*Symbiosis*, 8 (1990) 75–85
Balaban, Philadelphia/Rehovot



# Comparison Between Eight Symbiotic, Cultured *Nostoc* Isolates and a Free-Living *Nostoc* by Recombinant DNA

I. LEIZEROVICH, N. KARDISH and M. GALUN
*Department of Botany, The George S. Wise Faculty of Life Sciences*
*Tel-Aviv University, Tel-Aviv 69978, Israel*
Tel. 03-5459163, Telex 342171 VERSY IL, BITNET EEEM@TAUNOS,
Fax 972-3-5413752

Received October 25, 1989; Accepted January 28, 1990

## Abstract

The *Nostoc* symbiont of the lichens: *Nephroma laevigatum*, *Peltigera horizontalis*, *P. polydactyla* var. *dolichorrhiza* and *Collema* sp.; the liverworts: *Anthoceros laevis* and *Blasia pusilla*; the gymnosperm: *Cycas revoluta*; the angiosperm: *Gunnera kaalensis* and a free-living *Nostoc* have been isolated and maintained in liquid culture. DNA extracted from these cultured *Nostoc* strains was digested with endonucleases (*EcoR*I and *Hind*III), blotted (Southern) and hybridized to $^{32}$P-labelled fragments of *nif*K, *nif*H and the 11 kb DNA sequence intervening the nitrogenase structural genes of *Anabaena* sp. PCC 7120. The hybridization patterns obtained revealed similarities and diversities, which are not in correlation with the phylogenetic status of the hosts. *C. revoluta* and *P. polydactyla* var. *dolichorrhiza* seem to contain two symbionts, which appear as single symbionts in *N. laevigatum* and *G. kaalensis* and in *P. horizontalis* and *Collema* sp. respectively.

The blott-hybridization patterns of *A. laevis* and *B. pusilla* symbionts differ from each other and all the other patterns.

Keywords: symbiosis, *Nostoc*, DNA isolation and restriction

0334-5114/90 /$03.00 

## 1. Introduction

The plant-cyanobacteria symbiotic associations known in nature are represented in all phyla of the plant kingdom. These include the liverworts *Anthoceros, Blasia, Cavicularia* and a few other genera; *Azolla* is the sole member of the ferns. The gymnosperms are represented by 10 cycad genera with about 150 species; *Gunnera*, a genus with about 40 species, is the only angiosperm in symbiotic association with cyanobacteria (Rai, 1990; Stewart et al., 1983). About 10% of the approximately 15, 000 lichen species known are associated with cyanobacteria (Tschermak-Woess, 1988).

The cyanobionts of these symbiotic systems belong to the genus *Nostoc*, except for *Anabaena* which is the cyanobiont of *Azolla* and some lichens which contain other heterocystous and non-heterocystous cyanobionts (Stewart et al., 1980; Franche and Cohen-Bazire, 1987; Werner, 1987). When integrated in the intact symbiosis the cyanobionts become substantially modified, often to the extent that their origin is completely obscured. Their identity and taxonomic relatedness can therefore not be ascertained from the *in-situ* symbiotic complex (Marton and Galun, 1976; Stewart et al., 1983; Kardish et al., 1989).

Also, the knowledge for reestablishment of these symbioses from their separated and/or cultured components, under controlled conditions, has not yet been acquired for many associations. This presents a significant problem in host-symbiont range determination.

Comparison and determination of genetic relatedness can now be carried out with great reliability by recombinant DNA techniques.

In this study we used DNA-restriction patterns, followed by Southern blot hybridization with probes from the nitrogenase structural genes of the free-living *Anabaena* sp PCC 7120 (Mazur et al., 1980) to perform a comparison analysis of 8 symbiotic, cultured *Nostoc* isolates and one free-living *Nostoc*.

## 2. Materials and Methods

*Nephroma laevigatum* Ach. was collected from bark of oak trees at Har Meron, Upper Galilee (Israel); *Peltigera horizontalis* (Huds.) Baumg. collected from Goblin Combe, Avon (England) and *P. polydactyla* var. *dolichorrhiza* Nyl. from Kipahulu Valley, Mauri (Hawaii), were obtained from R.P. Beckett; *Collema* sp. was collected from soil at the Upper Galilee; *Anthoceros laevis* (L.) Prosk. was collected from soil near Nahal Bezet, Western Galilee (Israel); *Blasia pusilla* L. was collected from wet sand on the shore of a small lake near Rehlingen, Saarland (Germany) and obtained from R. Mues; *Cycas revoluta* Thunb. was purchased from Kibbutz Horshim (Israel); *Gunnera kaalensis* (Krajina) St. John was collected form Mt. Kaala, Oahu (Hawaii) and obtained from E.M. Towata; *Nostoc* sp. was isolated from a free-living colony growing on soil at Har Meron, Upper Galilee.



