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Lectin from the Lichen *Nephroma laevigatum* Ach. Localization and Function

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Abstract

A lectin termed *Nephroma laevigatum* agglutinin (NLA) that was extracted from the thallus of the cyanolichen *Nephroma laevigatum* is immunologically cross-reactive with an extracellular substance produced by the isolated fungal symbiont (mycobiont) in culture. In the intact thallus NLA is located on the mycobiont's cell walls. The interaction of NLA with the native *Nostoc* of *N. laevigatum* (symbiotic and cultured), as well as its interaction with other *Nostoc* symbionts and free-living *Nostoc* spp. was examined. Akinetes (the single-cell stage) of the native *Nostoc* possessed the highest binding capacity. A possible role of NLA as a determinant of specificity at the initial stage of symbiont interaction, is discussed.

Keywords: lectin, lichen, *Nephroma laevigatum*, recognition, specificity, symbiosis

Abbreviations: DDW = double distilled water, DMSO = dimethylsulfoxide, DTT = dithiothreitol, FITC = fluorescein isothiocyanate, IIA = indirect immunoassay, NLA = *Nephroma laevigatum* agglutinin, PAGE = polyacrylamide gel electrophoresis, PBS = phosphate buffered saline, SDS = sodium dodecyl sulfate, TBS = tris-buffered saline, TEM = transmission electron microscopy, UHT = ultrahigh temperature

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1. Introduction

Lichens are regarded as a highly selective symbiosis. Circumstantial evidence in support of this postulate has been provided by Galun and Bubrick (1984). The potential partners for an initiation of the lichen symbiosis are fungal spores and green algal or cyanobacterial cells. Those are, in nature, interdispersed among a myriad of microorganisms. Yet, only compatible associations lead to the formation of a functional lichen. Consequently, the question arises, how do the compatible partners recognize and select each other out of this large and diverse population. This basic question of mutual recognition is obviously not restricted to lichens but is of much broader relevance to symbiotic associations.

Lectins and lectin-like molecules have been isolated from many organisms, but their physiological role is still far from being understood (Van Damme and Peumans, 1989). However, the involvement of those isolated from symbioses, as cell-cell mediating agents between partners of symbiotic associations, has been determined for a number of systems. Legume root lectins recognized by bacterial receptor molecules are important determinants of specific selection in the *Rhizobium*-legume symbiosis (Bohlool and Schmidt, 1974; Dazzo et al., 1988; Diaz et al., 1989).

Reisser (1989) recently suggested that specificity in the *Paramecium bursaria-Chlorella* sp. association "is based on a lectin-type recognition mechanism between carbohydrate algal cell wall surface groups and proteinous ciliate membrane components".

Lockhart et al. (1978), Petit (1982) and Bubrick (1984) found that lectins extracted from several *Peltigera* species (cyanolichens) bound to their respective, cultured *Nostoc* photobionts and suggested that such lectins may be involved in recognition and initial interaction between compatible lichen symbionts. Recently we have shown that a glycoprotein produced by the fungal partner (mycobiont) of the lichen *Xanthoria parietina* binds to the cultured photobiont of this lichen and is able to distinguish between potential photobionts and other photobionts or free-living algae (Bubrick et al., 1981, 1985a,b).

In view of these previous findings we investigated the possible role of a lectin isolated from the cyanolichen *Nephroma laevigatum*, during the initial stages of reassociation between separated symbionts. We demonstrate that the lectin is produced by the lichen thallus as well as by the cultured mycobiont and binds preferentially to akinetes (the single-cell stage) of the homologous cyanobiont.

2. Materials and Methods

Organisms

Nephroma laevigatum Ach. was collected from bark of oak trees at Har Meron, Upper Galillee, Israel. *Nostoc* spp. were isolates from *N. laevigatum*, *Blasia pusilla* L., *Gunnera kaalensis* (Krajian) St. John, *Collema* sp. and from a free-living colony. The origin of these organisms as well as isolation procedures and culture conditions were as described by Leizerovich et al. (1990). *Nostoc muscorum* 7119 was obtained from E. Tel-Or, Faculty of Agriculture, Hebrew University, Jerusalem and *Synechocystis* sp. PCC 6803 from M. Gurevitz, Dept. Botany Tel-Aviv University. *Trebouxia* was isolated from *Xanthoria parietina* and cultured as described by Bubrick and Galun (1980); *N. laevigatum* and *X. parietina* mycobiont spores were isolated according to Ahmadjian (1967) and germinated on agar-distilled water medium (1.8% w/v) except that 1% soil extract was added to the *N. laevigatum* medium. *Penicillium* sp. was from our stock cultures. Akinetes were prepared in modified AA/8 medium as described by Skill and Smith (1987).

