The recognition pattern of green algae by lichenized fungi can be extended to lichens containing a cyanobacterium as photobiont

Mara Sacristán, Mercedes Vivas, Ana María Millanes, Blanca Fontaniella, Carlos Vicente and María Estrella Legaz.

Department of Plant Physiology, Faculty of Biology, Complutense University, 28040 Madrid, Spain.

Lichens are intimate and long-term symbioses of photosynthetic, unicellular algae or cyanobacteria and heterotrophic fungi joined to form a new biological entity different from its individual components. Specificity required for the lichen association can be defined in this context as the preferential, but not exclusive, association of a biont with another. Recognition of compatible algal cells is carried out by specific lectins produced and secreted by the potential mycobiont. However, lichen phenolics are not involved in the recognition process, in contrast to that found for other plant symbioses, such as mycorrhizal or *Rhizobium* legume associations. Some lectins from phycolichens have been characterized as glycosylated arginases which bind to an algal cell wall receptor identified as an α-1,4-polygalactosylated urease. The binding is improved by Ca^{2+} and Mn^{2+}, in a similar way to that described for legume lectins. Two forms of glycosylated arginases seem to be involved in the recognition process, one of them, particulated in the cell wall of fungal hyphae and involved in cell contact between phyco- and mycobiont, whereas a second, secreted arginase produces recruitment of compatible algal cells near the surface of fungal hyphae. When glycosylated urease is lacking from the algal cell wall, fungal arginase is internalized, increasing the levels of algal putrescine, which promotes chloroplast disorganization, activation of glucanases and breakdown of the cell wall with loss of the protoplast. Evolution of symbiotic relationships implies then the synchronization of cell division and lectin receptor production, probably as a consequence of the perception of environmental factors (light and temperature).

This recognition pattern can be now extended to cyanobiont-forming associations. *Leptogium corniculatum*, a cyanolichen containing *Nostoc*, also produces a secreted glycosylated arginase that binds to *Nostoc* as well as to *Trebouxia* cells from *Evernia prunastri*, a phycolichen species. In addition, *Leptogium* arginase is retained by *Evernia* urease bound to an activated agarose bead and it is desorbed by 50 mM D-galactose, in a similar way to that previously reported in the interaction arginase-urease from phycolichens.

Key words: Cyanolichens, discrimination, lectins, lectin ligands, phycolichens, recognition.

1. Lichens are symbioses where one or both symbionts appear obligate, specific and specialized

Lichens are intimate and long-term symbioses of photosynthetic algae or cyanobacteria and heterotrophic fungi joined to form a new biological entity different from its individual components. Both bionts appear in nature among a mixture of millions of non-symbiotic microorganisms, and mechanisms of compatible combination are required [1]. Thus, specificity is required for the lichen association. Specificity can be defined in this context as the preferential, but not exclusive, association of a biont with another [2]. For example, the mycobiont of *Cladonia cristatella* produces squamules with different species of *Trebouxia*, displaying a selective behaviour [3]. However, the mycobiont of *C. cristatella* cannot form squamules with green algae other than *Trebouxia* , showing high specificity [4]). Moreover, culture experiments to investigate the selectivity of the mycobiont of *Fulgensia bracteata* towards a variety of potential photobionts provide evidence for mycobiont selectivity and varying compatibility of the respective symbionts,

which can be interpreted as a cascade of interdependent processes of specific and non-specific reactions of the symbionts involved [5].

On the other hand, specificity needs mechanisms of recognition finely tuned. When the algal cells proliferate inside a growing thallus, daughter cells are enveloped by fungal hyphae, which recognize the new cells as compatible [4].Thus, recognition mechanisms are absolutely required, not only for de novo formation of new associations, but also for the maintenance of the symbiotic equilibrium in the lichen symbiosis. Ultrastructural or resynthesis studies aiming to investigate the relationship between lichen symbionts in the lichen thallus suggested that such relationship might involve cell surface recognition factors [6].

