Nota Corta: Endophytic fungi in healthy soybean leaves

S. Larran [1*], C. Rollán [1], H. Bruno Ángeles [2], H.E. Alipi [1], M.I. Urrutia [3]

[1] Centro de Investigaciones de Fitopatología (CIDEFI), Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata (UNLP). 60 y 119 (1900) La Plata, Buenos Aires, Argentina

[2] Laboratorio de Genética y Mejoramiento Animal y Vegetal. Fac. de Ciencias Agrarias y Forestales. UNLP

[3] Curso de Cálculo Estadístico y Biometría. Fac. de Ciencias Agrarias y Forestales. UNLP

mbalatti@fedpat.com.ar

SUMMARY

Fungal endophytes were isolated from healthy cultivated soybean leaves at two growth stages (R_2-R_3 and R_4-R_5) during the growing season 1997/98. Samples were obtained from a segregating population (F_3 generation) cultivated at the experimental field of the Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Buenos Aires, Argentina. Twelve genera of endophytic fungi were isolated and identified. In general, in both growth stages the same species were isolated and most of them did not show significant differences in their infection frequencies. Most of the fungi isolated are cited as soybean pathogens in different places of the world. The most commonly isolated endophytes included *Alternaria alternata* and *Glomerella cingulata* (= *Colletotrichum gloeosporioides*).

Key words: endophytes, *Glycine max*

Endophytes are fungi that form inapparent infections within leaves and stems of healthy plants (Carroll, 1988). Such infections are termed «endophytic» particularly when the association is believed to be mutualistic or at least non-pathogenic, or «latent infections», where a latent pathogen is involved. Agrios (1988) defined latent infections as the state in which a host is infected with a pathogen but does not show symptoms. Latent in-

* Autor para correspondencia
Recibido: 3-11-00
Aceptado para su publicación: 5-10-01

fections persist until signs or symptoms are prompted to appear by environmental or nutritional conditions or by the stage of maturity of the host or pathogen (Agrios, 1988).

Endophytic fungi have been found in all plant families thus far investigated, which represent many species in different climatic regions of the world (Spurr and Welty, 1975; Petrini and Carroll, 1981; Petrini *et al.,* 1992).

The study of endophytes distribution, biodiversity and their biochemical characteristics are of immense importance in plant biology to understand and to improve plant fitness (Sridhar and Raviraja, 1995). Endophytic fungi are of biotechnological interest due to their potential use as genetic vectors (Murray *et al.,* 1992), as biological control agents (Dorworth and Callan, 1996), and as a source of secondary metabolites (Strobel *et al.,* 1996). Some endophytic fungi are thought to produce compounds that render plant tissues less attractive to herbivores, while others strains may increase host plant drought resistance (Chanway, 1998).

Soybean (Glycine *max* (L.) Merr.) is one of the most important crops in Argentina, not only by its production but also because of the volume exported and is planted on about 8 million ha (Botta, 2000). Soybean crops are affected by several diseases which decrease the total production.

Preliminary investigations with soybean provide evidence that most fungal pathogens of seedlings, plants, pods and seed have an asymptomatic or latent period after infection or colonization. *Cercospora kikuchii* (Matsumoto & Tomoyasu) Gardner, *Colletotrichum truncatum* (Schw.) Andrus & Moore, *C. gloeosporiodes* (Penz.) Penz. & Sacc., *Diaporthe*/*Phomopsis* complex, *Macrophomina phaseolina* (Tassi) Goid. and *Fusarium* spp. have been reported by Sincalir (1991) causing latent infections on soybean plants.

In our investigation we used the term «endophytic fungus» in a wide sense. It only defines the fact that a fungus has been isolated from symptomless plant tissues.

The present study was undertaken in order to: i) document the species composition of endophytic fungi of healthy cultivated soybean leaves; ii) determine their infection frequencies and iii) verify possible quali-quantitative changes of species isolated at two growth stages (R_2-R_3 and R_4-R_5).

