatives of these two groups, respectively, are frequently subject to the action of cattle trampling and defoliation, as sequelae from grazing during large flooding periods. Our aim was to assess a series of response variables that characterize the water relations, the tissue aeration and the growth of the plants of *L. glaber* and *P. dilatatum* when they are simultaneously affected by grazing and flooding. In this hypothesis we propose that *L. glaber* does not tolerate the simultaneous action of trampling, defoliation and flooding, such as the gramineous *P. dilatatum*. We predict that *L. glaber* responds to these disturbances with a lower water potential, conductance, and foliar transpiration than *P. dilatatum*. Besides, it shows lower porosity in their tissues and it does not place its leaves above water level, as fast as *P. dilatatum* does.

In order to test this hypothesis, an experiment was carried out in 25 individuals of each species extracted from the grassland in soil blocks. After a two-month acclimatizing period, the treatments were initiated in an experimental garden: 1) flooding (F), 15 days at a 6 cm level, 2) simulated cattle trampling (T), at the onset of the experiment, 3) defoliation (D), at the onset, by cutting above 6 cm, 4) control without alterations (C). The flooding period was followed by a recovery period of 30 days. The design was a factorial arrangement with 5 treatments (C-F-T-D-FTD) and 5 replicates. The following response variables were measured: a) leaf waterpotential (Ψ_w), b) stomatal conductance (g_s), c) transpiration (E), at 3-5 day intervals, d) porosity of roots and shoots/sheaths, by pycnometry at the end of the flooding, e) time record of the height of 10 young shoots/tillers and f) number of green and dry shoots/tillers.

The results showed an increase in the porosity of the roots and sheaths of *P. dilatatum* in the treatments with flooding or in the FTD interaction. Besides, in this species the greater values of Ψ_w , g_s and E were recorded in the days of high atmospheric demand with respect to the control ones and, as from the 5th day of the flooding, the tillers were above water level. The number of tillers was lower with the trampling (T) and increased with the defoliation (D or FTD), compared to the control ones, but there were no differences with respect to the flooding. The *L. glaber* plants with FTD treatment died five days after the onset of the

experiment. The ones that were only subject to flooding increased their porosity, in the roots and shoots, however they showed lower values of Ψ_w , g_s and E along the time with respect to the control ones. Once the flooding was discontinued, such plants recovered. The control shoots of the *L. glaber* plants took three times less time than the flooded ones to reach the height that the water level had in the flooding treatment. In this species, trampling caused a decrease in the radical porosity and the quantity of shoots. This last variable was also reduced with the flooding but increased with the defoliation. It is concluded that flooding and grazing show a differential effect on these species, in their water relations and their growth, and that these responses would be related to the different tolerance that they express with respect to those grassland disturbances.

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Morpho-physiological characterization of *Lotus glaber* naturalized populations

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Lotus glaber Mill (*Lotus tenuis* Waldst. et Kit) is a legume that has become naturalized in lowlands of Argentinean pampas. The productive cycle of this perennial legume native from Europe is during spring and autumn and summer and is considered a great alternative for forage production in stressed soil conditions including flooding and phosphorous deficiency. In addition, *Lotus glaber* is well known for its anti-bloating and high nutritional properties. (Mazzanti *et al*, 1992).

Our principal aim in this work is to evaluate the morpho-physiological genetic variability looking to contribute to future breeding programs in the species.

During 2003 and 2004 were collected several genotypes of *Lotus glaber* in diverse environments of Buenos Aires Province. The places where the samples were obtained were evaluated for their edaphically properties as well as their historical cow managements and production. Using an experimental block randomized method; at least 50 seeds were collected for each genotype.

A biodiversity evaluation of vegetal species implanted around the *Lotus glaber* completed the study.

In May 2004 the seeds collected were sown in pots containing compost and maintained in controlled environmental conditions in a greenhouse. One month later, the plants obtained were transplanted to farm condition in the experimental field of EEA Pergamino. Eighty genotypes for each population were analyzed using the method described elsewhere (Turesson, 1922).

The variables evaluated were:

- Plant vigor (low, medium and high)
- Growth style (erect, prostrated and intermediate)
- Stem and blade numbers per plant
- Dry weight per plant
- Time flowering after farm transplantation.
- Seed production
- Existence or not of bacterial and fungal diseases

The results obtained will be presented and discussed.

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Influence of the arbuscular mycorrhizal fungus *Glomus intrarradices* on the saline stress physiology of *Lotus glaber*.

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Lotus glaber is a glycophytic, perennial legume from Europe that occurs widely in saline habitats. Previous observations indicate the occurrence of genotypes highly tolerant to salinity in different populations of *L. glaber* (Mujica and Rumi, 1997). Several workers have shown that AM fungi protect the plant against salinity (Al-Karaki *et al.*, 2001; Feng *et al.*, 2002). In turn, a high diversity of AM fungal colonization patterns in roots of *L. glaber* was found in fields characterized by their high salinity (Sannazzaro *et al.*, 2004).

Our aim was to evaluate the effect of mycorrhizal colonization on growth response to salt stress in two genotypes of *L. glaber* differing in their tolerance to salinity. We also hypothesized that polyamines, small organic cations that are thought to play a role in the plant responses to salt stress (Bouchereau *et al.*, 1999; Simon-Sarkadi *et al.*, 2002) are involved in such a process.

The experiment consisted of a randomized block design with two factors: (1) mycorrhizal treatments (with or without AM fungus) and (2) two salinity levels of 0 and 200 mM NaCl. *L. glaber* plants colonized by *G. intrarradices* grew better than non-AM ones, particularly under saline condition, where they showed higher values of net growth, shoot/root ratio, K/Na rate, and protein and chlorophyll contents. An increase in total free polyamine content of mycorrhized *L. glaber* plants compared to non-mycorrhized ones, suggests that these amines may be involved in the salt stress alleviation of this species. The increment in spermine levels in sensitive *L. glaber* plants grown under salt stress condition could be due to de novo synthesis from its metabolic precursors. In addition, high proline levels were observed under salt stress conditions in both genotypes. Our results interestingly indicate that *G. intrarradices* established a more efficient symbiosis with the tolerant than with the sensitive genotype. Results suggest that the fungal symbiont could play an important role in the adaptation of *L. glaber* plants to salt stress under field conditions.

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Molecular and biochemical approximation of polyamine roles in tolerance mechanisms to salt stress in *Lotus* spp.

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Lotus glaber is the most important legume in the saline-alkaline lowlands of the Salado River basin. This region (approximately 9,000,000 ha), located in Buenos Aires Province (Argentina) is devoted to the breeding of beef cattle. In order to increase forage yield and improve the quality of their pastures, regional farmers utilize *L. glaber*, whose adaptability to saline soils is well-known. The economic importance of this legume has led to an increasing number of studies regarding the physiological basis of its salt tolerance. Polyamines are aliphatic amines of low molecular weight charged positively at physiological pH. The distribution of these positive charges permits their interaction with proteins, membrane lipids and DNA. It's well known that the activity of the plant enzymes of polyamine biosynthesis is induced under abiotic stress, including salinization. With this idea in mind, we evaluated the effect of salt stress in polyamine pathway in *Lotus glaber*. As proline is a traditional stress marker in plants, we evaluate their levels under similar stress conditions. To understand the response of *L. glaber* to salt stress, we evaluated 15 day-old seedlings germinated and grown under 0, 25, 50 and 75 mM NaCl. We observed a progressive accumulation of Na⁺ and loosening of K⁺. Simultaneously we observed an increasing in proline levels and accumulation in Spm, coincidently with a diminishing in Spd levels.

In addition, we analyzed a natural population of *L. glaber* collected from lowlands of Salado River Basin. We isolated 103 genotypes of *L. glaber* and cloned them by nodal cutting. Thirty days old genotypes were exposed to 300 mM NaCl (n=5), and death days average was determined. A Gaussian distribution was obtained. The time survival varied from 12 till 30 days. Five genotypes of each extreme were selected and cloned and sub irrigated with 0 and 150 mM NaCl. Based on their differential relative growth rates the genotypes were classified in sensitive and tolerant. We concluded that the preliminary classification based on average days of "survival-time" under strong saline conditions are not representative of their tolerance physiological conditions because some genotypes identified firstly as sensible were tolerant and vice versa.

Analysis in polyamine content demonstrated that spermine accumulates less in tolerant genotypes than in sensitive. Simultaneously, proline presents a progressive accumulation in

both genotypes but was smaller in tolerant ones. Complementary and taking in to account the results obtained, we generate *Lotus corniculatus* transgenic plants that over express under control of the constitutive promoter CaMV35S, a putative gene of spermidine synthase previously cloned from tobacco. The lines obtained presented constitutive high levels of Spd and Spm than controls non-transformed. Moreover, we observed that these transgenic plants under saline stress showed a decrease in the spermidine concentrations and an increment in the spermine levels suggesting activation in the spermine synthase activity. Under similar conditions the proline levels diminished.

Actually, we are working in the generation of *Lotus spp* transgenic plants that potentially over express regulatory enzymes in the polyamine biosynthesis under control of a promoter inducible by stress. This promoter denominated RD29A was cloned from *Arabidopsis thaliana* and is under evaluation in our lab. Binary vectors harboring the sequence of arginine decarboxylase (ADC) under control of this promoter was assayed successfully in hairy roots of *Lotus corniculatus*, suggesting a conservative stress signaling pathway between species.

Adventitious shoot regeneration in *Lotus glaber* Mill.

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In order to get a suitable protocol of regeneration for the genetic transformation of *Lotus glaber* Mill., several experiments from different explants were conducted. Roots, cotyledons and leaves from seedlings grown *in vitro* were cut into pieces and used as source of explants. Subsequently, they were cultured on 11 cc glass tubes containing 3 ml of Murashige and Skoog (1962) (plus sucrose 3%) semisolid medium (agar 0.65%), supplemented with different combinations of auxins (either naphthalenacetic acid or indoleacetic acid; NAA and IAA, respectively) and cytokinins (benciladenine, kinetin or thidiazuron; BA, KIN and TDZ, in that order). The cultures were incubated under $116 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF (photoperiod 14 h) and $27\pm 2^\circ\text{C}$.

After 45 days of culture, *de novo* shoot organogenesis was noticed from all explants tested. The type of explants markedly influenced organogenesis and the growth of the regenerated shoots. The regeneration frequencies were higher with leaf and cotyledons explants while the number of shoots formed per responsive explant was greater with leaf and roots.

The number of shoots produced per responsive leaf explant increased from 4 to 23, as the percentage of leaf explants producing shoots increased from 10 to more than 40%. NAA in combination with BA induced the highest regeneration rate ($40.1\pm 18.3\%$) bringing 19.7 ± 3.3 shoots per responsive leaf explant. Histological examination confirmed the direct process of organogenesis. The regenerated shoots from the best induction treatment were transferred to a fresh medium of similar chemical composition and without plant growth regulators for 30 days; in which, the *in vitro* rooting was stimulated. In all cases, the morphogenetic process was characterized by a direct pattern of root formation without callus proliferation. Plantlets with fully expanded leaves and well-developed roots were acclimatized in pots with transparent covers that were subsequently lifted to reduce humidity. The acclimatized plantlets were successfully established in soil. All plantlets were phenotypically normal.

In conclusion, our study provides a practical technique for efficient plantlets production of *Lotus glaber*. The procedure described here for the direct shoot organogenesis from various explants, and subsequently plantlets regeneration, facilitates the rapid propagation of this species. It will also be of use in cryoconservation and genetic breeding aimed at improving the abiotic stress tolerance.

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Fire on native pastures - effects on soil and vegetation.

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The effects of fire and alternative managements on soil and vegetation of native pastures were revised from several research works, jointing personal observations developed during almost 40 years in range areas of Rio Grande do Sul, Brazil. Soil parameters such as potential acidity; aluminum concentration; basis saturation; and others were considered with vegetation parameters such as production and quality of green forage dry matter during the year; botanical composition; volumetric amount of water in soil; nutrients cycling through the forage and dead material; etc. Some results: Burning results in higher contents and saturation of aluminum, and higher potential soil acidity; Mowing reduces potential soil acidity and increases soil basis saturation; Lime, fertilization and mowing favor native species of higher forage value such as *Paspalum notatum*, *Paspalum plicatulum* and *Desmodium incanum*; Burning favor the andropogonea species in detrimental of prostrate grasses and legumes, and also ciperaceae, reducing the floristic diversity; Burning reduces the forage green dry matter and dead material, and the volumetric amount of water in soil, resulting in considerable proportion of uncovered soil surface; Higher nutrient cycling through forage and dead material in areas not burned; The regrowth of native species during the spring period is delayed in areas burned every two years for more than 100 years as compared with other alternative managements. *Eryngium horridum*, undesirable species, increases its participation in burned areas as compared with other alternative managements. The general conclusion, based on the results of the revised works, is that the burning of natural pastures, in the high altitude region of southern Brazil, must be avoided as a routine practice, because it is detrimental to the environment, reduces forage yield and quality, and it is not a sustainable practice.

Is arriving the *Lotus glaber* time in the Pampa Deprimida del Salado?

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The National farming situation showed a clear tendency to intensify agriculture to the detriment of cow production. This situation is induced by the clear differences that exist between the economic prices actually obtained by farmer's through agriculture and livestock. This occurs in part by the high price of the grains and the low livestock productive efficiencies in result to the absence of innovations.

In addition, there is an increasing demand of soils for agricultural activities producing a substantial reduction in the quality and the extension of lands devoted to cow feeding.

One alternative to increase forage capacity and productive efficiency is the implantation of pastures in soils that edaphically stressing, traditionally not utilized for forage production. *Lotus glaber* is ideal to grow in typical unfavorable conditions that exist in lowlands frequently associated with marginal soils in the region.

The *Lotus glaber* in normally sowed associate ie: a mix pasture with *Festuca arundinacea*. In our experimental field, the Chacra Experimental Manantiales, we analyzed this pasture that was sowed in 2002. This assay was development in typical "mosaic soils" using capacities class IV to VII.

The whole forage production was 12672 Kg dry weigh /ha; estimating a cow consumption of 4738 Kg we estimated a mean meat production of 600,5 Kg /ha. The results obtained and the economic equations in productivity will be shown and discussed.

In our opinion, this information is demonstrative that we still can "make something" for a more rentable livestock in the region in using *Lotus glaber* and modifying a farm characterized by only cattle breeding exploitation to cattle breeding and fattening.

Perspectives of utilization of native legumes in Rio Grande do Sul.