2. Molecular determinants of cell recognition

Several phytohaemagglutinins have been isolated from a number of lichen species. Lockhart *et al.* [7] found that haemagglutinins bind to the appropriate phycobiont using *Peltigera canina* and *P. polydactyla*, two cyanolichens containing *Nostoc* as photobiont. Moreover, a protein fraction isolated from the thallus of *Xanthoria parietina* labelled with fluorescamine showed strong binding to cultured phycobionts obtained from *X. parietina*, *Caloplaca auriantia* and *C. citrina* [8], whereas it did not bind to freshly isolated phycobionts from the same species or isolated or cultured algae from *Cladonia convoluta*, *Ramalina duriaei* and *R. pollinaria*, species from taxonomic families different from Teloschistaceae. Protein binding occurred at the cell wall surface of algal cells, which was further confirmed by binding of labelled protein to algal cell wall ghosts. A likely counterreceptor is ABP (algal binding protein), a polypeptide of 12.5 kDa [9], found in *Xanthoria* mycobionts cultured in vitro but ABP is restricted to the hyphal cell walls [10]. A protein with similar binding properties was later obtained from the cyanolichen *Nephroma laevigatum* [11] and purified as an heterodimer of 52 kDa and 55 kDa subunits. A new lectin has been isolated from an aqueous extract of the symbiotic phenotype of *Dictyonema glabratum* that contains *Scytonema* sp as cyanobacterial photobiont. Its native conformation is a dimer formed by two identical subunits of 16.5 kDa. The lectin is a glycoprotein with a low degree of glycosylation, containing galactose, xylose, glucose and mannose as neutral monosaccharides, in addition to glucosamine, which could indicate both *N*- and *O*-linkages [12]. Similar lectins have been found in *Peltigera membranacea* [13] and cephalodia of *P. aphthosa* [14].

3. Lichen lectins develop additional enzymatic activities to their binding ability

The ABP from *X. parietina* thallus has been characterized as a glycosylated arginase able to hydrolyze arginine into ornithine and urea [15]. The glycidic moiety of ABP contains equimolar amounts of N-acetyl-D-glucosamine and D-glucose. Another secreted arginase of *X. parietina* thalli (SAX, secreted arginase from *Xanthoria*) is identical to ABP in the amino acid composition but its glycidic moiety is composed of equimolar amounts of D-galactose and D-glucose [16]. Fluorescein-labelled ABP (glycosylated, cell wall-pelletable arginase) and SAX (secreted arginase) bind to the cell wall of isolated phycobionts of *X. parietina* (Fig. 1). Such binding is strongly enhanced after induction of a glycosylated urease located at the algal cell wall, which is almost identical to that secreted by the thallus. Such urease consists of a single polypeptide glycoprotein with a large polygalactose moiety [17]. Binding of glycosylated arginase to urease inhibited enzymatic activities of both proteins.

Evernia prunastri, a phycolichen from Parmeliaceae family also produces both ABP and SAE (secreted arginase from *Evernia*) but the polysaccharide moiety of the secreted enzyme is composed by fructose, mannose and glucose [18]. ABP, SAE and SAX exhibit Mn^{2+}-dependent arginase activity. Ca^{2+} does not overcome the absence of Mn^{2+}, and the addition of Ca^{2+} to reaction mixtures in the presence of Mn^{2+} significantly inhibits the arginase activity of both secreted enzymes [19]. However, simultaneous addition of both Ca^{2+} and Mn^{2+} to the incubation media of phycobionts recently isolated from thalli of *E. prunastri* and *X. parietina* strongly increased the binding efficiency of labelled lectins to the cell wall of

algal cells when detectable urease is previously found for this structure, in accordance with lectin dependence on Ca^{2+} for binding [20]. Binding occurs in both homologous and heterologous algae. This means that SAE binds efficiently to *Evernia* phycobionts, but it is also able to bind to *Xanthoria* phycobionts. Conversely, SAX preferently binds to *Xanthoria* phycobiont but also interacts with the cell wall of algae from *Evernia*, which suggests a certain degree of unspecificity. It is very interesting to note that the only requirement for binding is the occurrence of polygalactosylated urease in the cell wall of both