Nephroma laevigatum *agglutinin*

The lectin, designated *Nephroma laevigatum* agglutinin (NLA) was isolated from the lichen *N. laevigatum* and purified to homogeneity by gel filtration and hydrophobic chromatography as will be published elsewhere. The lectin consists of two subunits of 52 kDa and 55 kDa and is specifically inhibited by N-acetylglucosamine. All the experiments described here were carried out with partially purified lectin, after the first gel filtration step.

Fluorescence labelling of NLA

NLA (0.5 mg in 1 ml of 0.2 M potassium borate buffer, pH 9.3) was mixed by vigorous vortexing with 0.2 mg of fluorescamine (Hoffmann LaRoche, Nutley, NJ) freshly dissolved in 0.1 ml of acetone. The solution was used within 3 hr.

Binding of labelled NLA to algae

Homologous and heterologous *Nostoc* spp. and *Synechocystis* sp. were washed twice with the growth medium (BG-11, Stanier et al., 1971) and three times with PBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 150 mM NaCl, pH 7.2). About 2×10^6 algal cells were incubated with 50 μl labelled lectin for 40 min in Eppendorf tubes (1.5 ml), at room temperature. The tubes were shaken on the platform head of a vortex at 120 strokes/min. Controls were prepared in

parallel: (1) using fluorescamine in borate buffer instead of the labelled lectin; (2) preincubation with non-labelled lectin prior to incubation with labelled lectin. The cells were then washed twice with 100 μl PBS and suspended in borate-buffered glycerol (glycerol-0.2 M borate buffer, pH 9.5, 9:1). The preparations were examined with a Zeiss fluorescence microscope (BG-12 exiter filter, no. 50 barrier filter) using epiillumination optics. A Kodacolor 400 ASA film was used for photography.

Absorption of NLA with algal cells

Algal cells were washed as above. Approximately 6×10^6 cells were pelleted in polypropylene centrifuge tubes (1.5 ml), the supernatant removed and replaced with 100 μl NLA solution (protein 0.5 mg/ml). Cells were resuspended and the mixture shaken on a vortex as above for 30 min at room temperature. Cells were then pelleted and the remaining NLA transferred to a second tube containing fresh cells. The procedure was repeated once more. Twenty μl of each of the remaining NLA solutions, were then loaded on an acrylamide gel for electrophoresis.

Electrophoresis and immunoblotting

Samples were solubilized in sample buffer (2.2% SDS, 11% glycerol, 0.05 M Tris-HCl, pH 6.8 and 5.5% DTT) and boiled for 3 min. Separation was carried out with a Mighty Small II, 7×8 cm Vertical Slab Unit (Hoeffer Scientific Instruments) by SDS-PAGE according to Laemmli (1970) with a 1.5 mm thick 10% polyacrylamide gel. The gels were either stained with silver (BioRad, Richmond, CA), according to the manufacturer's instructions or electrotransferred to nitrocellulose membranes (pore size 0.45 μm). Electrotransfer was performed according to the modification by Yahalom et al. (1991) to the method of Scewezyk and Kozloff (1985). Transfer was for 1 hr at 170 V in ethanolamine (7 mM)/glycine (5 mM) buffer, pH 9.5, containing 20% methanol using a Mighty Small blotter (Hoeffer Scientific Instruments). Following electrotransfer, the blots were blocked for 1 hr with 5% milk powder in TBS buffer (200 mM NaCl, 50 mM Tris-HCl, pH 7.4) containing 0.02% sodium azide, incubated overnight at room temperature with anti-NLA diluted 1:2000 in the blocking solution and shaken in a shaker bath (Gesellschaft f. Labortechnik m.b.H., D-3006 Burgwedel) at 100 strokes/min. After a short wash with TTBS (TBS containing 0.1% Tween 20) followed by one short wash with TBS and two washes of 10 min each with TBS, the blots were incubated for 1 hr with goat-anti-rabbit IgG coupled to alkaline phosphatase (Jackson Immuno Res.)

at a 1:4000 dilution in TBS. Following three washes with TBS for 10 min each, the reaction product was visualized by treatment with 3.3 mg/10 ml of p-nitro blue tetrazolium chloride and 1.7 mg/10 ml of 5-bromo-4 chloro-3 indol phosphate (both from US Biochemical Co.) in development buffer (100 mM Tris-HCl, 100 mM NaCl, 2 mM $MgCl_2$, pH 9.5).