*Isolation of the symbiotic* Nostocs

Plant fragments containing cyanobiont cells were washed for 1 hr with running tap water and then either plated on BG-11 medium (Stanier et al., 1971) solidified with 1.2% agar in 9 cm petri dishes, or inoculated on a medium consisting of fine silica gel powder mixed with a modified Bristol liquid nutrient solution (1:2 w/v), as described by Galun et al. (1972). *Nostoc* filaments which grew out from the inoculi were transferred to agarized BG-11 medium, as above. After further purification by repeated restreaking on the same medium, cultures were transferred to liquid BG-11 medium. All cultures were grown at 20–22°C and illuminated with white light at an intensity of 6.5 $J.m^{-2}\cdot sec^{-1}$ at a 16:8 hr light:dark cycle. Liquid cultures were shaken on a gyratory shaker (New Brunswick, N.J. USA), at 100 rpm.

*DNA extraction*

DNA extraction was performed according to Breiman et al. (1987) with minor modifications. *Nostoc* cells (1 g f.w.) were crushed to fine powder with pestle and mortar in liquid nitrogen in the presence of a small quantity of acid washed sand. The powder was mixed with 3.5 ml DNA isolation buffer (0.1 M NaCl; 0.1 M Tris-HCl, pH 8.5; 0.05 M EDTA; 2% SDS; 0.15 mg/ml proteinase K). The homogenate was extracted once with phenol-chloroform (1:1 v/v) and once with chloroform-isoamyl alcohol (24:1 v/v) and the aqueous phase ethanol precipitated in the presence of 0.2 M sodium chloride. The DNA was redissolved in 1.5 ml of TE buffer (0.01 M Tris-HCl, pH 8.0; 0.001 M EDTA) and incubated for 30 min at 37°C with 100 $\mu$g/ml RNAse A and 60 units/ml RNAse T1. The aqueous phase was reextracted with phenol-chloroform (1:1 v/v) and chloroform-isoamyl alcohol (24:1 v/v). The DNA was ethanol precipitated in the presence of 0.2 M ammonium acetate and the precipitate was vacuum-dried (Speed Vac concentrator, Instr. Inc. N.Y.) and resuspended in 200 $\mu$l TE buffer.

*Restriction endonuclease digestion*

Approximately 5 $\mu$g extracted DNA were digested with 10 units/$\mu$g DNA of *EcoRI* or *Hind*III (purchased from IBI, New Haven, CT) in the buffer recommended by the supplier. Digestion reaction was carried out at 37°C for 18 hr.

*Agarose gel electrophoresis and blotting*

The digested DNA was run on 15 cm 0.8% horizontal agarose gels. Electrophoresis was performed overnight at 40 volts in TBE buffer (0.089 M Tris; 0.089 M boric acid; 0.002 M EDTA, pH 8.0). Lambda phage fragments (IBI) were used as molecular size standards. After electrophoresis, the gel was photographed under long-wave UV light, depurinated 10 min in 0.25 M HCl, denatured in 0.2 M NaOH and 0.6 M NaCl for 1 hr and then neutralized in 0.6 M NaCl and 1 M Tris, pH 7.5 for 1 hr. The separated DNA fragments were transferred on nylon filters (GeneScreen Plus, NEN Research Products, Boston, MA) according to Southern (1975). The filters were baked for 2 hr at 80°C.

*Hybridization procedure*

The nylon filters were prehybridized for 6 hr in a plastic bag at 65°C, according to the manufacturers instructions. For hybridization a $^{32}P$ labelled probe and 100 $\mu$g/ml sheared salmon sperm DNA were injected into the plastic bag and incubated for at least 16 hr. The probes were removed according to the manufacturers instructions for reuse of the membranes.