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The importance of legumes for animal production has been shown since a long time ago by many authors (Blaser, 1982; Petritz *et al.*, 1980). The inclusion of legumes on pastures promotes several advantages, such as an increase on animal production, as a result of better forage quality, a better forage distribution of forage yield along the year and an increase on soil fertility and microbiological activity, due to incorporation of N to the soil. Recently Dall'Agnol and Scheffer-Basso (2004) have related the general benefits of temperate and tropical legumes, as well as the situation of temperate and native legumes in the "Zona Campos" of South America (Dall'Agnol *et al.*, 2002).

Although temperate legumes are species considered important for many grazing systems, their lack of persistence has been pointed as the major limitation to their utilization, as well as bad management practices by the farmers (Beuselinck *et al.*, 1994). In Southern Brazil, the grazing systems used are typically extensive, with the native pasture being the base of most pastures. On those systems, legumes are not extensively used, but some of them have a relative importance. Among those, the most important are white clover (*Trifolium repens*), red clover (*T. pratense*), birdsfoot trefoil (*Lotus corniculatus*). These species are used as cultivated pastures or are over-sown on the native pastures, aiming an improvement on forage quality and yield distribution, but on both cases with poor persistence as earlier related.

Regardless of the importance that the maintenance and persistence of legumes on pastures have, very little attention has been given to their study and for the understanding of their relationships on the different grazing systems. This point is particularly true in relation to our native species, which demonstrates the lack of collaborative work among the different researchers and research institutes.

An increase in legume utilization is possible and one of the possibilities is through their inclusion on areas integrated with cultures, such as soybean-pastures, rice-pastures, etc. However, an increase on legume utilization, especially the natives, must be made with special care on conserving this valuable natural resource. This germplasm is unique, with species of excellent value of forage and it is our obligation to study and to preserve it for the next generations. Among the several genera and species with potential, it is worth mentioning: *Adesmia* (*A. bicolor* and *A. bicolor*); *Trifolium* (*T. polymorphum*, *T.*

riograndense and *T. argentinense*); *Desmodium* (*D. incanum*, *D. uncinatum*) and *Macroptilium*, *Vigna* and *Vicia*.

Finally, an increase on legumes utilization, natives or not, only will be possible if we can make a real integration of different areas of knowledge, such as plant pathology, microbiology and physiology, among others. Besides that we should make an effort to **decrease** the increasing number of burocratic barriers for germplasm exchange and collaborative work, including those existing in our home countries.

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***Lotus glaber* productivity changes under different management conditions.**

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Lotus glaber productivity changes pure and in mixtures with other species were analyzed. *L. glaber* productivity was high in the first year, but it declined in time (Colabelli and Miñón 1994; Quadrelli *et al.*, 1997). The changes in the productivity and quality of pasture sown in the Flooding Pampas (Buenos Aires, Argentina) have been studied with a succession viewpoint. The pasture was gradually replaced by native species, present in the seed bank (León and Oesterheld, 1982; Oesterheld and León, 1987). Pasture changes were caused by soil compaction (Oesterheld and León, 1993), plant mortality and nutrition deficiency (Guaita *et al.*, 1996), among other factors. *Lotus glaber* productivity declined in three years' time while the productivity of native and exotic species increased, some of them of poor quality (Colabelli and Miñón, 1994; Quadrelli *et al.*, 1997). *Lotus glaber* productivity changes may be due to reduction of stem density (Miñón and Refi, 1993; Acuña and Cuevas, 1999) and seedling mortality caused by *Fusarium spp.* (Monterroso *et al.*, 1998). *Lotus glaber* plant mortality caused by flooding (Vignolio *et al.*, 1994) and soil compaction (Striker *et al.*, 2005) was also reported. Plant mortality by mechanical shoot cut carried out at the beginning of the reproductive season also was recorded. *Lotus glaber* spreads by seed; therefore, if seed production is affected by grazing (Miñón and Refi, 1993; Acuña and Cuevas, 1999) or mechanical cut (Colabelli and Miñón, 1993; Quadrelli *et al.*, 1997), besides the aspects previously mentioned, it is possible to understand the reduction of its productivity in time. Bovine can spread *L. glaber* seeds, but, the number of seedlings dying in dung during the establishment phase is very high (Sevilla *et al.*, 1996). Furthermore, if the animal eats immature pods and seeds, the seed bank could exhaust and delay *L. glaber* population recovery. In order to recover pasture productivity is recommendable: (a) to control weeds, although some studies have reported that the weeds biomass was increased with fertilization (Quadrelli *et al.*, 1997.); (b) to fertilize with P (Guaita *et al.*, 1996); (c) to sow the species that are in low quantity and (d) to maintain the seed bank for natural reseeding (Taylor *et al.*, 1973). The persistence of *L. glaber* populations in pasture and in grassland could be possible by means of seeds bank. *Lotus glaber* has an important seedling emergence pulse at the end of winter (Sevilla *et al.*, 1996); therefore, if the farmers generate favorable conditions for its establishment, *L. glaber* population could be maintained.

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Epidemiological studies on crown and root rot of birdsfoot trefoil in Uruguay

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Abstract

Birdsfoot trefoil fields in 3 locations in Uruguay were surveyed to determine incidence and severity of crown and root diseases in 1- to 3-yr-old stands. Twenty-five plants in each of 12 permanent quadrats were evaluated at each site (n = 300 plants per site, 3 sites per location). Plants were scored for disease severity following a 5-class scale: 0 = no disease, 4 = dead plant. Crown and root rot occurred in every site, with average incidences (percent infected plants) of 43, 96, and 100% and average severities of 0.51, 1.51, and 1.86 in 1-, 2-, and 3-yr-old stands, respectively. *Fusarium oxysporum* was the primary pathogen associated with diseased plants. Variance to mean ratios for disease severity among quadrats within sites were consistently less than 1, suggesting that disease was not aggregated among quadrats in individual sites. Stand counts decreased with age, from 200 plants/m² in 1-yr-old to less than 50 plants/m² in 3-yr-old stands. Stand counts also decreased with increasing disease intensities among stands of the same age, suggesting a relationship between crown and root rot and plant persistence. Resistant cultivars and proper utilization practices should be considered as potential means for disease management.

Keywords: birdsfoot trefoil, crown and root rot, *Fusarium oxysporum*, *Lotus corniculatus*, persistence

Introduction

Birdsfoot trefoil (*Lotus corniculatus* L.) is the most important forage legume in Uruguay, where it is used for pasture, hay or silage. In addition, seed production is an important export enterprise. Birdsfoot trefoil is usually undersown with a cereal crop during the fall, and plays an important role in the sustainability of crop-pasture rotations. The major constraint for the use of birdsfoot trefoil is its relatively poor persistence. In Uruguay, significant plant losses are observed in pastures in stands two-years-old and older, especially following periods of environmental stress (i.e. summer drought) or under continuous grazing systems (Formoso, 1993). These stands become unprofitable and the farmer may decide to start a new crop, resulting in a short legume-phase in the rotation. A similar situation is reported by several authors in North America (Beuselinck *et al.*, 1984; Hoveland, 1989; Miller *et al.*, 1964;

Taylor *et al.*, 1973). The limited persistence is generally attributed to the interaction of several abiotic and biotic factors such as climatic and edaphic stresses, diseases and pests, and management practices that produce a cumulative stress load (Grau, 1996; Leath, 1989). The use of birdsfoot trefoil could be increased if highly productive stands could be maintained under intensive management for several years.

Diseases are a major cause of premature stand decline and reduced productivity in most temperate forage legumes (Leath, 1989; Leath *et al.*, 1996). Crown and root diseases have been identified as the most important limitations to birdsfoot trefoil production and persistence (Berkenkamp *et al.*, 1972; Beuselinck, 1988; Drake, 1958; Grant and Marten, 1985; Henson, 1962; Hill and Zeiders, 1987; Hoveland *et al.*, 1982; Hoveland *et al.*, 1987; Kainski, 1960; Miller *et al.*, 1964; Pettit *et al.*, 1966; Taylor *et al.*, 1973). Severe losses from these diseases are usually associated with warm weather and high humidity, and thus these are of greater importance in the South than in the Northeast or Northcentral U.S. (Beuselinck, 1988; Grant and Marten, 1985). However, these diseases have been recently reported in most regions where birdsfoot trefoil is grown (Altier, 1994; Bergstrom *et al.*, 1995; Viands *et al.*, 1994). In Uruguay, Altier (1994, 1997) found that 93% of birdsfoot trefoil plants from a space plant nursery died by the end of the second year, and 82% of plant losses were due to crown and root diseases.

The first symptom of crown and root infection is the failure of the plants to resume growth after harvest (Berkenkamp *et al.*, 1972; Grau, 1996; Henson, 1962; Kainski, 1960). Infected plants have a low tolerance to water stress during summer months and reduced vigor; if the invasion continues, plants become wilted and die. Diseased plants show necrosis and rotting of crown and root cortical tissues, but discoloration may be restricted to the central core and follow the vascular system (Figure 1). As the disease develops, both the cortex and central core may be invaded by the fungus. Necrotic areas are often associated with wounds in the crown or root surface (Altier, 1994; Leath *et al.*, 1971). Insect feeding injury by root curculio probably enhances infection by soil pathogens (Kalb *et al.*, 1994; Leath and Hower, 1993).



Figure 1. Symptoms of crown and root rot in a diseased birdsfoot trefoil plant.

Crown and root diseases are caused by a complex of soil organisms. Although several genera of fungi including *Rhizoctonia*, *Mycoleptodiscus*, *Macrophomina*, *Phoma*, and others have been isolated from diseased plants, *Fusarium* species make up the largest number of pathogens causing crown and root diseases of birdsfoot trefoil (Berkenkamp *et al.*, 1972; Beuselinck, 1988; Drake, 1961; Henson, 1962; Kainski, 1960; Ostazeski, 1967). The species of *Fusarium* most frequently associated with crown and root rots of forage legumes is *F. oxysporum*, followed by *F. avenaceum*, *F. solani*, *F. acuminatum*, *F. tricinctum*, and *F. moniliforme* (Grau, 1996; Leath, 1989). In addition, *F. oxysporum* has been reported as the causal organism of Fusarium wilt of birdsfoot trefoil (Gotlieb and Dorisky, 1983; Zeiders and Hill, 1988). More recently, Bergstrom and Kalb (1995) described a wilt organism of birdsfoot trefoil as a specific pathogen of this species, for which they proposed a new taxon, *F. oxysporum* f.sp. *loti*. In Uruguay, Altier (1994, 1997) studied the fungi associated with diseased birdsfoot trefoil plants in a nursery and found that the majority of fungi isolated from crown and root tissues were *Fusarium* spp. (72%), with the two most frequently isolated species being *F. oxysporum* (54% of total) and *F. solani* (9% of total). Similar results were found by Chao *et al.* (1994), who reported *Fusarium* as the main genus (>80%) associated with infected crowns and roots during a survey of diseases affecting birdsfoot trefoil in western Uruguay and the Entre Rios Province, Argentina.

While the information published on crown and root diseases of *Lotus* focuses on descriptions of pathogens, etiology, or yield impacts, studies on disease ecology and epidemiology are limited (English, 1999). Information on the ecological aspects of *Fusarium* crown and root disease comes from studies done with other plant hosts. Forage legume roots are most likely colonized by *Fusarium* species shortly after planting. However, disease symptoms may not appear for some time. This delay has been generally attributed to the weak pathogenicity of root rotting *Fusaria*, which cause more severe rot when plants are under stress (Grau, 1996; Leath, 1989). *Fusarium* rot then progresses gradually, increasing in severity with the age of the plant (Kalb *et al.*, 1994; Leath, 1989).

Knowledge of disease development on individual plants must be coupled with epidemiological studies to understand host population responses to pathogen population pressures in the field. Although gross estimates on the impact of crown and root rots on forage legumes are available, there is much less research concerning the quantitative measurement of disease incidence and severity and the dynamics of these diseases in time and space (Nutter and Gaunt, 1996). The understanding of root disease development in time and space is of critical importance for the management of a perennial forage crop. The longevity of each plant, and therefore the productivity and persistence of the crop, will be directly dependent on healthy root systems. Until epidemiological data are available, disease management in forages will be imprecise (Leath, 1989).

Quantitative characterization of root disease epidemics has been difficult because of the relative inaccessibility of the roots (Campbell, 1986). The progress of root disease epidemics is most commonly monitored as an increase in incidence and severity of root symptoms. Obtaining these measurements requires destructive sampling which does not allow repeated assessments on the same plant. Sampling strategies must be carefully designed in order to study the temporal development and spatial pattern of disease (Campbell and Madden,

1990).

The current study was aimed to assess the importance of crown and root rot complex on birdsfoot trefoil production in Uruguay and characterize disease epidemiology. This knowledge should provide insight into potential means of disease management to aid farmers in decision-making. Specifically, the major objectives of this research were to determine (1) the incidence and severity of crown and root diseases of birdsfoot trefoil as affected by stand age in diverse ecological regions of Uruguay, and (2) the main pathogens associated with diseased plants.

Materials and Methods

Field survey

Between September 1994 and March 1996, 12 birdsfoot trefoil fields were surveyed in three areas representing distinct physiographic regions of Uruguay, distinguished by soil type, topography, and prevalent production systems. These fields were located in areas near INIA La Estanzuela, Colonia, INIA Tacuarembó, Tacuarembó, and INIA Treinta y Tres, Treinta y Tres (INIA, National Institute for Agricultural Research, Uruguay) (Figure 2).



Figure 2. Location of three regions in Uruguay, in which birdsfoot trefoil was surveyed for crown and root diseases from 1994 to 1996.

Characteristics at each area are as follows; Colonia: clay-loamy, horizon B textural soils (O.M.=2.1-4.3%; pH=5.8-7.0; Al<0.1meq/100g; P>5ppm), sown pasture for very intensive production systems (livestock-crop and dairy farms); Tacuarembó: sandy/sand-loamy, deep soils (O.M.=1.1-3.3%; pH=4.5-5.5; Al=0.4-0.8meq/100g; P<5ppm), sown pasture for intensive to more extensive production systems (livestock, small area under crop rotation and dairy farms); Treinta y Tres: loamy/loam-sandy soils (O.M.=1.5-4.7%; pH=5.1-5.6; Al=0.1-0.5meq/100g; P<5ppm; high erosion risk), sown pasture for diversified production systems (livestock, hay-seed production of forage crop).

Each September (spring 1994 and 1995), birdsfoot trefoil fields were selected to compose a matrix of nine sites: three locations and three ages of stand (1-, 2-, and 3-yr-old). One-yr-old stands represented pastures sown during the fall months (April-May-June) of the current year. In September 1995 at each location, the 3-yr-old field was dropped while a new 1-yr-old field was included, in order to keep the nine site matrix. This new matrix was sampled at September 1995 and at March 1996.

