Phyton (Horn, Austria)	**Vol. 40**	Fasc. 2	323–328	27. 12. 2000

An Improved Method for the Separation of Lichen Symbionts

By

Blanca FONTANIELLA*), María Carmen MOLINA**) and Carlos VICENTE *)

With 2 Figures

Received March 10, 2000

Key words: *Evernia prunastri*, *Lichenes*, photobionts, symbionts, isolation technique.

Summary

FONTANIELLA B., MOLINA M. C. & VICENTE C. 2000. An improved method for the separation of lichen symbionts. – Phyton (Horn, Austria) 40 (2): 323–328, 2 figures. – English with German summary.

A novel method for the isolation of lichen photobionts by density gradient centrifugation has been assessed using the lichen *Evernia prunastri* as an experimental model. An initial sucrose-KI gradient was prepared in which algae and small hyphal fragments formed an interphase in a sucrose-KI gradient. Then, 10 mM phosphate buffer is added and the preparation centrifuged a second time. This partitioned the algal cells towards the buffer while the bulk of the fungal hyphae were retained in the sucrose solution. This method allowed the purification of algal cells with no contamination from the fungal partner.

Zusammenfassung

FONTANIELLA B., MOLINA M. C. & VICENTE C. 2000. Eine verbesserte Methode für die Trennung von Flechten-Symbionten. – Phyton (Horn, Austria) 40 (2): 323–328, 2 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Eine neue Methode zur Isolation von Flechten-Photobionten durch Dichtegradient-Zentrifugation wurde an Hand von Experimenten mit *Evernia prunastri* geprüft. Ein Sucrose-KI-Gradient wurde hergestellt, in welchem Algen und kleine Hyphenfragmente eine Zwischenphase bilden. Dann wurde 10 mM Phosphatpuffer zugegeben und neuerliche zentrifugiert. Dadurch gelangten die Algenzellen in den

*) Dr. Blanca FONTANIELLA, Prof. Dr. Carlos VICENTE, Laboratory of Plant Physiology, The Lichen Team. Faculty of Biology, Complutense University, 28040 Madrid, Spain.

**) Dr. Maria Carmen MOLINA, Department of Plant Biology II, Faculty of Pharmacy, Complutense University, 28040 Madrid, Spain.

Puffer während die Masse der Pilzhyphen in der Sucrose-Lösung verblieb. Diese Methode erlaubt die Abtrennung von Algenzellen ohne Verunreinigungen vom Pilzpartner.

Introduction

The physical separation of lichen symbionts is necessary to analyze certain processes in this mutualistic relationship, such as the transport of carbohydrates from the phycobiont to the mycobiont (RICHARDSON & al. 1968), nitrogen fixation by cyanobionts and partition of the biosynthesized amino compounds (MILLBANK 1972), distribution of lichen enzymes between both symbionts (LEGAZ & VICENTE 1981), axenic culture of the bionts and lichen resynthesis (AHMADJIAN 1993), and the role of lichen lectins in the recognition of compatible phycobionts (GALUN 1991; MOLINA & al. 1993).

Algal bionts have been usually isolated by the micropipette method (AHMADJIAN 1973), a convenient procedure to achieve axenic cultures of many phycobionts but very hard and tedious for biochemical and physiological analysis for which a considerable mass of algal cells is required (LEGAZ & VICENTE 1981). Very small quantities of algal cells are also obtained by filtration of lichen homogenates through Sepharose 2G (PÉREZ & al. 1985). Axenic cultures of mycobionts have successfully been performed from spores obtained from mature fruit bodies (AHMADJIAN 1973) although it has been suggested that this procedure cannot be applied to many lichen species (YAMAMOTO 1991). Most of these methods have been reviewed by BUBRICK 1988 and BAČKOR & al. 1998.

Quantities of a lichen photobiont sufficient for physiological analyses can be routinely obtained by centrifugation of disrupted thalli in a homogeneous medium (DREW & SMITH 1967) or in a density gradient (RICHARDSON & al. 1968; KERSHAW & MILLBANK 1970, BAČKOR & al. 1998). The gradients most frequently used for this purpose are sucrose/phosphate (KERSHAW & MILLBANK 1970, MILLBANK 1972), sucrose/potassium iodide or sucrose/caesium chloride (ASCASO 1980). However, algal preparations obtained by this method are invariably contaminated by hyphae fragments retained in the sucrose solution.

This paper describes a modification of the density gradient centrifugation method for isolating lichen bionts, which results in improved homogeneity of the samples.