*Recombinant DNA clone and $^{32}P$ labelling*

The following pAn plasmids were used as radiolabelled ($^{32}P$) probes: (a) pAn 154.3, containing *nif*H (1.8 kb); (b) pAn 207.8, containing *nif*K (0.8 kb); (c) pAn 207.3, containing an insert (1.8 kb) from the 11 kb DNA segment of *Anabaena* sp. PCC 7120 which is excised during heterocyst differentiation (Rice et al., 1982; Golden et al., 1985). Labelling was performed by a nick translation reagent kit (BRL-Bethesda Research Lab.) according to Rigby et al. (1977). The specific activity of the labelled DNA was $\sim 10^8$ cpm/$\mu$g DNA.

## 3. Results

The DNA of 8 cultured symbiotic *Nostoc* isolates and of one cultured free-living *Nostoc* were digested with *EcoR*I and *Hind*III, electrophorosed, blotted and hybridized with the labelled probes.

According to the hybridization patterns obtained with *nif*K and *nif*H on DNA digested with *EcoR*I and *Hind*III, the 9 *Nostoc* strains could be categorized into 3 groups (Figs. 1–4; Table 1), except that the *Nostoc* isolates of *A. laevis* and *B. pusilla* each showed a unique hybridization pattern with *nif*K on DNA digested with *Hind*III and *nif*H on DNA digested with both enzymes.



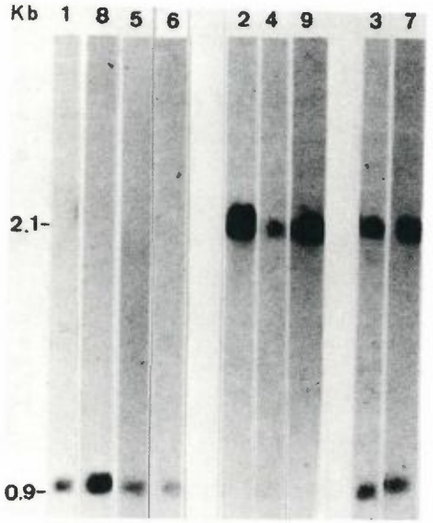


Figure 1. Hybridization of *nif*K to DNA from symbiotic, cultured *Nostoc*s and a cultured free-living *Nostoc*, digested with *EcoR*I. 1 = *Nephroma laevigatum*, 2 = *Peltigera horizontalis*, 3 = *P. polydactyla* var. *dolichorrhiza*, 4 = *Collema* sp., 5 = *Anthoceros laevis*, 6 = *Blasia pusilla*, 7 = *Cycas revoluta*, 8 = *Gunnera kaalensis*, 9 = free-living *Nostoc* sp.

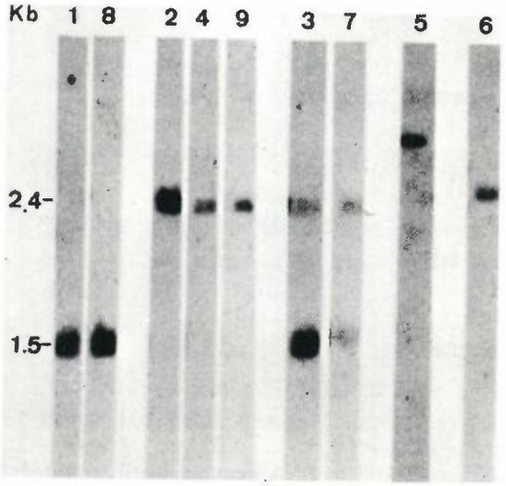


Figure 2. Hybridization of *nif*K to DNA from symbiotic, cultured *Nostoc*s and a cultured, free-livng *Nostoc*, digested with *Hind*III. Organisms as in Figure 1.

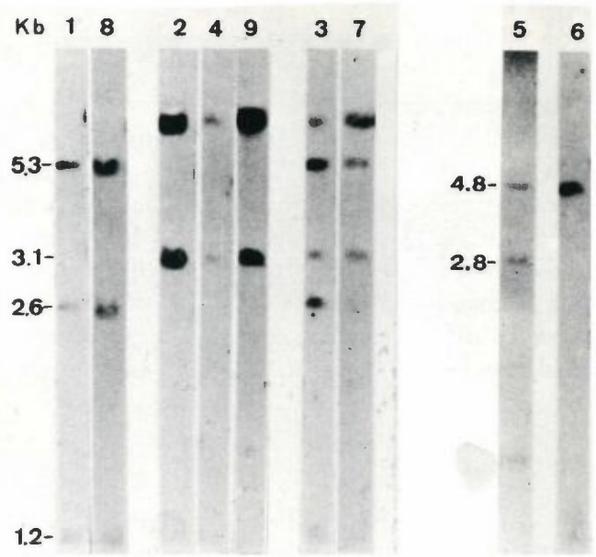


Figure 3. Hybridization of *nif*H to DNA from symbiotic, cultured *Nostocs* and a cultured, free-living *Nostoc*, digested with *Eco*RI. Organisms as in Figure 1.