A stratified sampling design was employed using twelve permanent 5x5 m quadrats per site. In each site, stand counts and plant samplings were performed twice a year, at the end of winter (September) and at the end of summer (March). Stand counts (no. of plants/m²) were performed in the central square meter of the quadrat, using a 1.0x0.1 m transect. Sample size in each quadrat was 25 plants, with one plant randomly sampled from each 1x1 m cell of the quadrat. Plants were dug and removed with the entire root system, placed in ice chests and taken to the laboratory. At the laboratory, roots and crowns were washed and split longitudinally. Each plant was scored for disease severity (crown rot and root rot separately) following a visual five-class scale: 0 = no disease, 1 = slight rot or discoloration (less than 30% affected tissue), 2 = moderate crown/root rot or discoloration (30-70% affected tissue), 3 = severe crown/root rot or discoloration (more than 70% affected tissue), 4 = plant dead. Disease incidence (crown rot and root rot separately) was calculated as percentage of diseased plants per quadrat. The assessed unit was the whole crown or root: a scale value of 1 or higher constituted disease. Variance-to-mean ratios were calculated for the crown and root severity data for each quadrat at each sampling time to provide insight into the spatial pattern of the disease among quadrats (Campbell and Madden, 1990).

Descriptive statistics and analyses of variance (general linear model procedure, SAS Institute) were performed on crown and root rot incidence (CRI, RRI), crown and root rot severity (CRS, RRS), stand counts (NP), and variance-to-mean ratios for crown and root rot severity (VMC, VMR). Mean separations were performed using Fisher's protected LSD test ($P < 0.05$). For the seven variables analyzed (CRI, RRI, CRS, RRS, NP, VMC, VMR), the variances were not homogeneous among different years (1994-1995 vs. 1995-1996) and different sampling seasons (September vs. March), therefore data were analyzed separately.

Fungal isolations

Subsamples of diseased roots from each quadrat of each site, sampled in September of 1994 and September of 1995, were used for fungal isolation. The roots were randomly selected from those representing the median severity class in the given quadrat, most commonly roots in classes 1 and 2. Pieces of 0.5-1.0 cm² from the interface of infected and non-symptomatic tissue were washed under flowing tap water overnight, surface-sterilized by soaking in 95% ethanol for 1 min, then soaking in 1% sodium hypochlorite for 3 min, followed by a rinse in sterile distilled water, and finally plated on PDA. Two and five pieces were plated per quadrat, for roots sampled in September of 1994 and September of 1995, respectively. The intention was to obtain at least one fungal isolate per quadrat per site (12 quadrats x 3 locations x 3 stand ages = 108 isolates). Hyphal tip growth of each different fungal colony (except for easily identified genera) was transferred to PDA plates and tubes for further

identification and storage. Each year (1994 and 1995) a collection of *Fusarium* spp. isolates was maintained on PDA slants at 4 C during the identification process (four months). Subsequently, selected isolates were stored on silica gel crystals at 5 C until needed (Windels, 1992). Identification of *F. oxysporum* was done using the procedures outlined by Nelson *et al.* (1983). Three randomly selected isolates were sent to the International Mycological Institute (IMI-CAB International, UK) for confirmation of identification (IMI No. 368015, 368016, 368017, report from Dr. D. Brayford). Two collections of *F. oxysporum* isolates were finally composed.

Results

Field survey

Crown and root rot occurred in every field surveyed, independent of location and stand age. Locations followed no consistent trend as a source of variation for crown and root rot incidence. Stand age had a large and significant effect on disease incidence (Table 1). At both sampling times (September and March), crown rot incidence and root rot incidence were significantly lower in 1-yr-old stands than in 2- or 3-yr-old stands. The largest increase in crown rot incidence was observed between September and March of 1-yr-old stands, i.e. after the first summer of the crop, when incidence reached levels close to 80% (Figure 3A). Root rot incidence increased more slowly than crown rot incidence, but by September, in two-yr-old stands, levels were very close to 100% (Figure 3A). From that time on, levels of crown and root rot incidence were always higher than 90% (Figure 3B) but differences in disease incidence between 2-yr-old vs. 3-yr-old stands were still usually significant (Table 1).

Disease severity data showed similar trends as disease incidence (Table 2, Figure 4). Location did not always have a significant or consistent effect on crown rot and root rot severity. Crown rot severity as well as root rot severity increased significantly with the age of the stand. As compared with disease incidence, disease severity continued to increase gradually from 2-yr-old stands to 3-yr-old stands, when there were no or few nondiseased plants left (disease incidence was close to 100%). The largest increase in disease severity occurred during the winter for both years. Crown rot severity was always higher than root rot severity, except in September in 1-yr-old stands, where crown rot severity was equal to or lower than root rot severity.

Stand counts were significantly affected by location and stand age (Table 3). Two and 3-yr-old stands at Tacuarembó generally had lower counts than the other two locations. The average number of plants per square meter declined as stands aged. The largest reduction was observed from 2- to 3-yr-old stands. Within each field, the stand counts indicated that most plants died during the summer, as determined by large differences between September counts and March counts (Figure 5). Data on stand counts showed the reverse trend from data on disease level, i.e. the older the stand, the higher the disease level and the lower the number of plants per square meter.

Table 1. Incidence¹ of *Fusarium* crown and root rot in birdsfoot trefoil, for each sampling time² as affected by stand age.

Stand Age	Crown Rot Incidence (%)				Root Rot Incidence (%)			
	S942	M95	S95	M96	S94	M95	S95	M96
1-yr-old	16 ³	78	2	80	17	43	26	79
2-yr-old	96	95	100	99	91	92	96	99
3-yr-old	99	99	100	100	99	98	99	100
LSD (0.05)	2	3	1	4	3	4	4	4
CV (%)	7	8	4	8	9	12	11	8

¹ Disease incidence: No. of diseased plants/total No. of assessed plants.

² September 1994 (S94), March 1995 (M95), September 1995 (S95), March 1996 (M96). Data were from the same 9 site matrix for Sept. 94 vs. March 95, and for Sept. 95 vs. March 96, so these two pairs of columns of values can be compared. However, variances were not homogeneous, therefore LSDs were not calculated.

³ Average of three locations.

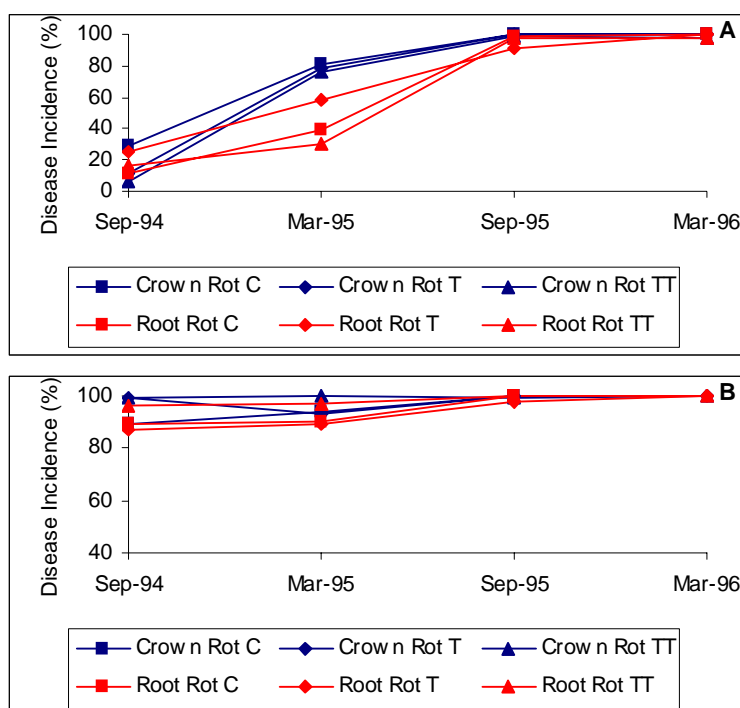
**Figure 3.** Progress of disease incidence of crown and root rot of birdsfoot trefoil in 1- and 2-yr-old stands (A) and 2- and 3-yr-old stands (B), surveyed in Colonia (C), Tacuarembó (T) and Treinta y Tres (TT). Disease incidence was calculated as No.diseased plants/total No. assessed plants.

Table 2. Severity¹ of *Fusarium* crown and root rot in birdsfoot trefoil, for each sampling time² as affected by stand age.

Stand Age	Crown Rot Severity				Root Rot Severity			
	S942	M95	S95	M96	S94	M95	S95	M96
1-yr-old	0.17 ³	0.90	0.02	1.06	0.18	0.48	0.28	0.97
2-yr-old	1.33	1.49	1.82	1.91	1.20	1.26	1.43	1.65
3-yr-old	1.67	1.75	2.22	2.39	1.52	1.49	1.80	2.00
LSD (0.05)	0.06	0.07	0.08	0.14	0.07	0.09	0.09	0.13
CV (%)	12.0	11.4	11.9	16.0	15.5	16.8	16.4	17.4

¹ Disease severity: 0 = no disease, 1 = slight crown/root rot (<30% affected tissue), 2 = moderate crown/root rot (30-70% affected tissue), 3 = severe crown/root rot (>70% affected tissue), 4 = plant dead. Since the scale included a class 0, crown and root rot severity are expressed as a disease index (DSI).

² September 1994 (S94), March 1995 (M95), September 1995 (S95), March 1996 (M96). Data were from the same 9 site matrix for Sept. 94 vs. March 95, and for Sept. 95 vs. March 96, so these two pairs of columns of values can be compared. However, variances were not homogeneous, therefore LSDs were not calculated.

³ Average of three locations.

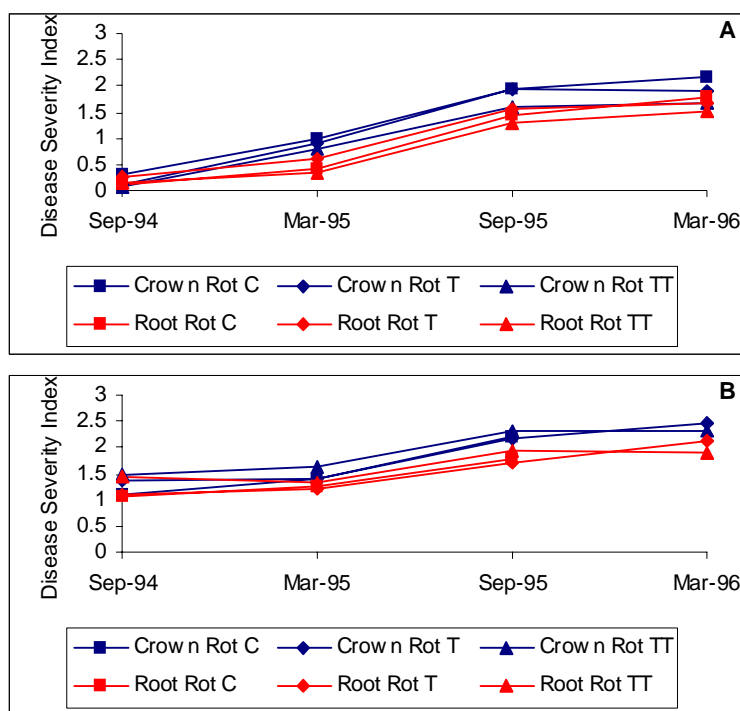


Figure 4. Progress of disease severity of crown and root rot of birdsfoot trefoil in 1- and 2-yr-old stands (A) and 2- and 3-yr-old stands (B), surveyed in Colonia (C), Tacuarembó (T) and Treinta y Tres (TT). Disease severity was rated using a visual 5-class scale: 0 = no disease, 1 = slight crown/root rot (<30% affected tissue), 2 = moderate crown/root rot (30-70% affected tissue), 3 = severe crown/root rot (>70% affected tissue), 4 = plant dead. Since the scale included a class 0, crown and root rot severity are expressed as a disease index (DSI).

Table 3. Number of plants of birdsfoot trefoil per square meter, for each sampling time¹ as affected by stand age.

Stand Age	No. of plants/m ²			
	S941	M95	S95	M96
1-yr-old	314 ²	168	188	106
2-yr-old	214	116	146	63
3-yr-old	89	46	86	1
LSD (0.05)	25	16	21	15
CV (%)	26	31	32	47

¹ September 1994 (S94), March 1995 (M95), September 1995 (S95), March 1996 (M96). Data were from the same 9 site matrix for Sept. 94 vs. March 95, and for Sept. 95 vs. March 96, so these two pairs of columns of values can be compared. However, variances were not homogeneous, therefore LSDs were not calculated.

² Average of three locations.

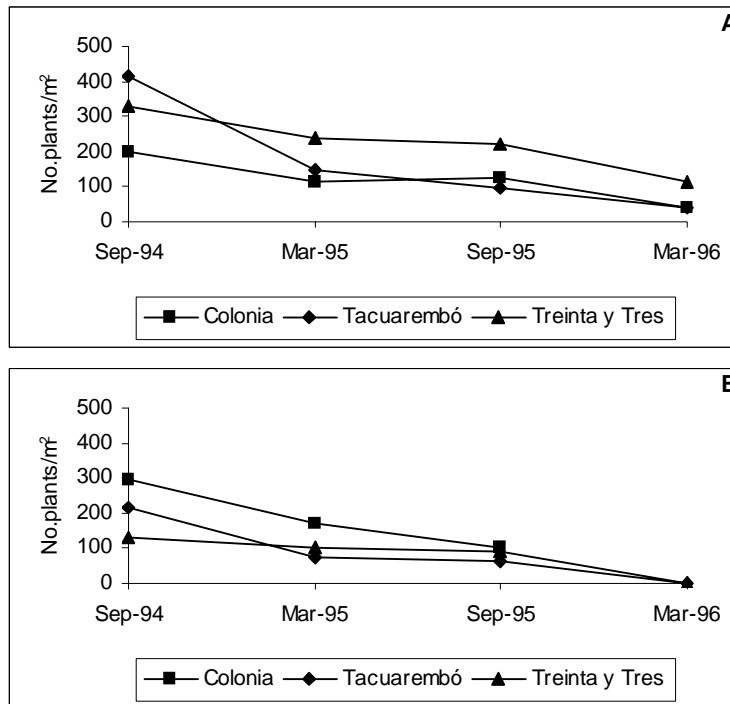


Figure 5. Reduction in the number of plants of birdsfoot trefoil in 1- and 2-yr-old (A) and 2- and 3-yr-old (B) stands surveyed in Colonia, Tacuarembó and Treinta y Tres.

The average variance-to-mean (VM) ratios were always less than 1, which suggests a rather uniform distribution of the disease independent of sampling time, stand age, and location (Table 4). Location did not have a significant effect on VM ratios, except for March 96. However, VM ratios were significantly affected by stand age, the younger the stand the higher the VM ratio. The highest average VM ratios recorded in September of 1-yr-old stands were closest to 1, which is indicative of a nearly random pattern of the disease in new stands. VM ratios for crown rot severity tended to be slightly lower than VM ratios for root rot severity, suggesting a marginally more uniform distribution of crown rot than root rot.