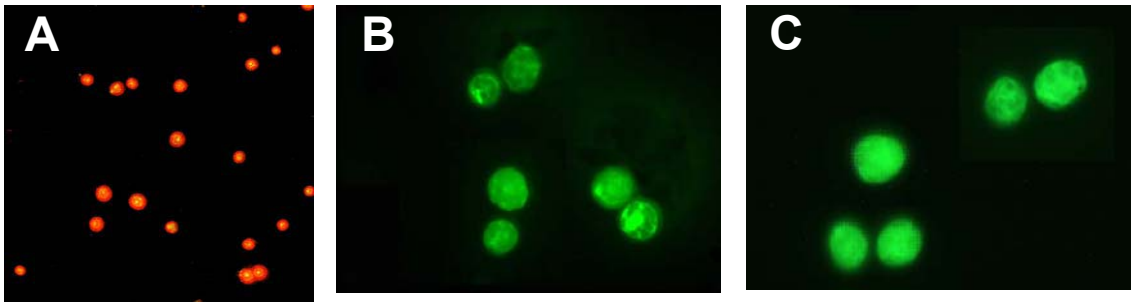

Fig. 1. Detection of the binding of a fungal lectin to the cell wall of potential, compatible phycobionts. A) Algal cells isolated from the lichen *Evernia prunastri* without added lectin, showing the red fluorescence of chlorophylls. B) Algal cells isolated from *E. prunastri* thalli after incubation for 1h with its lectin, SAE. C) Algal cells isolated from *Xanthoria parietina* thalli incubated for 1h with its lectin, SAX

homologous and heterologous algae. SAE and SAX binding can be reversed by galactose [19] in any condition, underlining the involvement of galactose residues in specific binding.

In summary, the fungal partner produces two classes of lectins with different roles: 1) ABP enzymes, which promote the physical interaction between the fungus and its specific algal partner, and 2) Secreted lectins, whose role may be to promote the recruitment of algal cells to the neighbourhood of the fungal mycelium. This may constitute a mechanism to optimize binding by increasing the odds of productive contact and also to prevent contact loss that would follow algal cell death that occasionally occurs during the early stages of the association.

4. The nature of the lectin ligand

Since arginase lectin binds to the polygalactoside moiety of a cell wall pelletable-urease [19], the nature of the galactosyl bond could be studied by treating phycobiont cells with α- or β-galactosidases. The occasional lost of the binding capability of the lectin could be then related to the partial or total enzymatic hydrolysis of the polygalactoside rest of the ligand. These hydrolases not only break the glycoside bond of disaccharides (lactose, melibiose) or tri- and tetrasaccharides (raffinose, stachyose) containing galactose but also the degalactosylation of some galactomanans and that of an agglutinin-glycoprotein from peanut and release galactose from galactosyl-fetuin [21]. Thus, it can be expected that the incubation of *Evernia* phycobionts with α- or β-galactosidases will produce degalactosylation of some galactosyl proteins in the surface of the cell wall. As Sacristán *et al.* [22] found, α-galactosidase removes three times more galactose than β-galactosidase from phycobiont cells naturally containing the lectin ligand and 1.5 times more from those containing experimentally induced ligand. In parallel to the loss of α-1,4-D-galactosides from the surface of the cell wall, SAE lectin does not bind to its specific ligand. Occasionally, lectin enters the cells in some extent even when their cell walls contained the unmodified ligand. This fact could be due to the occurrence of a limited number of ligand sites in the algal cell wall that saturates at low amount of labelled lectin. Thus, it can be concluded that glycosylated urease acting as the main (or unique) ligand for fungal arginase contains an α-1,4-D-galactoside attached to the polypeptide.