Material and Methods

Evernia prunastri (L.) ACH. growing on branches of *Quercus rotundifolia* WILLD., and collected from La Quinta (El Pardo, Madrid) was used through the study. Samples of dry thallus (0.5 g) were rinsed in distilled water to remove contamination. The sample was then macerated in a mortar with 10 ml distilled water. The homogenate was filtered through six layers of cheese-cloth and the filtrate centrifuged at $1{,}000 \times g$ for 10 min. The supernatant was discarded, the pellet was resuspended in 8.0 ml 0.25 M sucrose and then 4.0 ml of this suspension gently overlayed on the top

of 5.0 ml of 80 per cent (w/v) potassium iodide in a centrifuge tube which was centrifuged at $200 \times g$ for 45 s. Algal and hyphal fragments were found in a broad layer in the sucrose solution above the KI, whereas large fragments of non-disrupted thalli sedimented. The layer containing algal cells and hyphal fragments was recovered with a micropipette and placed on 5.0 ml KI solution.Then, 2.0 ml of 10 mM phosphate buffer were added and centrifuged at $800 \times g$ for 90 s. Algal cells formed an interphase between phosphate buffer and sucrose whereas small fragments of fungal hyphae were retained in the bulk of the sucrose solution. Large hyphal fragments were deposited at the bottom of the centrifuge tube as a pellet. The interphase containing algal cells was recovered with a micropipette, deposited on 5 ml KI and, then, 3.0 ml of phosphate were added and centrifuged at $1000 \times$ g for 3 min. This last step was repeated twice (Fig. 1).

The fungal fraction obtained as a pellet during the second centrifugation was also recovered and 80 per cent KI was added to a final volume of 4.0 ml. The mixture was strongly stirred and 4.0 ml 10 mM potassium phosphate buffer, pH 7.2, was added. The gradient was then centrifuged at $1000 \times g$ for 3 min. Small algal cells were recovered from the interphase between the buffer and KI solution whereas fungal hyphae sedimented at the bottom of the centrifuge tube. Algal contaminations were removed with a micropipette and added to the algal preparation. The protocol was employed twice, yielding a pure preparation of fungal cells (Fig. 1).

Results and Discussion

Algal and fungal preparations were examined by light microscopy. Algal preparations did not contain any fungal fragments (Fig. 2A). The integrity of the isolated cells was unaffected during the isolation process (Fig. 2C). The cell wall and the cytoplasm containing a large chloroplast appeared in perfect condition as well as some mother cells (Fig. 2D). Fungal cell preparations showed a complete absence of algal cells although they did contain amorphous aggregates probably consisting of broken cortex or substrate particles (Figs. 2B and D). Both fractions were twice washed with distilled water, completely dried at 80° C and weighted. Table I compared the mass recovery of alga and fungus using the present method with that using the method of ASCASO 1980.

The method proposed herein consists in the formation of a ternary gradient of phosphate buffer-sucrose-potassium iodide in contrast with previous published methods consisting of sucrose-KI (ASCASO 1980) or phosphate-sucrose (RICHARDSON & al., 1968). Separation in this system is based on the retention of algal cells in the interphase of the aqueous buffer and sucrose, whereas small segments of fungal hyphae were concentrated between sucrose and KI solutions. The largest hyphal fragments are recovered in the pellet. Algal cells are easily recovered employing this method and cleaned to remove contaminant hyphal fragments which can be achieved through two subsequent centrifugations in the same gradient, if necessary. This novel and easy method can be employed to perform routine separation of the phycobiont and the mycobiont from lichen thalli,