Adherence of blood cells to germinating N. laevigatum *spores*

Germinating spores of *N. laevigatum* grown on agar plates (as described above) were flooded with a suspension of 3% rabbit erythrocytes in PBS and attraction and adherence to the fungal hyphae was examined at 15 min intervals. A TMY-400 film was used for photography.

Preparation of antiserum

The two closely migrating subunits of NLA (52 kDa and 55 kDa) were excised from the nitrocellulose membrane (Nilson and Larsson, 1990) and extracted with DMSO (Harlow and Lane, 1988). The samples used for injection contained 50–60 μg protein. Antiserum was raised in female approx. 3 month old rabbits according to the procedure described by Bubrick et al. (1981).

Protein assay

Determination of protein content was done according to Marder et al. (1986).

Antiserum assays

Anti-NLA was assayed by an indirect immunoassay (IIA) using FITC-labelled goat anti-rabbit IgG (Biomakor, Kiryat Weizmann, Rehovot, Israel) on: (a) germinating spores, (b) thallus sections.

(a) Five mm discs of agar containing germinated fungal spores were cut from agar plates and put into wells (15 mm × 10 mm) of polysterene, flat-bottomed tissue culture plates. The discs were blocked with UHT sterilized and homogenized, 1% fat milk for 1 hr at room temperature and then incubated overnight with anti-NLA at a 1:100 dilution in milk. Following two washes, with PBS for 10 min each, the discs were incubated for 1 hr with FITC-goat anti-rabbit IgG at a 1:30 dilution in PBS. After 3 washes with PBS for 10 min each, the discs were mounted on slides in borate buffered glycerol and examined with a Zeiss epifluorescence microscope. A Kodak Tri-X film was used for photography.

(b) Thallus sections, prepared for TEM observations (see below) were used for NLA localization in the lichen thallus by the IIA, as described for hyphae of the germinating spores and examined by epifluorescence microscopy as above.

NLA localization by electron microscopy

Thallus material for thin section electron microscopy was fixed in 3% glutaraldehyde, 3% paraformaldehyde, 2% acrolein and 0.5% DMSO in 0.01 M Na-cacodylate buffer supplemented with 5 mM $CaCl_2$, shaken for 2 hr on a rotary shaker and then washed with the same buffer and postfixed with 1% OsO_4 in the same buffer. The samples were then dehydrated in a graded ethanol series (50%, 70% and 100%) for 2 × 10 min each and 2 × 15 min in 100% ethanol and transferred to a mixture of ethanol and LR-White (hard grade, Biorad, Cambridge, MA) 3:1 for 2 hr, then overnight at a 1:1 ratio of this mixture followed by several transfers to pure LR-White. Finally the samples were placed in pure LR-White in beam capsules and polymerized for 20 hr. Thin sections (900–1000 Å) were prepared with a diamond knife (Diatome, Biel, Switzerland) and attached to collodion coated nickel grids (200 mesh, Polysciences, Warrington, PA). Grids with sections down were passed from drop to drop in a moist atmosphere. All solutions used were filtered through 0.45 μm filters (FP 030/2, Schleicher and Schuell, USA). Non specific binding was blocked for 15 min with a blocking solution containing 0.1% of each of the following: gelatine, glycine, BSA, Tween 20 and 0.1 M PBS. The grids were then incubated for 90 min with anti NLA diluted 1:100 and then rinsed 5 × 5 min in PBS. Excess solution was removed and the grids then incubated with secondary antibody — goat anti rabbit IgG conjugated to colloidal gold (Auroprobe-EM, GAR-G15, Janssen, Belgium) diluted 1:20 in the blocking solution for 30 min. The sections were then washed 2 × 5 min in PBS and then thoroughly washed in DDW and post stained with 2% uranyl acetate for 30–60 min. Micrographs were recorded on a Phillips EM-410 (Eindhoven, Holland).