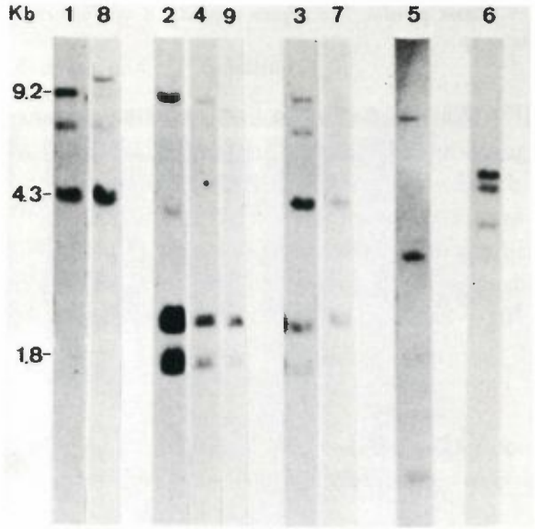


Figure 4. Hybridization of *nif*H to DNA from symbiotic, cultured *Nostocs* and a cultured, free-living *Nostoc*, digested with *Hind*III. Organisms as in Figure 1. (DNA of the isolate from *G. kaalensis* (8) was apparently partially cut, therefore 2 bands are seen near the 9.2 kb marker).



Table 1. Sizes of restriction fragments of *Nostoc* strains' DNA hybridizing to the *Anabaena* PCC 7120 *nif*K, *nif*H and pAn 207.3 probes

| | Probes | *nif*K | | *nif*H | | pAn 207.3 | |
|---|---|---|---|---|---|---|---|
| | Enzymes | *Eco*RI | *Hind*III | *Eco*RI | *Hind*III | *Eco*RI | *Hind*III |
| Group | Host species | | | | | | |
| I | *Nephroma laevigatum* (1) | 0.9 | 1.4 | 1,2; 2.6; 5.3 | 4.3; 8.9; 9.2 | 3.9 | — |
| | *Gunnera kaalensis* (8) | | | | | 5.6 | |
| II | *Peltigera horizontalis* (2) *Collema* sp. (4) free-living *Nostoc* sp. (9) | 2.1 | 2.4 | 3.1; 7.9 | 1.8; 2.1; 3.9; 9.2 | 8.9 | 2.5; 19.3 |
| III | *Peltigera polydactyla* var. *dolichorrhiza* (3) *Cycas revoluta* (7) | 0.9 + 2.1 | 1.5 + 2.4 | 1.2; 2.6; 5.3 + 3.1; 7.9 | 4.3; 8.9; 9.2 + 1.8; 2.1; 3.9 | 8.9 | 2.5; 19.3 |
| | *Anthoceros laevis* (5) | 0.9 | 3.6 | 1.8; 2.8; 4.8 | 1.5; 2.4; 9.0 | — | 4.3 |
| | *Blasia pusilla* (6) | 0.9 | 2.5 | 4.8 | 3.5; 5.5; 5.9 | 5.6 | 4.3 |

- The sizes of restriction endonuclease fragments are indicated in kilobases
- Numbers in parentheses correspond to the numbers in Figs. 1–6.

The *EcoRI* and *HindIII* restriction fragments hybridized to the *nifK* and *nifH* probes for DNA of *P. polydactyla* var. *dolichorrhiza* and *C. revoluta* (group III, Table 1), corresponded to those of both group I and group II.

When pAn 207.3 was used as probe one major band of 8.9 kb appeared in the patterns of the isolates from the organisms in group II and group III in the case of DNA digested with *EcoRI* (Fig. 5; Table 1). A weak band of 3.9 kb

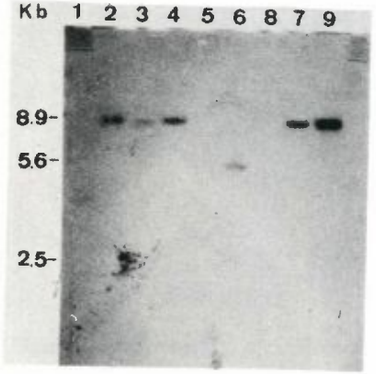


Figure 5. Hybridization of pAn 207.3 to DNA from symbiotic, cultured *Nostocs* and a cultured, free-living *Nostoc*, digested with *EcoRI*. Organisms as in Figure 1.