To achieve a higher genetic variability an F3 population resulting from a cross between G-3901 (Asiatic origin)   TGX 1740-6F (African origin) was selected as the sampling material for the trials. Plots were planted at the experimental field of the Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Buenos Aires, Argentina, during the 1997/98 growing season.

Fifty asymptomatic plants were randomly sampled at two defined growth stages: R_2-R_3 and R_4-R_5 (Fehr *et al.,* 1971). One leaf was excised from the middle of each plant. Leaves were washed under running tap water and cut into several disks of approx. 5 mm diameter. Small sections (subsamples) were surface-sterilized by dipping successively into 70 % ethanol for 1 minute, 2 min in sodium hypochlorite (5.5 % available chlorine v/v), and rinsed twice in sterile distilled water (according to Larran, S., unpublished). Five leaf disks were placed in each Petri dish containing 2 % Potato Dextrose Agar (PDA), acidified (pH 5.0) with 25 % lactic acid to suppress bacterial growth. Dishes were incubated in the dark at 26   2 C during 9 days in a growth chamber. Dishes were checked every 2 or 3 days until the 9^{th} day.

Identifications were made in the original dish, but when this was not possible, fungi were subcultured and incubated for further study in 2 % PDA. Fungal taxa were identified on the basis of cultural characteristics and the morphology of fruiting bodies and spores (Ainsworth *et al.,* 1973; Sinclair and Shurtleff, 1980).

The frequency of infection was calculated as the number of subsamples infected by a given fungus divided by the total number of subsamples incubated. Single and multiple infections were scored on each individual segment. The Student test "t" and Percentage Differences test were used to evaluate differences in infection frequencies for different fungi.

The endophytic species isolated from soybean leaves at R_2-R_3 and R_4-R_5 stages and their infection frequencies are listed in Table 1. Twelve genera of endophytic fungi were isolated and identified from leaves of field-grown soybeans. All isolates were identified at least to genus level. The identification of fungi of 12 genera shows the diverse nature of endophytic population. Most taxa isolated from soybean leaves belong to genera which have already been described as endophytes of plants from temperate zones and from the tropics (Spurr and Welty, 1975; Petrini *et al.,* 1992).

The endophytic fungi more frequently isolated from healthy leaves of soybean were *Alternaria alternata* and *Glomerella cingulata.* Other fungi less frequently isolated were *Phomopsis sojae, Phomopsis* sp. and *P. longicolla. Alternaria alternata* has been routinely isolated as endophyte of leaves from wheat and other plants (Sieber, 1985; Petrini, 1986) and also from maize (Wellacher, 1991). The results obtained in this investigation

Table 1

Mean percentage frequencies of endophytic fungi and their variations from soybean leaves at R_2R_3 and R_4-R_5 stages (Total segments sampled: 591)

Endophytes	Frequencies (%)			
	R_2-R_3 stage[a]	R_4-R_5 stage	Variation (%)	
Alternaria alternata (Fr.) Keissler	78.48	68.79	–12.34	NS
Alternaria tenuissima (Kunze ex Pers.) Wiltshire	0	1.60	–	NS
Bipolaris sorokiniana (Sacc.) Shoem.	0.94	0	–100.00	NS
Cladosporium sp.	0	2.06	–	*
Colletotrichum sp.	1.28	0	–100.00	NS
Curvularia lunata (Wakker) Boedijni	0	0.40	–	NS
Epicoccum nigrum Link	1.23	1.93	+56.90	NS
Glomerella cingulata (Stoneman) Spauld & Schrenk	17.20	14.04	–18.40	NS
Glomerella glycines Lehm. & Wolf	0.51	0.40	–21.60	NS
Nigrospora sphaerica (Sacc.) Mason	1.33	2.00	+50.40	NS
Penicillium sp.	0	1.86	–	NS
Phomopsis longicolla Hobbs	1.99	0	–100.00	*
Phomopsis sojae Lehman	3.48	3.86	+10.90	NS
Phomopsis sp.	2.89	5.50	+90.30	**
Pleospora herbarum (Pers. ex Fr.) Rabenh.	0.66	0.99	+50.00	NS
Stemphylium sp.	3.32	2.99	–9.90	NS