3. Results

NLA was labelled with fluorescamine to visualize its reaction with homologous and heterologus cyanobacteria (Table 1). NLA bound to hormogonia of *N. laevigatum* grown in culture (Fig. 1a), whereas the corresponding symbiotic *Nostoc* did not react with the lectin (Fig. 1b). Cultured *Nostoc* isolated from *G. kaalensis* and from *Collema* sp. also reacted positively with NLA (Table 1)

Table 1. Binding of NLA to *Nostoc* spp. and *Synechocystis* sp.

Nostoc from	*NLA binding
Nephroma laevigatum-cultured	++
N. laevigatum-symbiotic	–
Gunnera kaalensis-cultured	++
Collema sp.-cultured	++
Blasia pusilla-cultured	+
free-living *Nostoc*-cultured	+
Nostoc muscorum 7119	–
Synechocystis sp	–

– = no binding
\+ = faint binding
++ = intense binding
* = estimated microscopically

and that from *B. pusilla* showed occasional faint staining (Fig. 1c). A faint reaction was observed also on hormogonia of the free-living *Nostoc*, (Fig. 1d). *N. muscorum* 7119 and *Synechocystis* sp. showed no reaction (Table 1).

Since NLA bound to the cultured homologous cyanobiont, we assumed that by incubation in NLA, these cells would absorb the active fraction of the lectin, which consequently would be missing on the electrophoresis gel, thus indirectly indicating interaction with the lectin. NLA was therefore extensively absorbed with the cultured cyanobiont and in parallel with a number of other cells as control.

Cells tested for binding were: Cultured *Trebouxia* sp. — isolated from *X. parietina* (Fig. 2, lane 4); cultured filaments of *Nephroma-Nostoc* (lane 5); freshly isolated (symbiotic) *Nephroma-Nostoc* (lane 6); and akinetes of cultured *Nephroma-Nostoc* (lane 7). As shown in Fig. 2 (lane 7) the two bands corresponding to NLA on the gel were indeed missing when akinetes were incubated in NLA. The cultured filaments of the cyanobiont (Fig. 2, lane 5) as well as the green photobiont-*Trebouxia* (Fig. 2, lane 4) had no visible effect on NLA. Lanes 4 and 5 were identical to lane 3, which shows NLA treated as if incubated with cells. Freshly isolated (symbiotic) *Nostoc* cells did not interact with NLA. Moreover, after incubation of the freshly isolated cyanobiont in the NLA preparation the bands corresponding to NLA were more pronounced on the gel (lane 6) and were more intensively stained even than in lane 2 (the untreated partially purified NLA). This can be explained by the fact that the preparation of freshly isolated cells is always accompanied by remnants of the