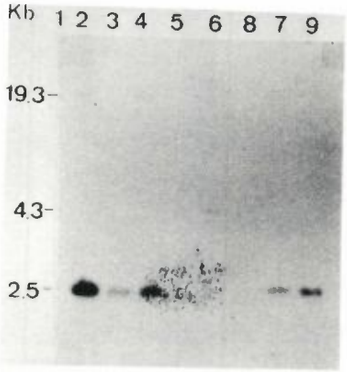


Figure 6. Hybridization of pAn 207.3 to DNA from symbiotic, cultured *Nostocs* and a cultured, free-living *Nostoc*, digested with *HindIII*. Organisms as in Figure 1.

was seen in the pattern of *N. laevigatum Nostoc*, a band of 5.6 kb appeared in this case in the isolate of *B. pusilla* (Fig. 5) and *G. kaalensis* (results not shown) and no bands could be detected in the *A. laevis* isolate. By *HindIII* digestion one major band of 2.5 kb and one minor band of 19.3 kb could be seen in groups II and III (Fig. 6; Table 1). The isolates of *A. laevis* and *B. pusilla* had one common band of 4.3 kb (Fig. 6). No radiolabelled bands could be detected in *Nostoc* of group I (*N. laevigatum* and *G. kaalensis*).

## 4. Discussion

The 9 *Nostoc* strains examined, of which 8 are symbiotic, cultured isolates and one a free-living strain, exhibit a rather great degree of diversity with respect to restriction sites in the *nif* gene region. There appears to be no correlation between the phylogenetic position of the host and the type of *Nostoc* symbiont. The two most unrelated organisms, the lichen *N. laevigatum* and the higher plant *G. kaalensis* host a *Nostoc* strain identical with respect to restriction sites in the region of the genes examined. On the other hand, *Anthoceros*



and *Blasia* which belong to the same taxonomic class (Hepaticae) each host a unique type of *Nostoc*, unrelated to any of the other examined *Nostocs*. The *Nostoc* of *P. horizontalis*, of *Collema* sp. and the free-living type exhibit the same hybridization patterns. The hybridization bands of the *P. polydactyla* var. *dolichorrhiza Nostoc* and that of *C. revoluta* are apparently, in all cases, the same and are in fact a combination of the patterns displayed by the symbionts of *N. laevigatum* and *G. kaalensis*, on one hand, and those of *P. horizontalis*, *Collema* sp. and the free-living *Nostoc*, on the other hand. It is possible that *P. polydactyla* var. *dolichorrhiza* and *C. revoluta* contain two symbiotic *Nostoc* strains. This assumption is in agreement regarding *C. revoluta*, with the recent suggestion by Lindblad et al. (1989) that more than one *Nostoc* strain can associate with the coralloid roots of a single cycad. In our case it seems that, except for *P. polydactyla* and the cycad, all the other organisms contain only one *Nostoc* type (see Figs. 1 and 2). The 4 lichen species examined are associated with 3 different *Nostoc* types. This is in full agreement with the polyphyletic origin of the lichens (Hawksworth, 1973).

The results obtained with the probe pAn 207.3 indicate that DNA was extracted from vegetative cells of the *Nostoc* isolates (Golden et al., 1985). The *Nostoc* strains examined here showed a relatively high degree of homology in this region, in contrast to the low degree of homology between the probe pAn 207.3 (located on the 11 kb segment excised in the course of heterocyst differentiation — Golden et al., 1985) and DNA of symbiotic and 7 free-living *Anabaena/Nostoc* strains (Franche and Cohen-Bazire, 1987).

Bonnett and Silvester (1981) and Enderlin and Meeks (1983) have reported successful reinfection of *Anthoceros* sp. and *Gunnear manicata* with each others symbionts and with *Nostoc* sp. isolated from cycads. This means that *Anthoceros* and *Gunnera* can form a symbiotic relationship with more than one type of *Nostoc*.

It has to be emphasized that differences in only one region of the genome is not sufficient to definitely determine the host-symbiont range of the symbiotic system examined. We cannot exclude the possibility that other potential symbionts exist. However, a great diversity among the *Nostoc* symbionts, which has no relation to the evolutionary status of the host, is obvious.