5. Compatibility as cell recognition versus incompatibility as cell discrimination.

When the glycosylated urease is lacking from the algal cell wall, fungal arginase is internalized, increasing the levels of algal putrescine, which promotes chloroplast disorganization, activation of glucanases and breakdown of the cell wall with loss of the protoplast [23, 24]. Algal cells do not produce arginase

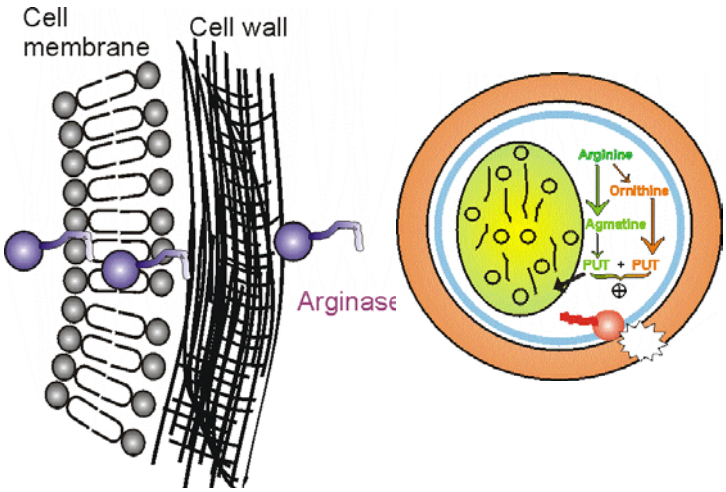

Fig. 2. Discrimination of incompatible algal cells by a fungal lectin developing arginase activity. The lack of the adequate, specific receptor (ligand) on the cell wall facilitates the entry of the lectin which increases the concentration of putrescine. The diamine disorganizes the chloroplast structure and activates glucanases that hydrolyze the cell wall and releases algal protoplast.

but they contain low amount of a mitochondrial ornithine decarboxylase which does not produce putrescine but it is probably involved in the production of pyrroline derivatives [25]. Synthesis of very low amounts of algal putrescine [26] is carried out by the sequential action of arginine decarboxylase and agmatine amidino hydrolase, but the affinity of the arginase for arginine is almost ten times higher than that of arginine decarboxylase. Both SAX and ABP entry into the cells produces plasmolysis, packing of thylakoids and development of storage bodies, as well as an increase in the number of polysaccharide secretion vesicles and multivesicular bodies (Fig. 2). As a function of vesicles structure, different types will be described: v_1, young Golgi vesicles, v_2, reorganized membrane vesicles, v_3, concentrated polysaccharide vesicles, v_4, exocytic vesicles, sequentially produced [27]. This can be explained as an attempt of algal cells to correct the

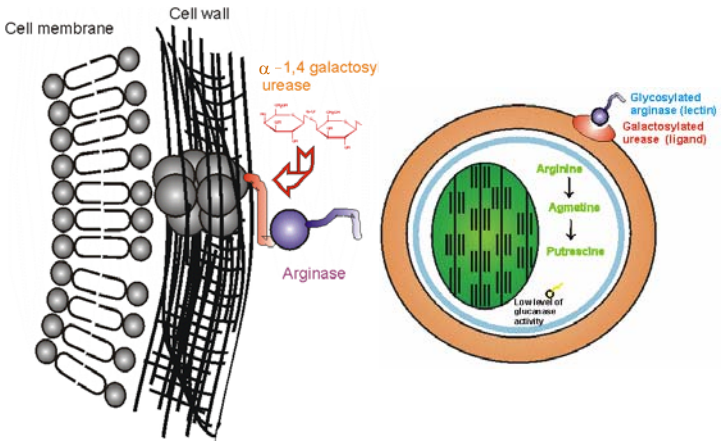

Fig. 3. Recognition by a fungal lectin developing arginase activity of the compatible phycobiont. The lectin is retained on the surface of the cell wall by an affinity reaction between the lectin and the cell wall ligand, a polygalactosylated urease synthesized by the compatible phycobiont. The concentration of algal putrescine remains then unchanged and the algal cell wall does not hydrolyzed by glucanases.