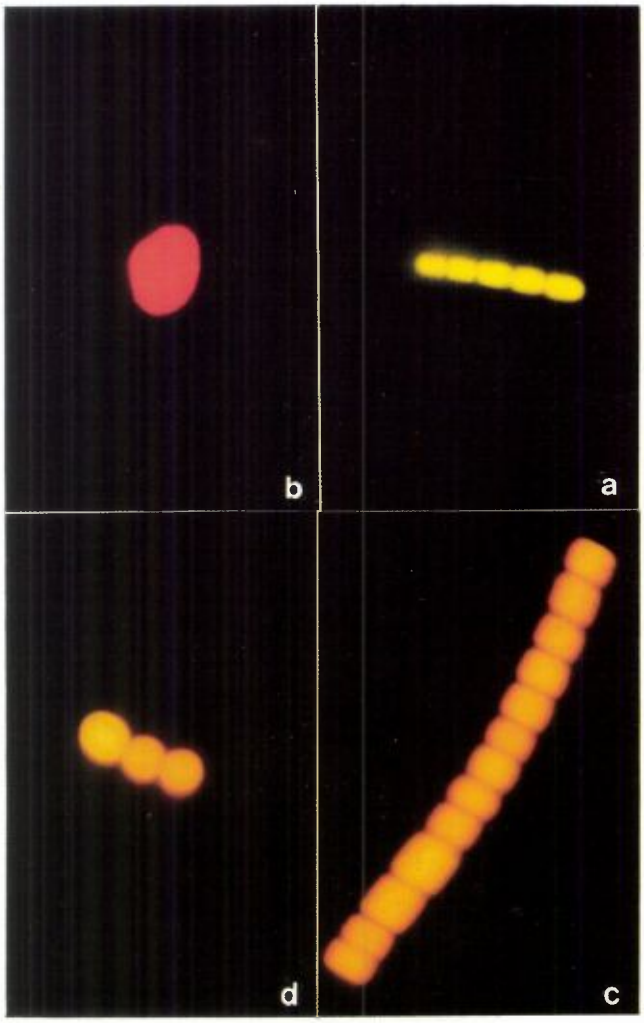

Figure 1. Fluorescamine-labelled-NLA staining of: (a) cultured *Nostoc* of *Nephroma laevigatum* – positive reaction; (b) symbiotic (freshly isolated) *Nostoc* of *N. laevigatum* – negative reaction; (c) cultured *Nostoc* of *Blasia pusilla* – faint reaction; (d) free-living *Nostoc* – faint reaction; (×4120) [yellow = positive reaction; red (autofluorescence) = negative reaction]

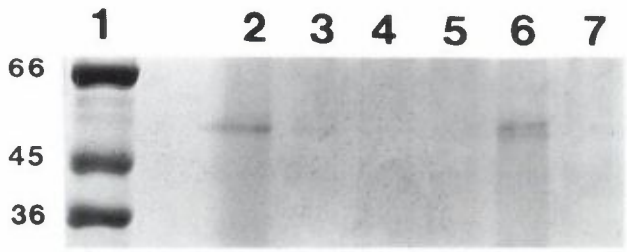

Figure 2. Silverstained SDS-polyacrylamide gel of partially purified NLA; lane 1 – molecular weight markers in kDa; lane 2 – partially purified NLA; lanes 4, 5, 6 and 7 show NLA preparations after incubating *Trebouxia* cells, cultured *N. laevigatum-Nostoc*; freshly isolated *N. laevigatum-Nostoc* and akinetes of cultured *N. laevigatum-Nostoc* respectively; lane 3 – NLA treated as if cells were incubated. Note that the bands of NLA are missing in lane 7.

mycobiont. As demonstrated below, NLA is located along the mycobiont's hyphae. This fraction, therefore, contains more lectin than the purified extract.

Further evidence for discriminative binding of NLA to akinetes of the homologous *Nostoc* is demonstrated in Fig. 3, showing a Western blot employing antibody to NLA labelled with secondary phosphatase conjugated antibody. Cultured filaments originating from *N. laevigatum*, *G. kaalensis* or *Collema* sp. (Fig. 3, lanes 2, 3 and 4 respectively) did not react with NLA, nor did akinetes of the two latter ones (Fig. 3, lanes 5 and 6). But, when akinetes of *N. laevigatum* were incubated in the NLA solution the band of NLA was missing on the Western blot (lane 7) indicating binding of NLA by these akinetes.

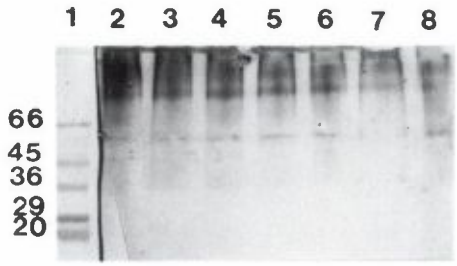

Figure 3. Western blot of a 10% SDS-polyacrylamide gel. Lane 1 – molecular weight markers in kDa; lanes 2, 3 and 4 show NLA that incubated cultured *Nostoc* filaments of *Nephroma laevigatum*, *Gunnera kaalensis* and *Collema* sp. respectively; lanes 5, 6 and 7 show NLA that incubated akinetes of *Nostoc* from *G. kaalensis Collema* sp. and *N. laevigatum* respectively; lane 8 – unabsorbed NLA (control). The NLA band of lane 7 is missing – indicating binding of NLA to the surface of *N. laevigatum* akinetes.