Table 4. Variance to mean ratios (V/M) of *Fusarium* crown and root rot severity in birdsfoot trefoil, for each sampling time¹ as affected by stand age.

Stand Age	V/M Crown Rot Severity				V/M Root Rot Severity			
	S941	M95	S95	M96	S94	M95	S95	M96
1-yr-old	0.89 ²	0.34	0.26	0.48	0.80	0.67	0.77	0.45
2-yr-old	0.24	0.28	0.23	0.28	0.31	0.32	0.26	0.28
3-yr-old	0.20	0.19	0.16	0.17	0.21	0.21	0.23	0.23
LSD (0.05)	0.08	0.04	NS	0.07	0.09	0.06	0.07	0.07
CV (%)	40.9	33.8	119.0	42.8	41.9	31.8	37.6	41.8

¹ September 1994 (S94), March 1995 (M95), September 1995 (S95), March 1996 (M96). Data were from the same 9 site matrix for Sept. 94 vs. March 95, and for Sept. 95 vs. March 96, so these two pairs of columns of values can be compared. However, variances were not homogeneous, therefore LSDs were not calculated.

² Average of three locations.

Fungal isolations

Fungal colonies were recovered from root and crown pieces of plants sampled in all three locations. Independent of the location, root and crown pieces from 1-yr-old plants yielded few fungal colonies (11.9% and 14.9% of the total, for 1994 and 1995, respectively), while fungi were readily isolated from root and crown pieces from 2- and 3-yr-old plants.

The majority of fungi isolated from diseased crown and root tissues of birdsfoot trefoil were *Fusarium* spp., with the most frequently and consistently isolated species being *F. oxysporum* (Table 5). The second most frequently isolated fungi included presumed saprophytic genera, *Penicillium*, *Aspergillus*, *Gliocladium*, *Epicoccum*, *Cladosporium*, *Rhizopus*, and *Mucor*. Unknown fungi included sterile hyphomycetes and coenocytic, nonsporulating species, and were recovered in relatively low frequencies (Table 5). One isolate recovered in September 1994, identified tentatively as *Mycoleptodiscus* spp., and two isolates recovered in September 1994, identified presumptively as *Rhizoctonia solani*, were counted as unknown fungi.

Table 5. Percent frequency of fungi isolated from diseased crowns and roots of birdsfoot trefoil plants from three locations (Colonia, Tacuarembó and Treinta y Tres) in Uruguay, for two sampling dates (September 1994 and September 1995).

Fungi isolated	Percent frequency	
	1994	1995
<i>Fusarium</i>	53	57
<i>F. oxysporum</i>	35	40
Other <i>Fusarium</i> spp.	18	17
Presumed saprophytic genera ¹	35	38
Unknown ²	12	5
Total number of isolates ³	120	365
Number of pieces examined	216	540

¹ Species of *Penicillium*, *Aspergillus*, *Epicoccum*, *Cladosporium*, *Rhizopus*, *Mucor*, *Gliocladium*, and others.

² Sterile hyphomycetes, coenocytic, nonsporulating fungi and others.

³ Total number of yielding colonies on PDA from 0.5-1.0 cm² pieces cut from surface-sterilized diseased birdsfoot trefoil crowns and roots.

Sixty four *Fusarium* spp. isolates were recovered from roots sampled in 1994, and 208 isolates from roots sampled in 1995, and composed the two *Fusarium* spp. collections. Forty two *F. oxysporum* isolates from 1994, and 146 isolates from 1995 composed the two *F. oxysporum* collections.

Discussion

Deterioration of roots and crowns of birdsfoot trefoil was demonstrated to occur in diverse ecological areas of the country, representing distinct physiographic regions distinguished by soil type, topography, and prevalent production systems. The range of edaphic conditions surveyed does not limit the development of crown and root diseases in birdsfoot trefoil. We confirm the hypothesis that the occurrence of the crown and root rot complex is a widespread phenomenon in Uruguay and has a negative impact on birdsfoot trefoil production and persistence (Altier, 1994; 1997). Our results also agree with preliminary information obtained by Chao *et al.* (1994) during a survey of diseases affecting 12 birdsfoot trefoil pastures in western Uruguay and the Entre Rios Province, Argentina, which reported that crown and root rot were the most prevalent diseases.

The repeated isolation of *Fusarium oxysporum* from symptomatic plants of birdsfoot trefoil suggests this species is frequently responsible for crown and root rot and stand decline. No other known pathogen that is alone capable of causing these disease symptoms was isolated

from diseased crown and root tissues. These results are consistent with previous findings when studying the fungi associated with crown and root rot complex of birdsfoot trefoil and other forage legumes (Altier, 1994; 1997; Beuselinck, 1988; Chao *et al.*, 1994; Grau, 1996; Kainski, 1960; Leath, 1989; Zeiders and Hill, 1988).

The random initial spatial pattern of crown and root rot (variance to mean ratios slightly less than 1) may result from the ubiquitous nature of *F. oxysporum* and indicates that the potential for disease development is independent of location and site. The fact that VM ratios decreased with the age of the stand suggests a gradual saturation of the system, where every plant sampled was diseased as a consequence of the dispersal over space and time. The observed lower VM ratios for crown rot severity as compared with VM ratios for root rot severity indicates that infection of crown tissue progresses faster than infection of root tissue and saturation of the system occurs early.

The large and significant effect of stand age on disease incidence and severity was expected and previously reported (Berkenkamp *et al.*, 1972; Beuselinck, 1988; Drake, 1958; Grant and Marten, 1985; Grau, 1996; Henson, 1962; Hill and Zeiders, 1987; Hoveland *et al.*, 1982; Hoveland *et al.*, 1987; Kainski, 1960; Leath, 1989; Leath *et al.*, 1971; Miller *et al.*, 1964; Pettit *et al.*, 1966; Taylor *et al.*, 1973). However, we did not expect the high disease incidence levels as early as March, when the plants had not completed one year in the field. Root rot incidence increased slower than crown rot incidence, but by September in the second production year most of the plants were symptomatic. Large areas of necrosis limit the amount of healthy tissue available to maintain the essential physiological functions of water and nutrient absorption, nitrogen fixation, carbohydrate storage and translocation to the growing points (Grau, 1996).

In the conditions of Uruguay, summer appears to be the critical season for plant survival. While the largest increases in crown and root rot severity occurred during the winter, stand count results indicate that most plants died during the summer. The high soil temperatures registered during that season, interacting with periods of drought, most likely accentuate the stress on plants already weakened by disease. Since data on stand counts supported data on disease level, i.e. the older the stand, the higher the disease level and the lower the number of plants per square meter, a relationship between disease level and persistence in the field is clearly established.

Fusarium crown and root rot is affected by environmental, biological and management factors that stress the plant and is a chronic rather than an acute disease (Grau, 1996; Leath, 1989). The utilization of perennial forage legumes is in itself the most serious stress repetitively imposed on the plants (Beuselinck *et al.*, 1984; Hoveland, 1989; Leath, 1989; Miller *et al.*, 1964; Taylor *et al.*, 1973). In the production systems of Uruguay, pastures are utilized during most of the year, therefore grazing animals are imposing a severe stress on the legume. Animals influence legume performance by selective grazing, trampling and excretion (Hoveland, 1989). Animal trampling causes direct injuries to the crown of the plants producing entry points for pathogens. Despite the fact that *Fusarium* species may directly penetrate unwounded tissues, wounding alters the host-pathogen interaction and favors fungal development in the tissues (Chi *et al.*, 1964; Stutz *et al.*, 1985). Wounding of

roots and crowns is a common phenomenon and *F. oxysporum* is a wound parasite that can readily invade tissues (Kalb *et al.*, 1994; Leath, 1989; Leath and Hower, 1993). *Fusarium* spp. are primarily cortical invaders which can survive and increase in the cortex until conditions favor pathogenicity (Kommedahl and Windels, 1979). Once the infection has taken place, the plant remains diseased. The impact of the disease and the rate at which it develops are functions of the environment and management. Because climate cannot be impacted, proper management becomes the prime strategy. Correct and timely application of crop management practices during the winter and summer months must contribute to reduce the stresses imposed on the plants and therefore, to reduce the rate of disease development and stand decline. Crop management practices, such as frequency and intensity of utilization, play a role in the development of *Fusarium* crown and root rot of red clover (Fezer, 1961; Fulton and Hanson, 1960; Rufelt, 1986; Siddiqui *et al.*, 1968) and alfalfa (Lukezic *et al.*, 1969). The effect of utilization management on the rate of disease development and consequently the impact on birdsfoot trefoil productivity need to be investigated.

Additionally, phenotypic selection has proved to be effective in increasing the level of resistance to *F. oxysporum*, when developing birdsfoot trefoil populations (Altier *et al.*, 2000; Rebuffo and Altier, 1997; Zeiders and Hill, 1988). The release of cultivars with enhanced resistance need to be coupled with improved management practices to provide an integrated management scheme for *Fusarium* crown and root diseases.

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Characterization of aggressiveness and vegetative compatibility diversity of *Fusarium oxysporum* associated with crown and root rot of birdsfoot trefoil

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Abstract

Birdsfoot trefoil (*Lotus corniculatus*) fields were selected in a 9-site-matrix of three locations and three stand ages, and surveyed twice a year during two successive years. Twenty-five plants in each of 12 permanent quadrats were sampled at each site and date. Samples of infected crown and root tissues were used for fungal isolation. *Fusarium oxysporum* was the primary pathogen associated with diseased plants (40% of isolations). Vegetative compatibility assessed using nitrate non-utilizing mutants was used as a measure of genetic relatedness of *F. oxysporum* isolates. No complementation was found among 18 isolates (630 pairings of 36 *nit* complementary mutants), indicating a high degree of genetic diversity in the pathogen population. A culture plate method was used to characterize isolate aggressiveness to birdsfoot trefoil seeds and seedlings, based on a 5-class scale: 1 = healthy seedling, 5 = dead seed. Most *F. oxysporum* isolates (36 out of 44) were pathogenic to birdsfoot trefoil, and were highly variable in aggressiveness (range: 1.44-3.85). The variability observed in the pathogen population needs to be considered when selecting isolates for resistance screening.

Additional keywords: crown and root diseases, forage legumes.

Introduction

Crown and root diseases are a major cause of premature stand decline and reduced productivity in birdsfoot trefoil pastures (Altier, 1997; Altier *et al.*, 2000; Bergstrom *et al.*, 1995; Berkenkamp *et al.*, 1972; Beuselinck, 1988; Henson, 1962; Hill and Zeiders, 1987; Kainski, 1960; Miller *et al.*, 1964; Pettit *et al.*, 1966; Taylor *et al.*, 1973). *Fusarium* species make up the largest number of pathogens causing crown and root diseases of birdsfoot trefoil (Berkenkamp *et al.*, 1972; Beuselinck, 1988; Drake, 1961; Henson, 1962; Kainski, 1960). The species of *Fusarium* most frequently associated with crown and root rots of forage legumes is *F. oxysporum*, followed by *F. avenaceum*, *F. solani*, *F. acuminatum*, *F. tricinctum*, and *F. moniliforme* (Grau, 1996; Leath, 1989; Leath *et al.*, 1971). More recently, Bergstrom and Kalb (1995) described a wilt organism of birdsfoot trefoil as a specific pathogen of this species, for which they proposed a new taxon, *F. oxysporum* f.sp. *loti*. In

Uruguay, Altier (1994, 1997) studied the fungi associated with diseased birdsfoot trefoil plants in a space-planted nursery and found that the majority of fungi isolated from crown and root tissues were *Fusarium* spp. (72%), with the two most frequently isolated species being *F. oxysporum* (54% of total fungi) and *F. solani* (9% of total fungi).

Authors disagree on the role that *Fusarium* spp. have in the development of the root and crown disease complex of forage legumes (Grau, 1996). Some contend that pathogenic forms only invade root tissues damaged or killed by other causes; others assert that the fungus plays a major role in root and crown disease development (Leath *et al.*, 1971). The pathogenicity of *Fusarium* species varies considerably among and within species depending on their ability to penetrate roots directly, their degree of host specificity, and their interaction with plant stress factors (Fezer, 1961; Leath, 1989; Leath and Kendall, 1978; Pederson *et al.*, 1980; Stutz and Leath, 1983; Venuto *et al.*, 1995). A number of environmental factors including soil moisture, drainage, air and soil temperature, nutrients, stand density, plant age, frequency and height of cutting, crop rotation, insect injury, and previous invasion by viruses and nematodes, have an effect on the expression of the disease and on plant susceptibility (Leath *et al.*, 1971).

Root rotting *Fusaria* may penetrate unwounded roots directly, but most species have limited ability to initiate root rot on their own (Chi *et al.*, 1964; Stutz *et al.*, 1985). The wounding of roots increases the frequency of penetration by disrupting the mechanical barrier imposed by the epidermis. Furthermore, Stutz *et al.* (1985) asserted that wounding alters the host-pathogen interaction to favor fungal development in the root. Wounding can be the result of insect damage or mechanical injuries by soil heaving, harvesting machinery, and trampling and soil compaction caused by animals.

Kommedahl and Windels (1979) asserted that *F. oxysporum* is mainly a wilt-inducing pathogen. However, it is so frequently isolated from necrotic roots that it is regarded as a root rot, wound-associated, pathogen (Kalb *et al.*, 1994; Leath, 1989; Leath and Hower, 1993). *F. oxysporum* has been described as an aggressive, pioneer colonizer of moribund tissues and it can readily invade roots (Leath, 1989).

An understanding of the evolutionary basis for the pathogenicity of *F. oxysporum* and the genetic diversity of the fungal population is critical and would help to develop, or improve, the effectiveness of strategies for disease management (Gordon and Martyn, 1997; Kistler, 2001). Classical genetics based on segregation and recombination is not possible with this anamorphic fungus, since it lacks a known perithecial state (Kistler, 1997). Among diverse approaches in genetic techniques, heterokaryosis or vegetative compatibility analysis provides the opportunity to study the genetics of *F. oxysporum* with greater precision than before (Bosland, 1988; Klein and Correll, 2001). The mechanisms available for genetic exchange in this species are still largely unknown, but numerous possibilities exist beyond simple sexual or clonal reproduction. Genetic evidence consistent with horizontal genetic transfer (transposable elements) and past genetic hybridization between lineages indicate that means for recombination and production of novel genotypes are effectively available (Kistler, 1997). Heterokaryosis and parasexual recombination have been postulated as mechanisms that play a role in explaining the diverse pathogenic potential of *F. oxysporum*

(Bosland, 1988). Heterokaryon formation is favored by vegetative compatibility, which is mediated by nuclear loci, called *het* or *vic* genes (Leslie, 1993). Two strains are vegetatively compatible if they have the same allele at each incompatibility locus. In an asexual population (as in *F. oxysporum*), differences at the *vic* loci are assumed to effectively limit the exchange of genetic information to those individuals that belong to the same vegetative compatibility group (VCG). Since sexual recombination does not occur, members of each VCG will form a genetically isolated subpopulation that will be subjected to standard population genetic forces such as selection, mutation, migration, and drift (Leslie, 1993). Vegetative compatibility can serve as a natural means to further subdivide closely related fungal populations and has been used to estimate genetic diversity within and among these same populations (Correll, 1991; Klein and Correll, 2001; Leslie, 1993).