[a] Based on the plant stages designated by Fehr *et al.* 1971.
[b] The infection frequency was calculated as the number of subsamples infected by a given fungus divided by the total number of subsamples incubated.
* Significant difference (P < 0.05); ** Highly significant difference (P < 0.01); NS: No significant difference.

are in almost perfect agreement with reports on endophytes from other hosts in which generally a large number of species can be isolated from a given host, but only very few species are present in significant amounts (dominant species) (Petrini *et al.,* 1992). These dominant taxa may be adapted to the particular microecological and physiological conditions present on soybean leaves.

In general, in both sampling dates the same fungal species were isolated. It should be pointed out that the following species appeared only in the first sampling date: *Bipolaris sorokiniana, Colletotrichum* sp. and *Phomopsis longicolla.* On the other hand, there were species that appeared only in the second sampling: *Alternaria tenuissima, Cladosporium* sp., *Curvularia lunata* and *Penicillium* sp.

Furthermore, Table 1 shows the fungal infection frequency variation (%) of each species during the time elapsed between both observations. Most of the species do not show significant differences, with exception of *Phomopsis longicolla, Cladosporium* sp. ($P < 0.05$) and *Phomopsis* sp. ($P < 0.01$). Fungi colonized 94 % and 89 % of surface-sterilized incubated leaf segments at each sampling date, respectively (Table 2). Multiple infections of individual segments were 17.98 % and 17.51 % at stages R_2-R_3 and R_4-R_5, respectively.

Table 2

Infection rate (%) of endophytic fungi from soybean leaves at R_2-R_3 and R_4-R_5

	R_2-R_3 stage[a]	**R_4-R_5 stage**
Total segments sampled	317	274
Total segments infected	298	243
Infection rate (%)	94	89

[a] Based on the plant stages designated by Fehr *et al.* 1971.

Most of the fungi isolated in this work are cited as soybean pathogens in different places of the world (Farr *et al.,* 1989). These fungi may be assigned to two groups: 1) well known and economically important pathogens of soybean, e.g. *Diaporthe*/*Phomopsis* complex and *Glomerella glycines*; and 2) minor pathogens such as *Alternaria alternata*, *A. tenuissima*, *Cladosporium* sp. and *Stemphylium* sp. (Sinclair and Shurtleff, 1980). The fungi isolated here could be either avirulent or hypovirulent, or are virulent but in a latent phase, with the pathogenic factors being triggered by exogenous or endogenous physiological or ecological changes as described by Petrini (1991). We do not know the behavior of the strains isolated in this work. An early detection in the field of the fungi that were identified as pathogen in a latent phase would contribute to the recognition of disease spread within soybean crop and to development of effective control measures.

Soybean leaves are hosts to an abundance of endophytic fungi but only *Alternaria alternata* was the dominant specie. Most of the endophytes isolated could be pathogens in a latent phase. Pathogenicity tests would be needed to investigate this hypothesis. Further studies will be carried out to evaluate the potential use of endophytes in biological control.

ACKNOWLEDGMENTS

This research was supported partly by Universidad Nacional de La Plata, CIC and CONICET. We thank Ing. Agr. Marina Sisterna for dematiaceus fungi identification.

RESUMEN

Hongos endófitos en foliolos de soja

Se aislaron hongos endófitos de hojas sanas de plantas de soja en los estadios de crecimiento R_2-R_3 y R_4-R_5 durante el período 1997-1998. Las muestras se obtuvieron de una población segregante (generación F_3) cultivada en el campo experimental de la Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Buenos Aires, Argentina. Se aislaron e identificaron 12 generos de hongos endófitos. En ambos estadios se aislaron las mismas especies y en general no presentaron diferencias significativas en sus frecuencias de infección. La mayoría de los hongos aislados han sido citados como patógenos de la soja en diferentes partes del mundo. *Alternaria alternata* y *Glomerella cingulata* (= *Colletotrichum gloeosporioides*) fueron las especies más frecuentemente aisladas.