Since NLA reacted with the homologous cultured cyanobiont we expected NLA to be a product of the fungal partner as in *X. parietina* (Bubrick et al., 1981) and therefore used immunofluorescence for visualization. Figure 4 shows fluorescence along the hyphal walls of the mycobiont colonies when treated

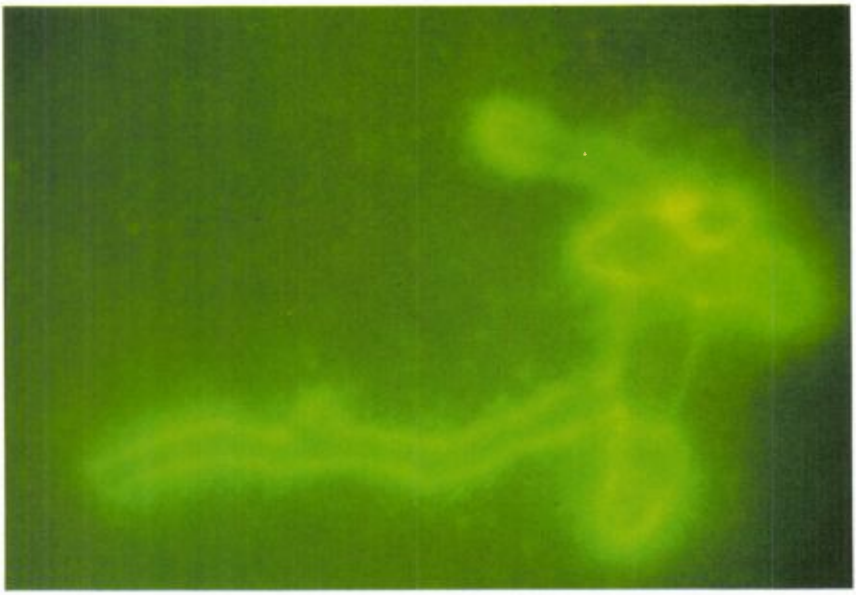

Figure 4. Cultured mycobiont colonies treated with rabbit anti-NLA-FITC-goat anti-rabbit IgG; ascospores negative; hyphae positive reaction, (×4120).

with anti-NLA and FITC goat anti-rabbit IgG. There was no reaction with the ascospore cell wall, but reaction was visible immediately with initiation of germination on both poles of the ascospores (not shown). Anti-NLA did not bind to the cell wall of *Penicillium* sp. nor to the germinating hyphae of the *X. parietina* mycobiont.

NLA agglutinates rabbit erythrocytes and the latter were used to assay NLA activity (unpublished). Adherence of rabbit erythrocytes, as shown in Fig. 5, further indicates that NLA is extracellularly located on the surface of the mycobiont-hyphae.

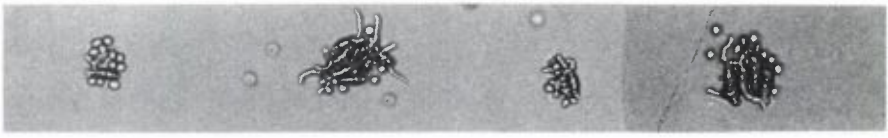

Figure 5. Rabbit erythrocytes adhering to the cultured mycobiont hyphae. (×480).

Immunofluorescence as well as immunogold labelling were performed for localizing the lectin in the thallus. Both the cortex and the medulla (Fig. 6a) were found to contain NLA, but the fungal "honey-comb-like" tissue, hosting the cyanobiont (see Figs. 1&2 in Kardish et al., 1989) seems to be the layer richest in NLA (Fig. 6b). Figure 6b shows a clear outline of gold labelling on the cell-walls of the cells constructing the fungal tissue around the cyanobiont cells.