initial stages of glucan hydrolysis carried out by activated glucanases in order to repair their damaged cell walls. However, the cell wall is finally broken and the algal cell dies. This complex mechanism can be interpreted as a process of fungal attack of the mycobiont on the phycobiont (consisting in arginase secretion) and algal resistance against this attack by mean of polysaccharide production that attempts to repair the broken cell wall. However,

this resistance mechanism is doomed, since the number of dead cells increases after glycosylated arginase penetration into the algal cell. Thus, expression of specific cell wall ligands to impede the uptake of arginases by the phycobiont cells constitutes a much more suitable defensive mechanism (Fig. 3).
In summary, fungal lectins provide two different outcomes depending on receptor availability; if the compatible algae expresses a specific cell wall ligand, these act as lectin ligands and the lectin is retained in the cell wall, preventing its penetration into the cell's cytoplasm; on the other hand, when the ligand is lacking they penetrate the cell and produce algal deterioration. This hypothesis is in agreement with the fact that the number of cell division cycles of algal cells is under strict control by the mycobiont [28, 29]. Supporting this notion, the growth zone of *X. parietina* thallus is mainly populated by small, young cells that can undergo division, whereas the mature zones, in which the mycobiont is predominant, contain preferentially bigger cells that are occasionally dead [30].
Compatibility in established thalli relies on expression of the ligand for the fungal lectin, which means that urease production and lectin secretion must be synchronized to warrant specific recognition of the daughter cells. The time-course of arginase secretion from *E. prunastri* thalli shows that the higher activity was obtained from thalli collected during July, but two secondary maxima were also shown, the first during April-May and another during October-November. However, urease secretion from *E. prunastri* thalli started in October and peaked during December to decrease thereafter [31]. Consequently, aplanosporangia were observed in sections obtained from thalli maintained under short-day photoperiod conditions, although the occurrence of these cells was not uniform. Distribution of cell size indicated that mature cells occurred more frequently in thalli under long-day conditions, whereas small-sized, young cells were more abundant in lichen thalli under short-day conditions [32]. In addition, cultures carried out at cold temperatures produced a higher frequency of average-sized cells as the number of the largest algae decreased considerably.

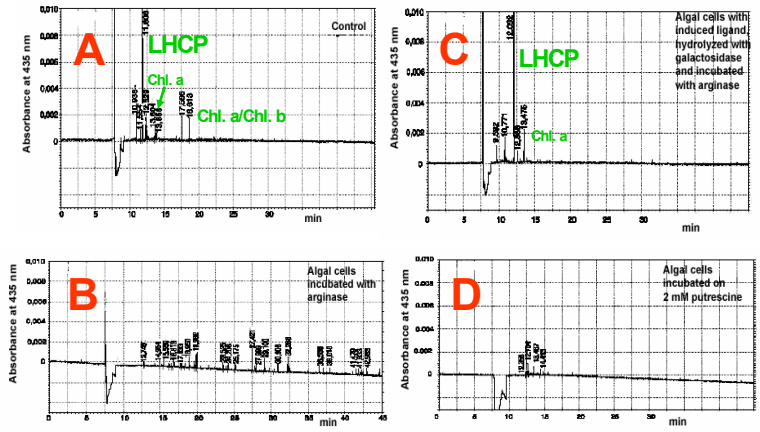

Fig. 4. Electrophoretic traces of chlorophyll-protein complexes isolated from *E. prunastri* phycobionts. In A), complexes obtained from control cells without any treatment. In B), complexes obtained from algal cells lacking the cell wall ligand for the lectin and incubated on SAE for 1h. In C), complexes obtained from algal cells containing the cell wall ligands, previously hydrolyzed with α-1,4-galactosidase and then incubated on SAE. In D), complexes obtained from algal cells lowaded with 2 mM putrescine.

The death of discriminated algal cells has been then defined as algal incompatibility. This secreted arginase is unequivocally defined as a fungal enzyme since activity assays in isolated partners show that arginase is preferentially found in the fungal component and, in addition, isolated phycobionts in axenic culture do not secrete detectable arginase [25]. Moreover, phycobionts isolated from *Xanthoria parietina* thalli and loaded with 28 mM arginine in liquid media do not secrete arginase and their content in putrescine does not increase significantly, indicating that diamine synthesis via arginine decarboxylase and agmatine amidino hydrolase is regulated by a feedback mechanism [23].