Puhalla (1985) initiated the idea of grouping isolates of *F. oxysporum* into VCGs based on the use of nitrate nonutilizing (*nit*) mutants generated on medium containing potassium chlorate. Mutants resistant to the salt are usually also *nit* mutants. On a minimal medium with nitrate as the sole nitrogen source, mutants have a radial growth comparable to wild type, but their colonies are very thin. If mutants produce a dense, wild-type growth when paired, they are vegetatively compatible (Puhalla, 1985). This author reported evidence for a correlation between VCG and forma specialis, and proposed an evolutionary model to explain the origin of formae speciales, races and VCGs. He asserted that vegetative compatibility may be a fast and easy way to distinguish pathotypes of *F. oxysporum* with unique virulence capabilities (Puhalla, 1985). Recently, Klein and Correll (2001) asserted that molecular markers and VCG are usually not independently associated, which may mean that VCGs in *F. oxysporum* represent clones, or closely related strains descended from a common ancestor (lineage). However, when pathotypes have been considered, there often has been no clear-cut association between these and molecular genotypes (Kistler, 1997).

Soil populations of some phytopathogenic fungi are extremely diverse with respect to VCG (Correll *et al.*, 1986; Gordon and Martyn, 1997; Gordon and Okamoto, 1991; 1992). Studies that have used *nit* mutants to differentiate strains in the nonpathogenic portion of a *F. oxysporum* population revealed a large number of distinct VCGs (Correll *et al.*, 1986; Gordon and Okamoto, 1991; Steinberg *et al.*, 1997). Moreover, most of the strains associated with a given host within the same geographic area, or even isolated from a single field in two consecutive years, belonged to different VCGs (Correll *et al.*, 1986; Gordon and Okamoto, 1991). Thus, the frequency with which anastomosis occurs within such populations is likely to be low.

Despite many references concerning VCGs in diverse formae speciales, few references are found concerning *F. oxysporum* that cause crown and root diseases of forage legumes. Venuto *et al.* (1995) studied the virulence, legume host specificity and genetic relatedness of *F. oxysporum* isolates from red clover, and reported that VCGs were not useful in predicting host reaction because isolates from distinct groupings elicited similar host reactions. Their results indicate that the number of genes controlling compatibility seems to be higher than the number of virulence genes (Venuto *et al.*, 1995).

Information on the pathogenicity and genetic diversity of *F. oxysporum* isolates from

birdsfoot trefoil is required if breeding for resistance is to be explored as a means of managing the *Fusarium* crown and root disease complex (Altier *et al.*, 2000). The major objective of our study was to characterize the *F. oxysporum* population associated with diseased plants of birdsfoot trefoil, in terms of aggressiveness and genetic relatedness.

Materials and methods

Fungal isolations

Birdsfoot trefoil fields were selected in a 9-site-matrix of three locations (Colonia, Tacuarembó and Treinta y Tres, Uruguay) and three stand ages (1-, 2-, and 3-yr-old), and surveyed during September of 1994 and 1995. A stratified sampling design was employed using 12 permanent 5x5 m quadrats per site and sample size was 25 plants per quadrat. At the laboratory, subsamples of five diseased roots from each quadrat of each site were used for fungal isolation. Pieces of 0.5-1.0 cm² from different areas of the root and crown (primarily from the interface of infected and non-symptomatic tissues) were washed under flowing tap water overnight, surface-disinfested by soaking in 95% ethanol for 1 min, then soaking in 1% sodium hypochlorite for 3 min, followed by a rinse in sterile distilled water, and finally plated on PDA. Two and five pieces were plated per quadrat, for roots sampled in September of 1994 and September of 1995, respectively. The intention was to obtain at least one fungal isolate per quadrat per site (12 quadrats x 3 locations x 3 stand ages = 108 isolates). Hyphal tips from each fungal colony (except for easily identified genera) were transferred to PDA plates and tubes for further identification and storage. Each year (1994 and 1995) a collection of *Fusarium* spp. isolates was maintained on PDA slants at 4 C during the identification process (four months). Subsequently, selected isolates were stored on silica gel crystals at 5 C until needed (Windels, 1992). Isolates were identified as *F. oxysporum* using the procedures outlined by Nelson *et al.* (1983). Three randomly selected isolates were sent to the International Mycological Institute (IMI-CAB International, UK) for confirmation of identification (IMI No. 368015, 368016, 368017, report from Dr. D. Brayford).

Two core collections of *F. oxysporum* isolates (composed of 15 out of 64 isolates of the 1994 *Fusarium* spp. collection, and 36 out of 208 isolates of the 1995 *Fusarium* spp. collection) were used to perform aggressiveness and vegetative compatibility tests. Isolates to compose the core collections were selected as follows: 1. the three geographical locations were represented; 2. different stand ages within locations were represented; 3. different quadrats within sites were represented, and if there were more than one isolate per quadrat within a site, one isolate was randomly selected.

Aggressiveness of isolates

A culture plate method was used to characterize *F. oxysporum* isolates for aggressiveness to seeds and seedlings of birdsfoot trefoil. The seedling test was not aimed to parallel the development of the *Fusarium* crown and root rot in the field, which primarily occurs in mature plants, but rather to compare the behavior of isolates on a potential host plant as

reported by Fulton and Hanson (1960), Kainski (1960), and Kilpatrick *et al.* (1954). The method is similar to one used to select alfalfa germplasm for resistance to *Pythium* seedling diseases (Altier and Thies, 1995).

Fungal inoculum, consisting of mycelia and conidia, was produced in a 9 cm-diameter petri plate containing PDA. A 3 mm-diameter disc of inoculum was removed from the periphery of the resulting 4 to 5-day-old colony, placed in the center of a 9 cm-diameter petri plate containing 1.5% water agar (WA) and incubated at 22 C for 7 days. Using a vacuum template, 25 surface-disinfested birdsfoot trefoil seeds were placed equidistantly to the inoculum disc in a radiate pattern on the agar surface. The plates were incubated in growth chambers at 22 C for 7 days under cool-white light (12-h photoperiod; 330 FT. candles). Noninoculated plates of WA containing 25 surface-disinfested seeds were used as controls to determine seed germination, and expected percentage of dead and hard seed.

Disease severity was used as a measurement of isolate aggressiveness and was rated using a five-class scale, in which 1 = healthy seedling, primary root free of necrosis or with slight discoloration; 2 = infected seedling, primary root tip necrotic but firm, cotyledons free of disease; 3 = severely infected seedling, primary root tip and/or cotyledons rotted and soft, seedling will die as infection progresses; 4 = dead seedling, germinated seed with emerged radicle rotted; 5 = dead seed, nongerminated seed rotted. Aggressiveness was expressed as disease severity index (DSI), calculated as the numerical value of each class times the number of individuals in the class, divided by the number of seeds expected to germinate as determined in the noninoculated control, and percentage of surviving plants (PSP), calculated as the total of classes 1 and 2 divided by the number of seeds expected to germinate as determined in the noninoculated control.

Eight *F. oxysporum* isolates from the 1994 core collection were tested against San Gabriel and Estanzuela Ganador birdsfoot trefoil cultivars in June 1995, and 36 *F. oxysporum* isolates from the 1995 core collection plus one isolate from 1994 (used as a control) were tested against San Gabriel birdsfoot trefoil in June 1996. The experimental design was a randomized complete block with four replications over time totaling 100 seeds per treatment, with a factorial arrangement of treatments (isolates x birdsfoot trefoil cultivars). For both experiments, data on DSI and PSP were subjected to analysis of variance (general linear model procedure, SAS Institute) and means were separated using Fisher's protected LSD test ($P < 0.05$).

Genetic relatedness of isolates

Vegetative compatibility was used as a measure of genetic relatedness using the methodology developed by Puhalla (1985). Nitrate-nonutilizing (*nit*) mutants were recovered by plating the *F. oxysporum* isolates on a chlorate-containing medium (KPS), and complementation tests were performed on a minimal agar medium (MM) that contained sodium nitrate as the sole source of nitrogen (Puhalla, 1985).

For the recovery of *nit* mutants, each isolate of *F. oxysporum* was grown on MM at 22 C for 3-4 days. Four 3 mm-diameter mycelial plugs were taken from each colony and spaced well

apart on each plate of KPS. The KPS plates were incubated at 22 C for 14 days, during which time they were inspected for fast-growing, chlorate-resistant sectors. Different sectors from the same isolate were then transferred to MM. Very thin, but normally expansive growth on MM indicated that the sectors were also unable to reduce nitrate (*nit* mutants). Different *nit* mutants of a given isolate were plated on MM as follows: 1 mm³ mycelial block of one of them, arbitrarily designated *nitA*, was placed at the center of a plate of MM, and five of the other *nit* mutants of that isolate were spaced in a circle of radius 15 mm around *nitA*. Plates were incubated at 22 C for 7 days and then examined. Any outer *nit* mutant that developed a line of dense growth where it contacted the central *nitA* colony was designated *nitB*. Based on Puhalla's results concerning efficiency of recovery of *nit* mutants (number of *nit* mutants per inoculum plug = 0.58, number of *nitB* mutants per inoculum plug = 0.13), we estimated that for each isolate, at least 16 wild type inoculum plugs should be plated on KPS (four KPS plates per isolate), to obtain nine *nit* sectors and two *nitB* mutants (Puhalla, 1985). The number of *nit* mutants tested for each isolate ranged from 12 to 24.

The number of isolates from which *nit* mutants were tested, the number of isolates from which *nitA* and *nitB* complementary mutants were obtained, and the number of isolates from which no complementary mutants were obtained, were recorded. Complementary *nitA* and *nitB* mutants from each of 18 *F. oxysporum* isolates were then paired on MM in all possible combinations to perform complementation tests among isolates (Puhalla, 1985). Nine isolates from the 1994 core collection and eight isolates from the 1995 core collection, plus the isolate 067NY-94 of *F.o.* f.sp. *loti* provided by Dr. G.C. Bergstrom (Dept. of Plant Pathology, Cornell University, Ithaca, NY 14853) were characterized for vegetative compatibility. Complementation tests for the 18 isolates (630 pairings of 36 *nit* complementary mutants) were repeated once during 1995 and 1996.

Results

Fungal isolations

Fungal colonies were recovered from root and crown pieces of plants sampled at the three locations. Independent of the location, root and crown pieces of 1-yr-old plants yielded few fungal colonies, but they were isolated readily from root and crown pieces of 2- and 3-yr-old plants.

The majority of fungi isolated from diseased crown and root tissues of birdsfoot trefoil were *Fusarium* spp., with the most frequently and consistently isolated species being *F. oxysporum* (66% and 70% of isolates, n=42 and n=146, in 1994 and 1995, respectively). Taxonomic identification was not done for the other *Fusarium* spp. Additional frequently isolated fungi included presumed saprophytic genera, *Penicillium*, *Aspergillus*, *Gliocladium*, *Epicoccum*, *Cladosporium*, *Rhizopus*, and *Mucor*. Unknown fungi (that were recovered in relatively low frequencies) included sterile hyphomycetes and coenocytic, nonsporulating species. One fungal isolate recovered in September 1994, identified tentatively as *Mycoleptodiscus* spp., and two isolates recovered in September 1994, identified tentatively as *Rhizoctonia solani*, were counted as unknown fungi.

Sixty-four isolates of *Fusarium* spp. were recovered from roots sampled in 1994, and 208 isolates from roots sampled in 1995, and composed the two *Fusarium* spp. collections. Fifteen *F. oxysporum* isolates from 1994, and 36 isolates from 1995, were selected to compose the two *F. oxysporum* core collections.

Aggressiveness of isolates

The eight *F. oxysporum* isolates tested from the 1994 core collection were pathogenic to seed and seedlings of birdsfoot trefoil, but significant differences in aggressiveness were observed among the isolates (Table 1). The effect of host cultivar was significant, with Estanduela Ganador being more susceptible than San Gabriel (data not shown). Results could be due to differences in the germplasm reaction and/or to differences in the seed vigor of the seed lots. However, there was no interaction between isolates and cultivars and therefore, data on DSI and PSP were averaged over the two cultivars. The ranges for average DSI and PSP among all isolates were 1.44-2.25 and 58.7-85.7, respectively (Table 1). One isolate from Treinta y Tres (TT1C8) was significantly more aggressive than all the rest, as determined by the highest DSI and the lowest PSP. The other isolates showed a continuous range in variation for aggressiveness.

Table 1. Aggressiveness on birdsfoot trefoil of *Fusarium oxysporum* isolates from the 1994 core collection, as determined by disease severity and percentage of surviving plants.

Isolate ¹	Disease severity ² (DSI)	Percentage of surviving plants ³ (PSP)
TT1C8	2.25 ⁴	58.6 ⁴
C2C1	1.73	76.9
T2C1	1.68	79.5
C2C2	1.64	80.9
T3C1	1.61	80.1
T3C8	1.57	83.8
TT3C8	1.46	85.7
T2C6	1.44	85.6
LSD (0.05)	0.30	9.8
CV (%)	17.28	12.1

¹ The first letter(s) refer(s) to the geographic location of an isolate; TT= Treinta y Tres, C= Colonia, T= Tacuarembó. The first number refers to the age in years of the diseased plant from which an isolate was derived; the second number refers to the quadrat in the field from which the diseased plant was sampled (1-12).

² DSI, disease severity index based on a 5-class scoring system of individual seedlings: 1= healthy seedling, 2= primary root tip necrotic and firm, 3= primary root tip and cotyledons rotted and soft, 4= dead seedling, 5= dead seed.

³ PSP, percentage of surviving plants: percentage of seedlings in classes 1 and 2.

⁴ Values were averaged over birdsfoot trefoil cultivars San Gabriel and Estanduela Ganador.

Since the results from the first aggressiveness test (1994 isolates) indicated that the interaction between isolates and cultivars was not significant, and the number of isolates to be tested from the 1995 core collection was large, the second aggressiveness test (1995 isolates) included only birdsfoot trefoil cv. San Gabriel.