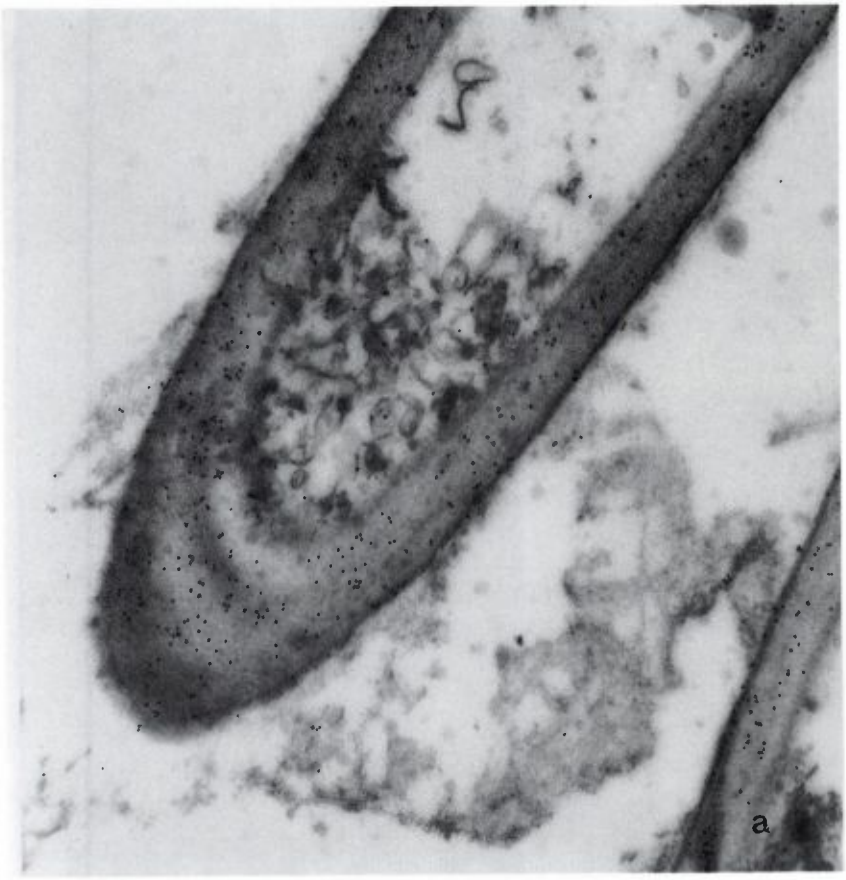

Figure 6. Immunogold localization of NLA in thallus sections: (a) immunogold-labelled NLA of medullarian hyphal cell walls; (b) immunogold-labelled NLA of the fungal tissue surrounding the cyanobiont cells; the gold particles are uniformly distributed in the fungal cell walls; (c) control-tissue treated with preimmune serum. (×14500).

(see next page for Figs. 6 (b) and (c))