Since the lectin entry into compatible algal cells, containing the specific ligand in the cell wall, is avoided, level of algal putrescine does not increase by the lack of additional, fungal arginase activity to the algal arginine decarboxylase. The maintenance of endogenous levels of PUT did not induce significant structural changes in the phycobiont cells. Cell area of *Evernia* phycobionts remained constant, although the chloroplast area was slightly diminished after cell treatment with SAE and SAX. The size of

the pyrenoid area was largely decreased, whereas the area of storage bodies significantly increased [19]. Inversely, both cell and chloroplast areas slightly increased after lectin treatment whereas the area of pyrenoid and storage bodies decreased in *Xanthoria* phycobionts.

Analysis of chlorophyll-protein complexes by capillary electrophoresis has been performed after incubation on fungal arginase of *Evernia* phycobionts lacking the lectin receptor as well as those in which the α-1,4-galactosyl moiety has been previously hydrolyzed with α-1,4-galactosidase. The different complexes were disaggregated by octylglucoside before electrophoresis. Both treatments dramatically decrease the amount of LHCP and chlor. a/chlor. b complexes whereas those containing only chlor. a remain unchanged. These last complexes, however, completely disappear after incubation of algal cells on 2 mM putrescine, the diamine concentration that would accumulate into the cells which internalize the fungal lectin (Fig. 4).

6. The theory about the recognition or discrimination of phycobionts by their potential fungal partner can be extended to cyanobionts-forming cyanolichens.

The mechanism of cell recognition described for phycobionts of *X. parietina* and *E. prunastri* must be extended to other phycolichen species. For this purpose, a consensus has been looked among the sequences of different fungal arginases and a combination of primers has been constructed that works properly. Then, we have tried to hybridise it with DNAs isolated from lichenized fungi, *E. prunastri*, *E. divaricata, Lecanora muralis* and *X. parietina*. In turn, we have assayed different temperatures of annealing and constructing several temperature gradients. By trying with another polymerase and a different gradient of temperatures, two combinations of primers were obtained to originate pure products in PCR to be cloned in *Escherichia coli* to obtain the most polymer to be sequenced.

Reactivity of several lichen cyanobionts against legume lectins, such as concannavalin A (Con A), was found by Marx and Peveling [33]. This implies that cyanobacteria able to form cyanolichens possess mannose-specific ligands on their cell wall, that each one of them behaves as a single protein different from the urease previously involved in galactose-dependent binding, and that these proteins bore no urease activity. But the ability to ligand ConA has also been described for *Evernia* and *Xanthoria* phycobionts [34], indicating that the cell wall of these algal cells contains multiple ligands for different lectins.

However, lichen species containing cyanobacteria as specific photobionts also develop lectins bearing affinity for polysaccharides containing galactose. Lehr *et al.* [35] purified one of such lectins from *Peltigera membranacea* as well as from cephalodia of *Peltigera aphthosa* [36]. The latter was a glycoprotein with a molecular mass of 20 kDa bearing high cytoagglutinin activity, which was reversed by addition of lactose to the cell suspension.

At present, we attempt to validate this arginase-urease interaction as the universal base not only for phycobionts but also for cyanobionts recognition by using *Leptogium corniculatum*. It has been found that recently collected thalli of *L. corniculatum* secrete arginase after 2h of incubation on 40 mM arginine. The enzyme is efficiently retained by activated agarose beads on which galactosylated urease from *Evernia* has previously been attached. *Leptogium* arginase is completely eluted from the bead using 50 mM D-galactose. This implies that an interaction between the cyanolichen lectin and the phycolichen ligand would justify the recognition process when the cyanobiont was able to synthesize urease and to retain a part of this galactosylated enzyme attached to its cell wall.

Acknowledgements This work was supported by a grant, BFU2006-14263, from the Ministerio de Educación (Spain). We are grateful to Miss Raquel Alonso for the excellent technical support.