Palabras claves: Endófitos, *Glycine max*

REFERENCES

AGRIOS G.N., 1988. Plant Pathology. 3 rd Edition. Academic Press, New York. 803 pp.

AINSWORTH G.C., SPARROW, F.K., SUSSMAN, A.S., 1973. The fungi. An advanced treatise. Academic Press, Inc. New York, Vol IV, 621 pp.

BOTTA G., 2000. Enfermedades de la soja. In: VI Curso de diagnóstico y manejo de enfermedades de soja. Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria Pergamino, Buenos Aires, Argentina. pp. 7-20.

CARROLL G.C., 1988. Fungal endophytes in stems and leaves: from latent pathogens to mutualistic symbiont. Ecology 69, 2-9.

CHANWAY C.P., 1998. Bacterial endophytes: ecological and practical implications. Sydowia 50, 149-170.

DORWORTH C.E. AND CALLAN B. E., 1996. Manipulation of endophytic fungi to promote their utility as vegetation biocontrol agents. In: Endophytic fungi in grasses and woody plants, eds. Redlin, S.C. and Carris, L.M. APS Press, St. Paul. pp. 209-216.

FARR D.F., BILLS G.F., CHAMURIS G..P., ROSSMAN A.Y., 1989. Fungi on plants and plant products in the Unites States. APS Press. The American Phytopathological Society. St. Paul, Minnesota. USA. 1252 pp.

FEHR W.R., CAVINESS C.E., BURMOOD D., PENINGTON J.S., 1971. Stage of development descriptions for soybeans, *Glycine max* (L.) Merr. Crop Science 11, 929-931.

MURRAY F.R., LATCH G.C.M., SCOTT D.B., 1992. Surrogate transformation of perennial ryegrass, *Lolium perenne,* using genetically modified *Acremonium* endophyte. Molecular and General Genetics 233, 1-9.

PETRINI O., 1986. Taxonomy of endophytic fungi of aerial plant tissues. In Microbiology of the Phyllosphere, ed. Fokkema, N.J. and Van den Heuvel. Cambridge. University Press. pp. 175-187.

PETRINI O., 1991. Fungal endophytes of tree leaves. In: Microbial ecology of tree leaves, ed. Andrews, J.H. & Hirano, S.S. Springer Verlag, New York. pp. 179-197.

PETRINI O., CARROLL G.C., 1981. Endophytic fungi of some Cupressaceae in Oregon. Can. J. Bot. 59, 629-636.

PETRINI O, SIEBER T. N., TOTI L., VIRET O., 1992. Ecology metabolite production, and substrate utilization in Endophytic Fungi. Natural toxins 1, 185-196.

SIEBER T.N., 1985. Endophytische Pilze von Winterweizen *(Triticum aestivum* L.). Zürich, Switzerland. Dissertation ETH N.º 7725, Swiss Federal Institute of Technology.

SINCLAIR J.B., 1991. Latent infection of soybean plants and seeds by fungi. Plant Disease 75, 220-224.

SINCLAIR J.B., SHURTLEFF M.C., 1980. Compendium of soybean diseases. American Phytopathological Society. St. Paul. Minnesota, USA. 69 pp.

SPURR H.W. JR., WELTY R.E., 1975. Characterization of endophytic fungi in healthy leaves of *Nicotiana* spp. Phytopathology 65, 417-422.
SRIDHAR K.R., RAVIRAJA N.S., 1995. Endophytes. A crucial issue. Current Science 69, 570-571.
STROBEL G.A., HESS W.M., FORD E., SIDHU R.S., YANG X., 1996. Taxol from fungal endophytes and the issue of biodiversity. Journal of Industrial Microbiology 17, 417-423.
WELLACHER M., 1991. Die Pilzkolonisation von Maispflanzen. Diplomarbeit. Baarn, The Netherlands. Centraalbureau voor Schimmelcultures.