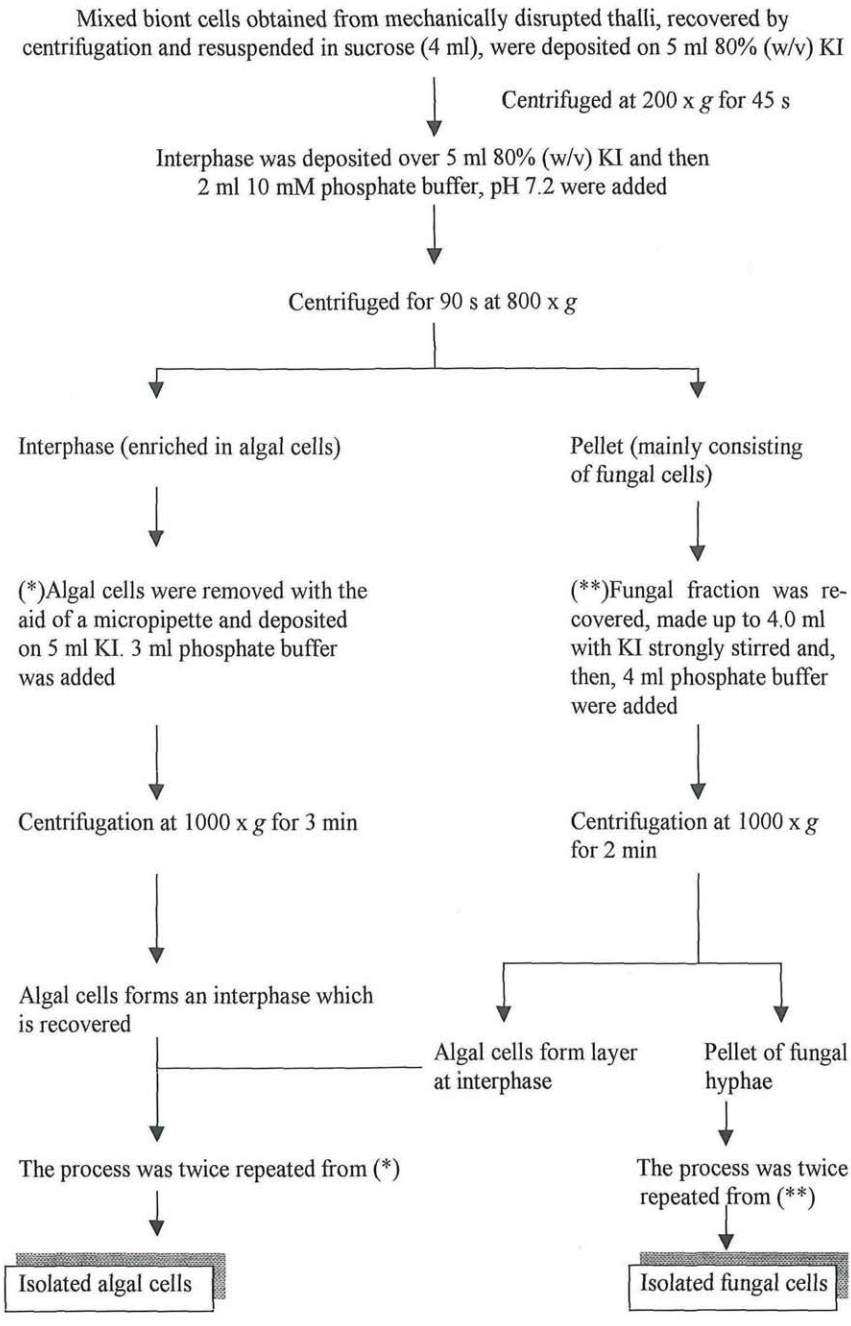

Fig. 1. Scheme for the isolation of lichen symbionts from *Evernia prunastri*.