References

[1] M. Galun and N. Kardish, Cryptogamic Botany **5**, 144 (1995).
[2] P. Bubrick, Effects of symbiosis on the photobionts, in Handbook of Lichenology, M. Galun (Ed.), CRC Press, Boca Raton, FL, 1988, Vol. 2, pp. 133.
[3] V. Ahmadjian and J.B. Jacobs, Nature 289, 169 (1981).
[4] V. Ahmadjian, The Lichen Symbiosis, John Wiley & Sons, New York, 1993
[5] T. Schaper and S. Ott, Plant Biology **5**, 441(2003).
[6] V. Ahmadjian, J.B. Jacobs and L.A. Russell, Science **200**, 1062 (1978).
[7] C.M. Lockhart, P. Rowell and W.D.P. Stewart, FEMS Microbiological Letters **3**, 127 (1978).
[8] P. Bubrick and M. Galun, Protoplasma **104**, 167 (1980).
[9] P. Bubrick, M. Galun and A. Frendsdorff, Symbiosis **1**, 85 (1985).
[10] P. Bubrick, M. Galun and A. Frendsdorff, Protoplasma 105, 207 (1981).
[11] N. Kardish, L. Silberstein, G. Flemminger and M. Galun, Symbiosis **11**, 47 (1991).
[12] S.L. Elifio, M.L.C.C. da Silva, M. Iacomini and P.A.J. Gorin, New Phytologist **148**, 327 (2000).
[13] H. Lehr, G. Fleminger and M. Galun, Symbiosis **18**, 1 (1995).
[14] H. Lehr, M. Galun, S. Ott, H.M. Jahns and G. Fleminger, , Symbiosis: **29**, 357 (2000).
[15] M.C. Molina, E. Muñiz and C. Vicente, Plant Physiology and Biochemistry **31**, 131 (1993).
[16] M.C. Molina and C. Vicente, Journal of Liquid. Chromatography **19**, 2101 (1996).
[17] E. Pérez-Urria and C. Vicente, Journal of Plant Physio*logy* **133**, 692 (1989).
[18] V. Planelles and M.E. Legaz, Plant Sci.ence **51**, 9 (1987).
[19] M.E. Legaz, B. Fontaniella, A.M. Millanes and C. Vicente, Eurpean Journal of Cell Biology **83**, 435 (2004).
[20] K. Zhu-Salzmann, P.K. Hammen, R.A. Salzmann, H. Koiwa, R.A. Bressan, L.L. Murdock and P.M. Hasegawa, Comparative Biochemistry and Physiology. B. Biochemistry and Molecular Biology 132, 327 (2002).
[21] H. Shibuya, H. Kobayashi, S. Yoshida, S. Kaneko, G.G. Park and I. Kusakabe, Bioscience Biotechnology and Bioengeenering. **67**, 1465 (1999).
[22] M. Sacristán, A.M. Millanes, M.E. Legaz and C. Vicente, Plant Signaling and Behaviour **1**, 23 (2006).
[23] M.C. Molina, E. Stocker-Wörgötter, R. Turk, C. Bajon and C. Vicente, Cell Adhesion and. Communication **6**, 481 (1998).
[24] M.C. Molina and C. Vicente, Cell Adhesion and Communication **3**, 1 (1995).
[25] C. Vicente and M.E. Legaz, Lichen enzymology, in Handbook of Lichenology, M. Galun (Ed.), CRC Press, Boca Raton, FL, 1988, Vol. 1, pp. 239.
[26] M.I. Escribano and M.E. Legaz, , Plant Physiology **87**, 519 (1988).
[27] M.C. Molina, C. Bajon, A. Sauvanet, D. Robert and C. Vicente, Journal of Plant Research **111**, 191 (1998).
[28] D.J. Hill, Symbiosis **14**, 325 (1992).
[29] D.J. Hill, Cryptogamic Botany 4, 270 (1994).
[30] D.J. Hill, New Phytologist 112, 175 (1989).
[31] M.E. Legaz, A. Avalos, M. de Torres, M.I. Escribano, A. González, A. Martín-Falquina, E. Pérez-Urria and C. Vicente, Environmental and Experimental Botany 26, 385 (1986).
[32] M. Sacristán, A.M. Millanes, C. Vicente and M.E. Legaz, J. Hattori Bot. Lab. **100**, 739 (2006).
[33] M. Marx and E. Peveling, Protoplasma **114**, 52 (1983).
[34]B. Fontaniella, A.M. Millanes, C. Vicente and M.E. Legaz, Plant Physiology and Biochemistry **42**, 73 (2004).