All of the 36 *F. oxysporum* isolates tested from the 1995 core collection were pathogenic to seed and seedlings of birdsfoot trefoil, but significant differences in aggressiveness were observed among the isolates (Table 2). The range for average DSI was 1.69-3.85 and for average PSP, it was 4.8-78.9. With few exceptions, isolates from Colonia were more aggressive than isolates from the other two locations. One isolate from Colonia (C2C7) was significantly less aggressive than all other isolates, as determined by the lowest DSI and the highest PSP. Isolates from Tacuarembó and Treinta y Tres showed a large variability in aggressiveness (DSI = 2.44-3.76). There was a tendency for isolates from different quadrats from the same site to have similar DSI and PSP values. The control isolate TT1C8, from the 1994 core collection, had low aggressiveness as compared to isolates from the 1995 core collection (DSI = 2.61, PSP = 48.9). However, these values are similar to those obtained from the previous test (DSI = 2.25, PSP = 58.7) (Table 2).

Genetic relatedness of isolates

Nit mutants were obtained for 45 out of 52 isolates: 15 of 15 isolates that composed the 1994 core collection, 29 of 36 isolates of the 1995 core collection, and for the isolate of *F.o. f.sp. loti* provided by Dr. Bergstrom. The number of *nit* mutants tested for each of the 45 isolates ranged from 12 to 24. Complementary *nitA* and *nitB* mutants were obtained for only 17 out of 45 isolates: 9 of 15 isolates of the 1994 core collection, 8 of 29 isolates of the 1995 core collection and for the *F.o. f.sp. loti* isolate (Figure 1). No complementary mutants were obtained from the rest of the isolates.



Figure 1. Complementary *nitA* and *nitB* mutants derived from *Fusarium oxysporum* isolates on minimal medium. The plate in the upper left corner is used as an example: one mutant, arbitrarily designated *nitA*, was placed in the center of the plate; then five other *nit* mutants obtained from the same isolate were spaced in a circle of radius 15mm around the *nitA* block. In this case the five outer *nit* mutants were complementary with the *nitA* mutant and were designated *nitB* mutants.

Table 2. Aggressiveness on birdsfoot trefoil cv. San Gabriel of *Fusarium oxysporum* isolates from the 1995 core collection, as determined by disease severity and percentage of surviving plants.

Isolate ¹	Disease severity ² (DSI)	Exp. Surv. Plants ³ (%)	Isolate ¹	Disease severity ² (DSI)	Perc. surv. plants ³ (PSP)
C2C11	3.85	4.8	C2C10	3.53	14.7
C2C2	3.82	7.6	TT1C9	3.48	15.6
C3C1	3.77	7.4	TT3C1	3.48	16.1
TT3C3	3.76	7.5	T2C9	3.44	14.5
C3C5	3.75	11.4	T3C5	3.44	18.4
TT3C10	3.73	8.8	T3C4	3.43	16.6
C3C4	3.73	9.9	T3C7	3.35	19.3
C3C10	3.73	10.5	TT1C10	3.29	21.3
C3C11	3.73	12.3	T3C9	3.25	24.2
C2C9	3.71	8.6	T3C12	3.25	25.4
T3C10	3.70	14.3	TT3C4	3.17	25.8
TT3C12	3.67	11.5	TT3C5	3.15	19.0
TT2C9	3.67	12.5	C3C2	3.14	26.4
T3C8	3.64	12.2	TT3C8	3.11	25.5
C3C8	3.64	12.7	T1C2	3.10	35.8
TT1C4	3.63	14.2	TT2C3	3.03	30.2
TT2C6	3.58	12.1	TT2C11	2.44	53.4
T3C2	3.58	12.3	C2C7	1.69	78.9
			TT1C8/94 ⁴	2.61	48.9
LSD (0.05)	0.42	16.1	LSD (0.05)	0.42	16.1
CV (%)	8.77	58.9	CV (%)	8.77	58.9

¹ The first letter(s) refer(s) to the geographic location of an isolate; C= Colonia, TT= Treinta y Tres, T= Tacuarembó. The first number refers to the age in years of the diseased plant from which an isolate was derived; the second number refers to the quadrat in the field from which the diseased plant was sampled (1-12).

² DSI, disease severity index based on a 5-class scoring system of individual seedlings: 1= healthy seedling, 2= primary root tip necrotic and firm, 3= primary root tip and cotyledons rotted and soft, 4= dead seedling, 5= dead seed.

³ PSP, percentage of surviving plants: percentage of seedlings in classes 1 and 2.

⁴ Control isolate from the 1994 core collection.

No complementation was found either among the nine *F. oxysporum* isolates from the 1994 core collection or among the eight isolates from the 1995 core collection. Pairings among the nine 1994 isolates and the eight 1995 isolates also resulted in no complementation (Fig. 2). When the 17 isolates from Uruguay were tested with the *F.o.* f.sp. *loti* isolate, no complementation was obtained. Based on these results, the 17 isolates should be assigned to different VCGs. Since each plate contained two complementary *nit* mutants for at least one isolate, we were assured that the methodology was adequate to detect compatibility among isolates if it did exist (Fig. 2). In these tests, complementation always and only occurred between complementary *nitA* and *nitB* mutants from the same isolate, used as controls.

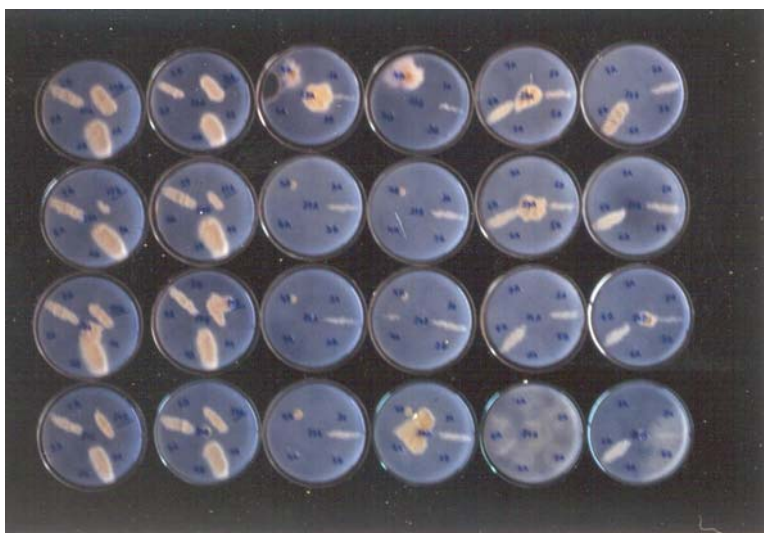


Figure 2. Complementation tests among *Fusarium oxysporum* isolates from which complementary *nitA* and *nitB* mutants were obtained, on minimal medium. No complementation occurred among different isolates. The observed complementation only and always occurred between complementary *nitA* and *nitB* mutants of the same isolate.

Discussion

The repeated isolation of *F. oxysporum* from diseased roots of birdsfoot trefoil suggests this species is primarily responsible for crown and root rot and stand decline, consistent with previous findings (Altier, 1994; 1997; Berkenkamp *et al.*, 1972; Beuselinck, 1988; Henson, 1962; Kainski, 1960). In addition, *F. oxysporum* has been reported as the causal organism of Fusarium wilt on birdsfoot trefoil (Bergstrom and Kalb, 1995). No other known pathogen that is alone capable of causing these disease symptoms was isolated from diseased crown and root tissues.

All the examined isolates of *F. oxysporum* incited a host reaction in birdsfoot trefoil. This means they may have similar genetic factors that determine pathogenicity and host disease reaction. However, isolates showed a continuous range in aggressiveness to birdsfoot trefoil seeds and seedlings. The observed variability was expected, and was consistent with previous reports (Leath and Kendall, 1978; Venuto *et al.*, 1995). Aggressiveness is defined as a property of the pathogen reflecting the relative amount of damage caused to the host without regard to resistance genes (Shanner *et al.*, 1992). With few exceptions, the highest aggressiveness was expressed by isolates from sites with long legume pasture history (e.g., Colonia as compared with the other two locations). There was a tendency for isolates from

the same site to have similar values for DSI and PSP; however, the reason for this is unknown. Results in our tests were similar to those of Fulton and Hanson (1960), Kainski (1960), and Kilpatrick *et al.* (1954), who used seedling tests to compare aggressiveness of *Fusarium* spp. isolates causing crown and root rot on forage legume hosts, and reported variability in the pathogen. Kainski (1960), while studying the fungi involved in root rots and seedling diseases of birdsfoot trefoil, showed that most of the fungi that were pathogenic to seeds and seedlings were also pathogenic to established plants but differed in their relative aggressiveness. Kilpatrick *et al.* (1954) studied the pathogenicity of 72 isolates of *F. oxysporum* associated with root rots of red clover and observed a wide variation in disease severity as measured by percentage dead plants (range among isolates: 26-95%). This variable is the complement of the one we used, percentage of surviving plants (PSP).

Despite obtaining *nit* mutants for 45 out of 52 isolates, complementary *nitB* mutants were obtained for only 17 out of 45 isolates. There are two plausible explanations for these results. First, the efficiency of recovering *nitB* complementary mutants is low and isolate dependent (average 0.13 per inoculum plug, range 0-0.25; Puhalla, 1985). Therefore, a large number of inoculum plugs per isolate should have been plated on KPS to recover more *nitB* mutants. Secondly, some isolates could have been heterokaryon self-incompatible (HSI), as defined by Correll *et al.* (1987), though HSI strains usually occur at low frequency in *F. oxysporum* populations (1-2%, J.F. Leslie, Kansas State University, U.S.A., 1996, pers. comm.; 4%, Steinberg *et al.*, 1997).

The lack of complementation among the isolates that compose the *F. oxysporum* population associated with birdsfoot trefoil indicates a large genetic diversity, as measured by vegetative compatibility. Isolates do not share genes for complementation and thus, they belong to different VCGs. However, they may share genetic factors that induce host disease reaction, since all of them have the ability to cause disease symptoms in birdsfoot trefoil seeds and seedlings. We may conclude that similarity or dissimilarity in *vic* genes does not reflect pathogenicity and aggressiveness capabilities. Our results agreed with those of Venuto *et al.* (1995) who showed that VCGs were not useful in predicting host reaction of red clover to isolates of *F. oxysporum*, because isolates from distinct groupings elicited similar host reactions. This indicates that the number of genes controlling compatibility seems to be greater than the number of virulence genes. Correll (1991) reported that over 46 distinct VCGs have been identified among a collection of *F. o.* f.sp. *asparagi* isolates pathogenic to asparagus in greenhouse pathogenicity tests, and that race 1 isolates of *F. o.* f.sp. *lycopersici* were found to belong to at least 41 VCGs.

Some genetic diversity studies have been consistent with Puhalla's initial generalization and have stated a correlation between pathogenic phenotype and genotype (Puhalla, 1985). However, the examination of numerous formae speciales of *F. oxysporum* has revealed that the relationship between host specialization (formae speciales), virulence capabilities (races) and VCGs can vary from simple to complex (Correll, 1991; Gordon and Martyn, 1997; Kistler, 1997; 2001; Klein and Correll, 2001). Based on pieces of evidence, authors assume that virulence and VCG phenotypes change independently of one another and at different rates (Correll, 1991; Klein and Correll, 2001). Kistler (2001) asserted that horizontal gene transfer could explain the partitioning of host specificity into genetically distant lineages.

From this hypothesis, the prediction is that genes for host specificity (as determined by pathogenic phenotype) may be more closely related than can be accounted for by the underlying phylogeny (as determined by molecular markers or VCGs). He concluded that strains pathogenic to a given host may emerge rapidly in a genetic background preadapted for fitness on any plant species currently colonized by *F. oxysporum* (Kistler, 2001). Several working models have been proposed to help explain the degree of VCG diversity thus far observed in *F. oxysporum* (Correll, 1991; Gordon and Martyn, 1997; Klein and Correll, 2001). It has been suggested that the parasitic, but nonpathogenic portion of the population may represent some primitive or basal population structure of this species and a largely unexplored reservoir of genetic diversity. From this primitive population, which has a high degree of VCG diversity, mutations to virulence may occur among isolates of the various VCGs. If selection of existing variants or a mutation occurred in isolates that are brought into proximity with a susceptible host (e.g., the roots), then they may proliferate and lead to an epidemic. This will likely result as a consequence of the intense selection pressure imposed by agricultural practices.

Soils in Uruguay support the growth of a wide range of leguminous species, either introduced crops, native species (including woody trees and shrubs) or weeds. In areas of intensive livestock production, forage legumes are used in short rotations with cereals and grasses. Under this situation, in most agricultural soils, *F. oxysporum* populations do find conditions conducive to development of host specialization, and survive, at least in part, by colonizing leguminous-host plants. Furthermore, results from a study to characterize soil populations of *F. oxysporum* under different rotation systems have demonstrated that even soil under continuous agriculture without any legume crop supports high population densities of this fungus (Altier, 2003). We could speculate that this population may represent what Correll (1991) designates the primitive or basal population structure of this species. Given the global distribution of *F. oxysporum* and its pervasive association with plants, this gives reason for concern. Future work should focus especially on the role of alternate hosts in maintaining pathogen populations in soil and in the study of factors that influence the dynamics of isolate competition within a heterogeneous *F. oxysporum* population.

Characterization of the pathogen population has practical implications for the success of breeding for resistance to Fusarium crown and root rot in birdsfoot trefoil. The high degree of variability for aggressiveness among *F. oxysporum* isolates supports using isolates from different locations in the inoculation and selection procedures of a breeding program for cultivar development for the region. If the primary variation in the reaction of the host is due to variation in the pathogen, selection for resistance to one or a few isolates would not result in resistance to other isolates (Pederson *et al.*, 1980; Venuto *et al.*, 1995). Developing germplasm with increased resistance to *F. oxysporum* should involve the screening of birdsfoot trefoil against several genetically divergent isolates of the pathogen, if the resistant cultivars are to be widely used.

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The Flavonoids of *Lotus corniculatus*

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Introduction

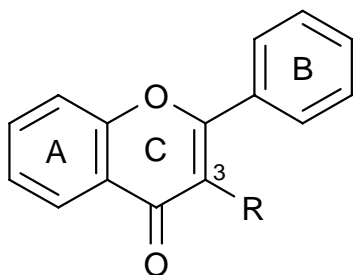
Since the first studies published in the fifties and sixties (Nakaoki *et al.*, 1956; Harney and Grant, 1964; Bate-Smith, 1965), many authors have investigated the flavonoid chemistry of *Lotus corniculatus* (Table 1 and Table 2) and demonstrated the richness and diversity of flavonoid compounds in this species. Some authors have examined the variation with altitude of the flavonoid content of *Lotus corniculatus*. Others have used flavonoids as speciation markers within the *Lotus corniculatus* complex.