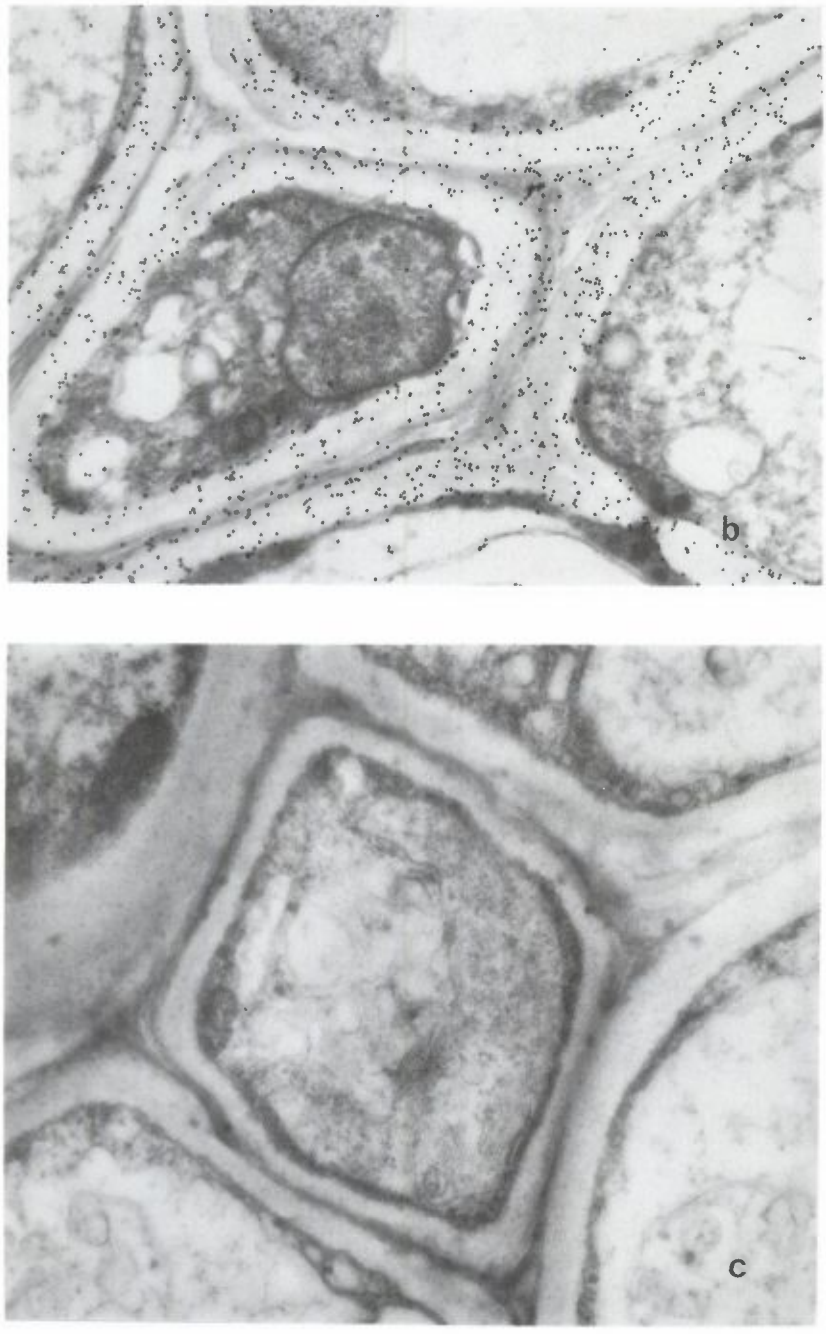

Figure 6 (b) and (c)

4. Discussion

The experimental evidence presented here, leads to the assumption that the lectin NLA, produced by the lichen *N. laevigatum* functions as a determinant of specificity at the initial stage of symbiont interaction. NLA is present on the surface of the germinated mycobiont spores, thus accessible to potential cyanobionts in the immediate environment. The lectin binds preferentially to akinetes of the homologous *Nostoc* (Figs. 2 and 3), strongly suggesting its capacity to distinguish between compatible and incompatible algal partners. Akinetes are the single-cell stage of *Nostoc* (as well as of other filamentous cyanobacteria). They may be considered as resting bodies of the cyanobiont in nature, and therefore the suitable form to be recognized by the fungal component via the extracellular lectin.

The number of binding sites seems to change during the life-cycle of the cyanobiont in culture. On hormogonia and mature filaments of the cultured cyanobiont, the number of binding sites suffice for a positive staining reaction with the fluorescamine-labelled lectin (Fig. 1), but appear insufficient to absorb substantial amounts (Figs. 2 and 3), necessary for interaction between the fungal lectin and algal surface receptors. Quantitative variability in binding sites on cells at different life-cycle stages have been observed also in plant-microbe interactions (Hardham and Suzaki, 1990).

Several of the *Nostoc* strains examined also share some common surface molecules with the homologous *N. laevigatum* cyanobiont, such as the *Nostoc* from the lichen *Collema* sp. and the angiosperm *G. kaalensis* (Table 1). Thus, the positive recognition between the partners apparently depends on quantitative as well as qualitative criteria. We like to emphasize here, as we did previously (Galun and Garty, 1988), that an initial positive interaction between the lichen symbionts does not guarantee lichenization upto a mature and functional lichen thallus, but is an essential event for further compatible development. The fungus continues to produce the lectin NLA in the intact thallus. We do not know if the lectin in the thallus and the lectin produced by the fungus in culture, are identical or slightly different variants of NLA, since no difference can be detected by polyclonal antibodies. By analogy to other symbiotic systems, antisera raised against pea-seed isolectin cross-reacted with non-seed (pea root and seedling cotyledons) isolectin, although different in amino acid composition (Diaz et al., 1990). Similarly, cross-reactivity with soybean-root lectin was the result when using antibodies raised against soybean-seed lectin with somewhat different biochemical traits (Vodkin and Raikhel, 1986).

The *Nostoc*-cyanobiont is positioned in a distinct layer in *N. laevigatum* (see Fig. 2 in Kardish et al., 1989) and is controlled by the mycobiont as in most

lichens with complex, stratified thalli (Honegger, 1988). A role of NLA in the control and regulatory processes of thallus formation and symbiont balance stability may be assumed, but requires further investigation.

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