It is not known why there are relatively few plant-cyanobacteria associations. Thus it appears that they arised at different epochs.

## Acknowledgements

This work was financially supported by a grant from the National Council for Research and Development, Israel and the European Economic Community. The authors are greatly indebted to Ms. H. Lehr and Dr. P. Keller for skillful assistance. We are very grateful to Dr. R. Haselkorn for the gift of *nif* probes.

## REFERENCES

Bonnet, H.T. and Silvester, W.B. 1981. Specificity in the *Gunnera-Nostoc* endosymbiosis. *New Phytol.* **89:** 121–128.

Breiman, A., Rotem-Abarbanell, D., Karp, A., and Shaskin, H. 1987. Heritable somaclonal variation in wild barley (*Hordeum spontaneum*). *Theor. Appl. Genet.* **74:** 1021–1112.

Enderlin, C.S. and Meeks, J.C. 1983. Pure culture and reconstitution of the *Anthoceros-Nostoc* association. *Planta* **158:** 157–165.

Franche, C. and Cohen-Bazire, G. 1987. Evolutionary divergence in the nifH.D.K gene region among nine symbiotic *Anabaena azollae* and between *Anabaena azollae* and some free-living hetercystous cyanobacteria. *Symbiosis* **3:** 150–178.

Galun, M., Marton, K. and Behr, L. 1972. A method for the culture of lichen thalli under controlled conditions. *Arch. Microbiol.* **83:** 189–192.

Golden, J.W., Robinson, S.J., and Haselkorn, R. 1985. Rearrangement of nitrogen fixing genes during heterocyst differentiation in the cyanobacterium *Anabaena*. *Nature* **314:** 419–423.

Hawksworth, D.L. 1973. Some advances in the study of lichens since the time of E.M. Holmes. *Bot. J. Linnean Soc.* **67:** 3–31.

Kardish, N., Kessel, M., and Galun, M. 1989. Characterization of symbiotic and cultured *Nostoc* of the lichen *Nephroma laevigatum* Arch. *Symbiosis* **7:** 257–266.

Lindblad, P., Haselkorn, R., Bergman, B., and Nierzwicki-Bauer, S.A. 1989. Comparison of DNA restriction fragment length polymorphisms of *Nostoc* strains in and from cycads. *Arch. Microbiol.* **152:** 20–24.

Marton, K. and Galun, M. 1976. *In vitro* dissociation and reassociation of the symbionts of the lichen *Heppia echinulata*. *Protoplasma* **87:** 135–143.

Mazur, B.J., Rice, D., and Haselkorn, R. 1980. Identification of blue-green algal nitrogen-fixation genes by using heterologous DNA hybridization probes. *Proc. Natl. Acad. Sci. U.S.A.* **77:** 186–190.

Rai, A.N. 1990. *Handbook of Cyanobacterial Symbiosis.* A.N. Rai, ed. CRC Press, Inc., Boca Raton, FL (in press).

Rice, D., Mazur, B.J., and Haselkorn, R. 1982. Isolation and physical mapping of nitrogen fixing genes from the cyanobacterium *Anabaena* PCC 7120. *J. Biol. Chem.* **257:** 13157–13163.



Rigby, P.W.J., Dieckmann, M., Rhodes, C., and Berg, B. 1977. Labelling deoxyribonucleic acid to high specific activity by nick translation with DNA polymerase. I. *J. Mol. Biol.* **113:** 237–251.

Stewart, W.D.P., Rowell, P., and Rai, A.N. 1980. Symbiotic nitrogen-fixing cyanobacteria. In: *Nitrogen Fixation.* W.D.P. Stewart and J.R. Gallon, eds. Academic Press, New York, pp. 239–277.

Stewart, W.D.P., Rowell, P., and Rai, A.N. 1983. Cyanobacteria-eukaryotic plant symbioses. *Ann. Microbiol. (Inst. Pasteur)* **134B:** 205–228.

Tschermak-Woess, E. 1988. The algal partner. In: *Handbook of Lichenology.* Vol. 1. M. Galun, ed. CRC Press, Inc., Boca Raton, FL, pp. 39–92.

Werner, D. 1987. *Pflanzliche und mikrobielle Symbiosen.* Georg Thieme Verlag, Stuttgart-New York, 241 pp.