Flavonoids

Flavonoids are a large class of secondary plant metabolites of widespread occurrence in higher plants (more than 6000 known structures; Harborne and Baxter, 1999). Of the two most frequent subclasses, flavones and flavonols (Figure 1), only derivatives of flavonols have been identified in *Lotus corniculatus* (3-OH free or substituted by a sugar).

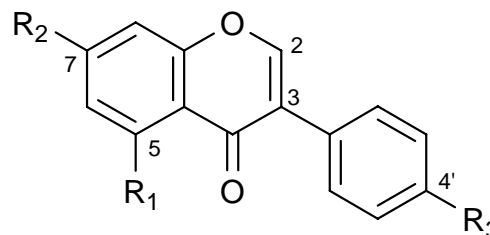
A recent study (Sarelli *et al.*, 2003) has revealed that *Lotus corniculatus* also contained insignificant amounts of two isoflavonoids at budding and flowering stages: formononetin and biochanin A (Figure 2). These two phytoestrogens are in too small a quantity to have adverse effects on reproductive functions.

Figure 1. Structure of flavones and flavonols



Flavones: R₃=H
Flavonols: R₃=OH

Figure 2 Structure of isoflavonoids

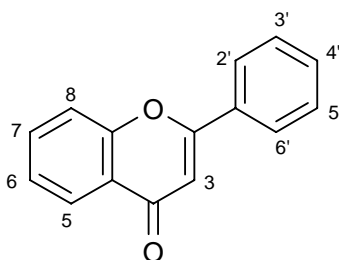


Formononetin: R₁=H, R₂=OH, R₃=Ome
Biochanin A: R₁=R₂=OH, R₃=OMe

Aglycones (Table 1)

As in most plants, flavonoids of *Lotus corniculatus* are not present as free aglycones: in the different studies reported in Table 1, the aglycones were obtained after acid hydrolysis of the plant material (leaves or flowers). The 10 compounds mentioned in the different studies are all derivatives of kaempferol and quercetin. The species is particularly rich in 5-desoxyflavonols (structures characteristic of the polyphenolic profile of *Fabaceae*) and, in flower material only, in 8-hydroxy or 8-methoxy flavonols. A LC-MS (Liquid Chromatography-Mass Spectrometry) study with different detection modes recently published by de Rijke *et al.* (2004) did not reveal the presence of these methoxy and desoxy derivatives as free aglycones.

Table 1. Flavonoid aglycones identified in *Lotus corniculatus* (after acid hydrolysis of the plant material).



	Plant organs and references			
	Seeds	Leaves	Flowers	Aerial parts
Kaempferol (3,5,7,4'-tetrahydroxyflavone)		1,2,3,4	3,4	
Quercetin (3,5,7,3',4'-pentahydroxyflavone)		1,2,3,4	3,4	
Isorhamnetin (3,5,7,3'-tetrahydroxy-4'-methoxyflavone)		3,4	3,4	
Desoxy-5-Kaempferol (3,7,4'-trihydroxyflavone)		3,4	3,4	
Desoxy-5-Quercetin (Fisetin) (3,7,3',4'-tetrahydroxyflavone)		3,4		
Desoxy-5-Isorhamnetin (Geraldol) (3,7,3'-trihydroxy-4'-methoxyflavone)		3,4	3,4	
Methoxy-8-Kaempferol (Sexangularetin) (3,5,7,4'-tetrahydroxy-8-methoxyflavone)			3,4	
Methoxy-8-Quercetin (Corniculatusin) (3,5,7,3',4'-pentahydroxy-8-methoxyflavone)			3,4	
Methoxy-8-Isorhamnetin (Limocitrin) (3,5,7,3'-tetrahydroxy-8,4'-dimethoxyflavone)			3,4	
Hydroxy-8-Quercetin (Gossypetin) (3,5,7,8,3',4'-hexahydroxyflavone)			3,4	

Monosides and Diosides (Table 2)

Lotus corniculatus is particularly characterized by the great diversity of its flavonol glycoside content (12 monosides and 10 diosides have been reported to date). In 1969, Harborne reported on the presence of 7-O-methyl-gossypetin, but the information was inaccurate and was further rectified; in 1978, the same author corrected the identification to 8-O-methylgossypetin (or 8-methoxyquercetin). Although the presence of isorhamnetin has been reported in the literature (Hasan, 1976; Jay *et al.*, 1978), no glycoside based on this molecule has been evidenced to date. The plant seeds are particularly rich in flavonol glycosides (5 monosides and 6 diosides). The recent study performed by de Rijke *et al.* (2004) has shown that the 2 major compounds present in *Lotus corniculatus* are two isomers of 3-O-rhamnoglucosyl-kaempferol.

Flavonoids and flower color

For Jay and Ibrahim (1986), the predominant flavonoids (present as glycosides) in the flower buds of *Lotus corniculatus* are kaempferol and quercetin. Small amounts of gossypetin are also present. The yellow coloration of flower petals is concomitant with the accumulation of large amounts of gossypetin and corniculatusin and much smaller amounts of sexangularetin. For these authors, gossypetin and corniculatusin are mostly responsible for the intensity of the yellow color during flower development.

In some individuals, the flowers have entirely yellow keel petals ("light-keeled *Lotus*"). In other, less common individuals, the keel petals are red-brown ("dark-keeled *Lotus*"). Several authors, like Jones and Crawford (1977), have shown a cline in keel color frequencies in different parts of Western Europe (England and Wales, Denmark, West Germany, the Netherlands, Austria, France, Spain and Sweden). These authors have also shown the lack of relationship between the color of keel petals and cyanogenesis.

A study by Jones *et al.* (1986) of the relation between keel color, insect visits and reproductive output has indicated that "keel color does not influence pollinator foraging behavior nor colonization by flower insects". Their data show that the phenotypes do not differ in pod and seed production.

Relation between flavonoids and altitude

An article published in 1972 by Ceruti *et al.* investigated the total flavonoids of *Lotus corniculatus* flowers collected at various altitudes in Northern Italy. After extraction, they quantified their flavonoid content by measuring the Optical Density (OD) at 350 nm (the wavelength corresponding to maximum absorption of kaempferol and quercetin glycosides). Their measures revealed that variations of the flavonoid content of the plant (OD, maximum value = 1) correspond to 3 areas:

- * from 230 to 600m, OD increased from 0.2 to 0.4
- * from 600 to 1600m, OD remained stable at approximately 0.4
- * from 1600 to 2600m, OD increased from 0.4 to 0.7

Table 2. Flavonoid glycosides identified in *Lotus corniculatus*

	Plant organs and references			
	Seeds	Leaves	Flowers	Aerial parts
Monosides				
Glucosyl-3-Kaempferol		5		
Rhamnosyl-3-Kaempferol		5		
Glucosyl-7-Kaempferol	6	5		
Rhamnosyl-7-Kaempferol		5		
Arabinosyl-3-Quercetin	7			
Galactosyl-3-Quercetin	7	5		
Rhamnosyl-3-Quercetin (Quercitrin)	7	4, 5		
Rhamnosyl-7-Quercetin	6	5		
Galactosyl-3-Gossypetin			8	
Galactosyl-3-Corniculatusin		5	9	
Glucosyl-3-Corniculatusin		5		
Glucosyl-3-Sexangularetin		5		
Diosides				
Diglucosyl-7-Kaempferol	7			
Diglucosyl-3,7-Kaempferol	7	5		
Dirhamnosyl-3,7-Kaempferol	6,7			6,10
Glucosyl-3-Rhamnosyl-7-Kaempferol	6,7			6
Rhamnosyl-3-Glucosyl-7-Kaempferol		5 (*)		
Dirhamnosyl-3,7-Quercetin	6	5		
Glucosyl-3-Rhamnosyl-7-Quercetin	6	(*)		
Rhamnosyl-3-Glucosyl-7-Quercetin		5 (*)		
Rhamnosyl-3-Glucosyl-7-Sexangularetin		5		
Rhamnoglucosyl-3 or 7-Quercetin		(*)		

(*) for these diosides, the aglycone and the sugars have been identified but the exact positions of the sugars on the aglycone skeleton remain to be determined (3 or 7).

References for Table 1 and Table 2.

- | | |
|--------------------------------|---------------------------------|
| 1 Harney and Grant, 1964 | 6 Waleska and Strzelecka, 1984 |
| 2 Bate-Smith, 1965 | 7 Gorski <i>et al.</i> , 1975 |
| 3 Jay <i>et al.</i> , 1978 | 8 Harborne, 1969 |
| 4 Hasan, 1976 | 9 Nielsen, 1970 |
| 5 Reynaud <i>et al.</i> , 1982 | 10 Nakaoki <i>et al.</i> , 1956 |

From these findings, they concluded that the upregulation of the flavonoid content is related with the quantity and the quality of sun radiations received by *Lotus corniculatus* individuals as a function of altitude. For these authors, the stability observed between 600 and 1600m would be due to the fact that individuals were collected in forest habitats.

Several years ago, in my thesis work, I assessed the ratio of flavonoid diosides to monosides (D/M) in *Lotus corniculatus* samples collected at various altitudes in two French regions (Massif Central and Alps). Variations of the D/M ratio were not similar in the two areas. In plants collected between 600 and 1400m in the Massif Central (ancient hercynian massif), the D/M ratio varied from 2.3 at 600m to 14.8 at 1600m, whereas in the Alps (a recent mountain range) the ratio varied from 5.5 at 1200m to 0.6 at 1800m. My conclusion was that *Lotus corniculatus* populations of the Massif Central correspond to early plant settlements, probably all tetraploids, with more evolved flavonoid chemistry and a strong capacity to synthesize diosides. In the Alps, the plant populations are more recent (recolonization after the last ice age), with tetraploid individuals at lower altitudes and diploid individuals (sometimes named *Lotus alpinus*) at higher altitudes. The capacity of these high altitude diploid populations to synthesize diosides is reduced.

Flavonoids as speciation markers

We have studied 412 individuals collected from diploid and tetraploid populations of *Lotus corniculatus* growing in the Southern French Alps (Mercantour, Ventoux and Lure Mountain). After extraction and HPLC analysis of their polyphenolic content, a polyphenolic "fingerprint" of each individual was obtained. A statistical analysis of the 412 HPLC profiles led us to the following conclusions:

- in this geographic area, at low altitudes, there are tetraploid plants with a rich and diversified polyphenolic content.
- at higher altitudes, where conditions are more unstable, we find two poor and homogeneous polyphenolic profiles corresponding to two types of diploid *Lotus corniculatus*: one type is characteristic of the inner Alps and the other one of the western Alps (Mont Ventoux, for instance).

Results of the different studies described above have been published in this journal and elsewhere (Reynaud and Jay, 1989; 1990; 1991; Jay *et al.*, 1991; Reynaud *et al.*, 1991).

Conclusion

Lotus corniculatus, a plant with high agronomic value in some regions of the world, is also of particular interest for more theoretical research due to its rich flavonoid content. Though only based on kaempferol and quercetin flavonols, the rich and numerous flavonoid compounds synthesized by the plant can be used to study speciation in the *Lotus corniculatus* complex or variations of flavonoid chemistry as a function of altitude. The increasing sensitivity of isolation and identification methods should make it possible to identify occurrences of new, yet undisclosed flavonoids in this species.

Acknowledgements

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Nitrogen metabolism in relation to drought stress responses in cultivated and model *Lotus* species

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Abstract

Amino acid profiles of *Lotus corniculatus*, *L. glaber*, *L. japonicus*, *L. subbiflorus* and *L. uliginosus* in response to drought stress were studied. All *Lotus* species accumulate proline, derived from *de novo* synthesis, in response to drought stress. Asparagine analyzed by HPLC showed the highest abundance accounting for 20 to 25 % of total amino acids, without revealing any change in response to drought stress. Additionally, an increase in the amount of photorespiratory pathway intermediates, serine and glycine, was observed in some species as a consequence of drought stress, but intracellular ammonium non change in response to drought stress. The differential effect of drought stress on amino acid profiles and proline accumulation in *Lotus* species plants is discussed.

Introduction

There are four species of *Lotus* that have been domesticated and improved by selection and plant breeding: birdsfoot trefoil (*Lotus corniculatus*), greater lotus (*L. uliginosus*), narrow-leaf trefoil (*L. glaber*) and hairy birdsfoot trefoil (*L. subbiflorus*) (Blumenthal and McGraw, 1999; Díaz *et al.*, 2005a). Besides its agronomical attributes, there is a great deal of interest in *Lotus* because the species are extremely amenable to tissue culture, in particular *L. corniculatus* and *L. japonicus* (Webb *et al.*, 1990; Handberg and Stougaard, 1992). In spite of the fact that *L. japonicus* is not used as forage legume, this species could be a good model for a wealth of genetic, biochemical, molecular biological (Orea *et al.*, 2002) and symbiotic studies which cannot be carried out in other model species as *Arabidopsis thaliana*.

Drought is the major limitation on crop productivity worldwide. In broad terms, drought can be permanent, seasonal or random. Another type of drought to be considered is named non-apparent drought and is observed in hot summer days when high temperature or wind, induce an increase on transpiration rates that exceeds water root absorption rates (Sánchez-Díaz y Aguirreolea, 1993).

Lotus species are sowed in geographical zones where the plants can be exposed to random or

non-apparent drought. They are adapted to temperate and humid environments, wherein these types of drought are frequent, and therefore, they could have acquired mechanisms to tolerate the drought.

For several years it has been known that plants respond to drought stress by undergoing biochemical adaptative processes such as ion transport and by accumulating different compounds, named compatible osmolytes. These osmolytes, which are known to increased there synthesis under osmotic stress, include proline, they accumulate to high concentrations without interfering with cell metabolism (Bray, 1993). As well as proline, other nitrogen compounds could be accumulating in plants in response to drought stress (Good and Zaplachinski, 1994; Chiang and Dandekar, 1995). The amino acid metabolism may play an important role in plant stress tolerance, by osmotic adjustment through to accumulation of compatible osmolytes; by detoxification of active oxygen species, xenobiotics and heavy metals; and by intracellular pH regulation (Rhodes *et al.*, 1999; Alia *et al.*, 2001).

During drought stress, protein residues may be altered by chemical processes; some proteins are irreversibly damaged by the effects of drought stress and are degraded by proteases. It has been suggested that proteases mobilize amino acids from proteins to the synthesis into compatible osmolytes (Campalans *et al.*, 1999). In our study the analyses were focused in amino acid profiles and in drought-induced proline accumulation in *Lotus* agronomical species and in the model species *Lotus japonicus*.