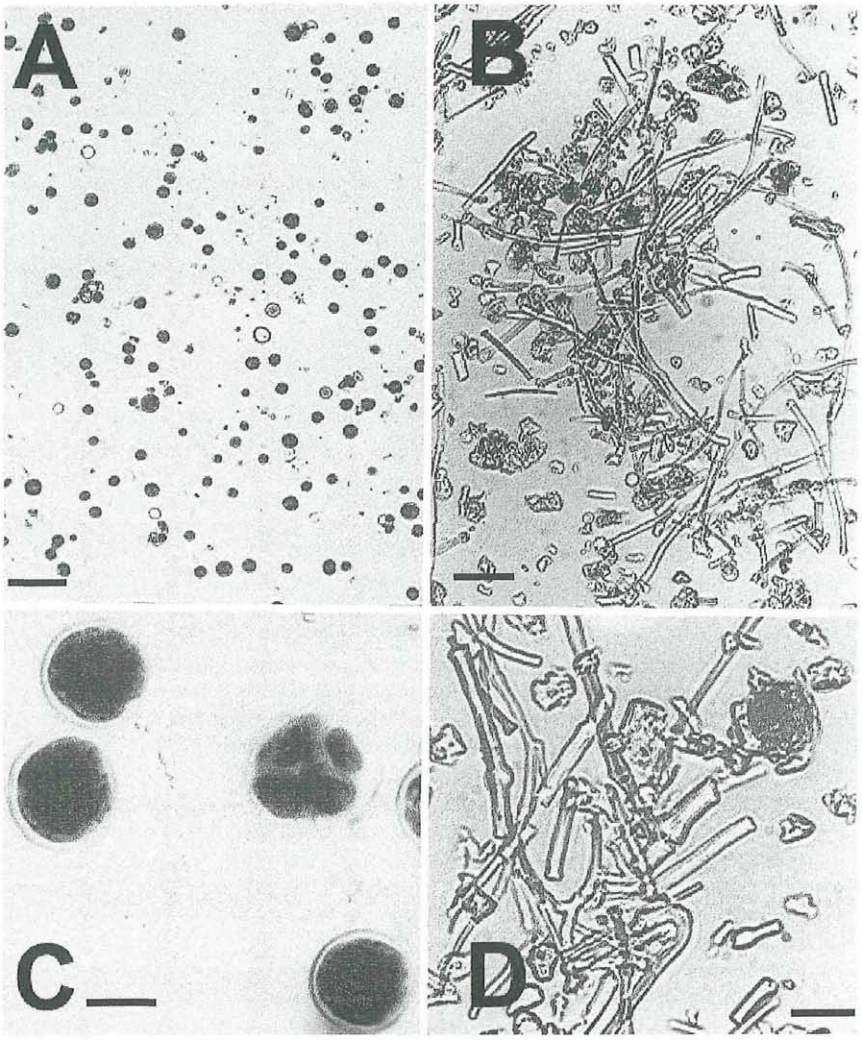

Fig. 2. Light micrographs of isolated phycobiont (A and C) and mycobiont (B and D) of *Evernia prunastri*. – Bar = 20 μm in A and B, and 5.0 μm in C and D.

Table I

Recovering of algal and fungal cells after symbiont isolation

Method	Amount of thallus (g)	Recovery of algal cells (mg g^{-1} thallus)	Recovery of fungal cells (mg g^{-1} thallus)
Ascaso 1980	2 g	50 mg wet weight	–
Proposed	0.5 g	7.1 ± 0.82 mg dry weight	85.4 ± 7.5 mg dry weight

yielding high homogeneous samples which can be employed in cellular or biochemical protocols.

In addition, the expression of yield as mg dry weight per g dry thallus is much more quantitative and realistic than the units mg fresh weight per g of thallus, as used by ASCASO 1980 and other authors.

Acknowledgements

We wish to thank Dr. P. CRITTENDEN for editing and Dr. M. V. MANZANARES for critical reading of the manuscript. This work was supported by a grant from the Dirección General de Investigación Científica y Tecnológica (Ministerio de Educación y Cultura, Madrid) PB 96 0662.

References

AHMADJIAN V. 1973. Methods of isolating and culturing lichen symbionts and thalli, pp. 653–659. – In: AHMADJIAN V. & HALE M. E. (Eds.), The Lichens. – Academic Press, New York.

— 1993. The lichen symbiosis. – John Wiley & Sons, Inc. New York.

ASCASO C. 1980. A rapid method for the quantitative isolation of green algae from lichens. – Ann. Bot. 45: 483.

BAČKOR M., HUDÁK J., Ziegler W. & BAČKOROVA M. 1998. Methods for isolation and cultivation of the eukaryotic lichen photobionts – looking for a general method. A review. – Thaiszia – J. Bot., Košice 8: 1–16.

BUBRICK P. 1988. Methods for cultivating lichens and isolated bionts, pp. 127–138. – In: GALUN M. (Ed.), Handbook of lichenology, 3. – CRC Press, Boca Raton.

DREW E. A. & SMITH D. C. 1967. Studies on physiology of lichens. VII. The physiology of the *Nostoc* symbiont of *Peltigera polydactyla* compared with cultured and free-living forms. – New Phytologist 66: 379–388.

GALUN M. 1990. Lichen research: an overview with some emphases, pp. 161–168. – In: NARDON P., GIANINAZZI-PEARSON V., GRENIÈRE A. M., MARGULIS L. & SMITH D. C. (Eds.), Endocytobiology. – INRA, Paris.

KERSHAW K. A. & MILLBANK J. W. 1970. Nitrogen metabolism in lichens. II. The partition of cephalodial-fixed nitrogen between the mycobionts and phycobionts of *Peltigera aphthosa*. – New Phytologist 68: 721–729.

LEGAZ M. E. & VICENTE C. 1981. Location of several enzymes of L-arginine catabolism in *Evernia prunastri* thallus. – Z. Naturforsch. 36c: 692–693.

MILLBANK J. W. 1972. Nitrogen metabolism in lichens. IV. The nitrogenase activity of the *Nostoc* phycobiont in *Peltigera canina*. – New Phytol. 71: 1–10.

MOLINA M. C., MUÑIZ, E. & VICENTE C. 1993. Enzymatic activities of algal-binding protein and its algal cell wall receptor in the lichen *Xanthoria parietina*. An approach to the parasitic basis of mutualism. – Plant Physiol. Biochem. 31: 131–142.

PÉREZ, M. J., VICENTE, C. & LEGAZ M. E. 1985. An improved method to isolate lichen algae by gel filtration. – Plant Cell Reports 4: 210–211.

RICHARDSON D. H. S., HILL D. J. & SMITH D. C. 1968. Lichen Physiology. XI. The role of the alga in determining the pattern of carbohydrate movements between lichen symbionts. – New Phytol. 67: 469–486.

YAMAMOTO Y. 1991. Production of lichen substances, pp. 58–71. – In: KOMAMINE A. (Ed.), Plant Cell Culture in Japan. – CMC Co., Ltd, Japan.