Material and methods

Plant material, growth conditions and drought treatment

L. corniculatus cv San Gabriel (AGROSAN S.A.), *L. uliginosus* cv Grassland Maku (Ing. Agr. D. Formoso), *L. subbiflorus* cv El Rincón (AGROSAN S.A.), *L. glaber* cv Herminia (PAS S.A. Montevideo Uruguay) and *L. japonicus* (Regel) Larsen ecotype Gifu (Prof. A. Márquez) were treated according to Orea *et al.* (2002) and germinated at 28 °C for 2 days. The plants were grown under controlled conditions: 16/8 h light/dark with photosynthetic photon flux density of 250 $\mu\text{mol. m}^{-2} \cdot \text{s}^{-1}$, 22/18 °C and relative humidity 70/80%. The plants were grown during 28 days in hydroponic assemblies (Borsani *et al.*, 1999) with a modified Hornum nutrient solution described by Handberg and Stougaard (1992) with 8 mM of KNO_3 . The drought stress was induced as described by Borsani *et al.* (1999) the measurements of leaf tissues were performed 0 and 12 h after and this is know as fast drought stress (Díaz *et al.*, 2005b).

Analytical determinations

The relative water content (RWC) was calculated according Antolín *et al.* (1995). Total protein was quantified according to Bradford (1972), chlorophyll concentration according to Wellburn (1994) and proline concentration according to Borsani *et al.* (1999). Nitrate, ammonium and total free amino acids were extracted as proposed by Izaguirre-Mayoral *et al.* (1992) with potassium phosphate buffer 10.0 mmol L^{-1} – ethanol (1 – 1). Nitrate was

analysed according to Cataldo *et al.* (1975), ammonium according Solorzano (1969), and total amino acids were quantified with ninhydrin reagent according to Moore and Stein (1948). The identification of amino acids was achieved by an extraction derivatised with o-phthaldialdehyde and the samples were separated-analysed by HPLC (Díaz *et al.*, 2005b in press). Proteolytic activity was measured according to Roy-Macauley *et al.* (1994).

Results

Biochemical responses to drought stress in plants are usually evaluated through the osmolyte accumulation and detoxification enzymes of reactive oxygen species, among others. Our work was focussed in nitrogen osmolytes as proline, GABA, amino acids, nitrate and ammonium.

Leaf RWC in different *Lotus* species averaged as follows: control 84 % and 12 h drought stress 63 % (Table 1). *L. subbiflorus* showed the lowest difference in RWC between the control and the drought stressed plant in our assay conditions. This fact could be explained since this is the most pubescent plant species and this feature could diminish the water loss by the leaf.

Photosynthetic pigments were used to determine the physiological status of the plants. No changes were observed in chlorophyll a and b concentration in response to 12 h of drought stress in the *Lotus* species studied, and also chlorophyll a/b ratio was largely unaffected by drought stress (Table 1).

Table 1. RWC, chlorophyll (clo), proline, total free amino acid (tot aa), protein, nitrate and ammonium in one month old plants control (c) and subjected to drought stress for 12 h (ds).

	<i>L. corniculatus</i>		<i>L. glaber</i>		<i>L. japonicus</i>		<i>L. subbiflorus</i>		<i>L. uliginosus</i>	
	c	ds	c	ds	c	ds	c	ds	c	ds
RWC ^a	83.5	63.4*	81.0	58.3*	87.5	66.8*	82.5	70.2*	85.9	58.5*
clo a ^b	7.1	6.7	8.8	7.3	10.6	10.7	7.8	8.7	5.3	5.9
clo b ^b	4.7	3.9	5.6	5.3	5.9	5.7	4.1	4.8	3.5	3.6
a/b	1.8	1.9	1.9	1.8	1.9	1.9	1.8	2.0	1.8	1.9
Proline ^b	2.5	12.2*	1.3	10.2*	2.6	6.2*	1.3	2.9	1.2	9.7*
Tot aa ^b	164.8	173.5	100.9	93.9	131.0	169.3	103.8	107.2	113.7	145.8
Protein ^c	119.2	104.0	110.4	129.7	135.3	148.5	89.7	92.3	118.4	95.3
nitrate ^b	330.5	360.2	174.4	94.1	365.5	310.9	123.4	51.9	419.5	230.3
Ammonium	21.5	14.1	0	0	0	0	0	0	24.4	30.8

The * represent a significant difference between drought stressed and control plants at 5 % of Duncan's method. ^a, %; ^b, $\mu\text{mol g}^{-1}\text{DW}$ and ^c, $\text{mg g}^{-1}\text{DW}$.

L. corniculatus, *L. japonicus* and *L. uliginosus* plants showed higher nitrate levels than *L. glaber* and *L. subbiflorus* plants and we did not found changes in response to drought stress.

On the other hand, ammonium was detected in *L. corniculatus* and *L. uliginosus* and no changes were observed in response to drought stress. Ammonium was not detected in the other three species (Table 1).

Proline content increased in drought stressed plants; these increases were between 3 and 7 fold (Table 1). Based on the Van't Hoff equation, this proline increase could be enough to account for only -0.1 to -0.5 MPa of osmotic adjustment in leaf tissue. However, results obtained by our group showed that in *L. corniculatus* subjected to 9 days of slow drought stress, the proline content accounts for -2.5 MPa of osmotic adjustment (Díaz *et al.*, 2005b; P. Díaz and M. Sainz, unpublished data).

Total free amino acids and protein content did not change significantly when the plants were subjected to drought stress (Table 1); so, the increase in proline content was also with respect to total amino acids. Additionally, protein content did not change in response to drought stress conditions; this could suggest that there is no protease increase. This point was verified assaying proteolytic activity with azocasein as substrate at different pH. The proteolytic activity was found to be similar in control and drought stressed plants (Figure 1).

Free amino acid composition of control and drought stress *Lotus* species was analyzed by HPLC and is showed in Table 2. The amino acids analyzed represent the 80% of total free amino acids in leaf tissues. Asparagine was present in high amounts, and accounted for 20 to 25 % in the different *Lotus* species, and arginine was prevalent in *L. uliginosus* (Table 2).

Table 2. Major free amino acid composition (expressed as $\mu\text{mol g}^{-1}$ DW) of one month old plants control (c) and subjected to drought stress for 12 h (ds).

	<i>L. corniculatus</i>		<i>L. glaber</i>		<i>L. japonicus</i>		<i>L. subbiflorus</i>		<i>L. uliginosus</i>	
	c	ds	c	ds	c	ds	c	ds	c	ds
ala	14.9	10.4*	10.3	8.6	7.6	9.7	4.3	11.5	4.3	15.5*
asp	17.3	6.1*	9.3	2.8*	6.2	3.1*	1.3	2.7	15.5	9.8*
asn	38.6	46.0	31.5	34.6	40.9	42.6	31.0	32.1	29.5	53.0
glu	23.8	18.6*	11.6	14.5	17.2	15.6	9.5	12.1	20.2	19.9
gln	1.8	5.4*	0.4	2.7*	3.2	3.3	1.3	2.1	2.1	5.4
gly	6.3	16.1*	4.6	7.0*	5.7	8.6	4.3	7.9	10.4	17.5
ser	12.5	20.5*	8.9	12.4*	17.1	15.0	12.5	14.8	7.4	13.3*
arg	6.6	4.9	5.7	5.4	2.7	3.2	3.4	4.1	9.5	34.6*
GABA	13.0	11.1	45.5	46.5	32.4	27.3	25.2	31.9	6.6	30.3*

The * represent a significant difference between drought stressed and control plants at 5 % of Duncan's method.

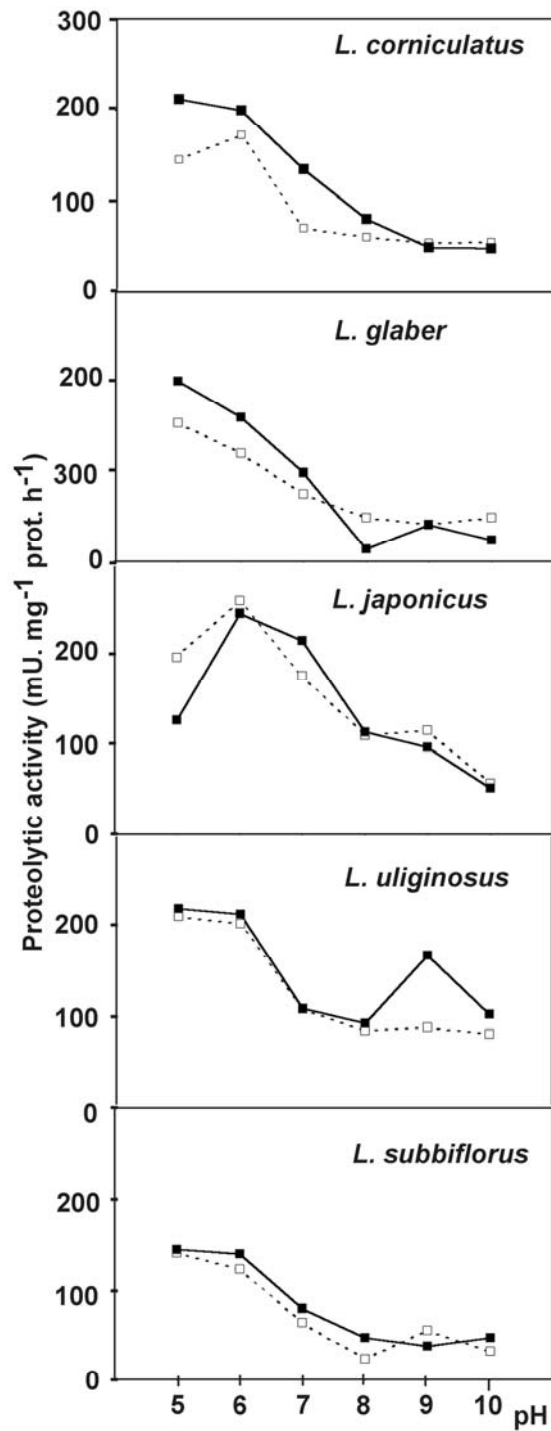


Figure 1. Proteolytic activity in response to drought stress in different *Lotus* species. Close point, control and open point, drought stress treatment. No difference between drought stressed and control plants at 5 % of Duncan's method were observed.

No changes in asparagine concentration were observed in *Lotus* species subjected to drought stress. *L. corniculatus* plants showed the most significant changes in the amino acid profile as a consequence of drought stress. For instance, a decrease in aspartate, alanine and glutamate, and an increase in glutamine, serine and glycine were observed in this species. Some similar changes in the amino acid profiles found in *L. corniculatus* could be observed in *L. glaber*, *L. japonicus* and *L. uliginosus*. The latter showed a decrease in aspartate concentration and *L. glaber* also exhibited an increase in serine and glycine concentration. A significant increase in GABA concentration was observed only in *L. uliginosus* as a consequence of 12 h of drought stress.

Discussion

The present study outlines the changes in proline and free amino acid concentration in several *Lotus* species during the onset of drought stress in plants. Proline was negatively correlated with RWC in *Lotus* species (Tables 1 and 2). Previous works showed that *L. corniculatus* and *L. japonicus* under drought stress conditions decreased RWC of leaves and this was accomplished by an increase in proline concentration (Borsani *et al.*, 1999; Díaz *et al.*, 2002). The amount of accumulated proline is too low to account for osmotic adjustment according to the Van 't Hoff equation, so proline may be regarded as a scavenger of hydroxyl and singlet oxygen radicals (Smirnoff and Cumbes, 1989; Alia *et al.*, 2001).

Our work shows that proline accumulation in all *Lotus* species results from *de novo* synthesis and not from protein hydrolysis, since total amino acids and protein content remained unaltered (Table 1). Additionally, no changes in proteases activity were detected (Figure 1). As well, no differences in protein content were found in *Lotus* species subjected to fast drought stress. The decreases in protein content are associated with slow drought stress which has been found to occur in tomato (Bauer *et al.*, 1997), *Phaseolus vulgaris* and *Vigna unguiculata* (Roy-Macauley *et al.*, 1992).

Lotus species showed different nitrate concentrations, which may probably accumulate in the vacuoles, and so nitrate might be regarded as having a role as an osmoregulator (Blom-Zandstra and Lampe, 1985; Márquez *et al.*, 2005). A decrease in nitrate concentration was observed in tobacco during dehydration. This decrease was accompanied by a general decrease in total free amino acids content (Ferrario-Méry *et al.*, 1998), but this metabolic event was not found in the *Lotus* species studied.

Different amino acids were prevalent in the different *Lotus* species studied: for instance, asparagine and glutamate were found to be the most abundant in *L. corniculatus* and *L. uliginosus* control treatment, and asparagine and GABA were abundant in the others species (Table 2). *Lotus* genus is similar to *Medicago sativa* in that asparagine is the principal amide (Girousse *et al.*, 1996). In temperate legumes, asparagine is the main molecule used to transport reduced nitrogen within the plant, and in that case can account high content. No changes in asparagine content were observed in response to drought stress in *Lotus* species. In *A. thaliana* and *Brassica napus* leaves an increase in asparagine content has been reported in response to osmotic stress (Chiang and Dandekar, 1995; Good and Zaplachinski, 1994).

High glutamate concentration is necessary for proline synthesis through the pyrroline 5-carboxylate synthetase and pyrroline 5-carboxylate reductase (Rhodes *et al.*, 1999). An increase in Fd-GOGAT responsible of glutamate supply for proline accumulation has been reported in *L. corniculatus* leaves under stress conditions (Borsani *et al.*, 1999; Díaz *et al.*, 2005b in press).

A decline in alanine content and an increase in serine and glycine content were observed in *Lotus* species, and may be due to higher rates of photorespiration in the drought stress condition, since alanine is a major donor of amino groups in photorespiratory metabolism.

A special feature show *L. uliginosus* which increased GABA, and this fact could lead to the reduction in the cytoplasmatic concentration of glutamate (Cayley *et al.*, 1992). This reduction could be carried out through a transport into the vacuole, or by GABA synthesis. Similarly, elevated GABA levels are observed under conditions when glutamine synthesis is limited, protein synthesis is inhibited and protein degradation is induced, all these metabolic alterations occur under drought stress conditions in plants (Bray, 1993; Shelp *et al.*, 1999). Also significant increases of arginine were observed in *L. uliginosus*, which may be associated to polyamines metabolism (Rhodes *et al.*, 1999).

Similar results obtained on proline accumulation and amino acid profiles in the *L. corniculatus*, *L. glaber* and *L. japonicus* showed that the latter species could be used as an excellent model to understand and therefore improve drought stress tolerance in cultivated *Lotus* species through osmolyte synthesis.

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