BIOCELL
2006, 30(3): 491-496

ISSN 0327 - 9545
PRINTED IN ARGENTINA

Brief Note

Cytochemical localization of acid phosphatase in *Stigeoclonium tenue* (Chaetophorales, Chlorophyceae)

KARINA M. MICHETTI[1,2], PATRICIA I. LEONARDI[1,3] AND EDUARDO J. CÁCERES[1,4]

1. Laboratorio de Ficología y Micología, Dpto. de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, Bahía Blanca, Argentina.
2. Fellow of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).
3. Researcher of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).
4. Researcher of Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, Argentina (CIC).

Key words: Chaetophorales, acid phosphatase, *Stigeoclonium tenue*, ultrastructure

ABSTRACT: Nonspecific acid phosphatases are a group of enzymes whose activity increases the availability of exogenous and endogenous orthophosphate either through extra- or intracellular hydrolysis of phosphate compounds. Our study demonstrates the activity of acid phosphatases in the filamentous freshwater alga *Stigeoclonium tenue*. These enzymes were detected following a cerium-based method in which cerium was used as an orthophosphate-capture reagent. In thalli from *S. tenue* from the natural environment, acid phosphatases were found in the longitudinal cell wall, plasmalemma, and vacuole. In thalli from Bold's Basal Medium culture, these enzymes were found mainly in the plasmalemma; they were scarce in the cell wall. In the thalli grown in phosphate-enriched culture medium, enzymes were found only in the plasmalemma. The low availability of orthophosphate in the medium seems to induce the transport of these enzymes to the cell wall. Its abundance, on the contrary, seems to attenuate this response without affecting the localization of acid phosphatases in the plasmalemma.

Introduction

Phosphorus (P) is one of the most important mineral nutrients required by algae for growth and development (Lee *et al*., 1999). Orthophosphate anion (P_i) is preferentially assimilated by organisms such as algae which obtain their mineral nutrients directly from the environment (Moraes, 1991).

Natural water contains different organic P compounds which degradation is low. Enzymatic hydrolysis is, therefore, the only mechanism by which biologically active orthophosphate is released from organic P (Gilbert *et al.,* 1999; Wasaki *et al*., 1999).

Acid phosphatases (APases, orthophosphoric-monoester phosphohydrolases) are a group of enzymes that catalyze the hydrolysis of a wide spectrum of orthophosphate monoesters and anhydrides with an optimum pH ranging between 5.0 and 6.0 to finally release phosphate ions (Duff *et al*., 1994; Gilbert *et al*., 1999; Ferreira

Address correspondence to: Dra. Karina M. Michetti. Laboratorio de Ficología y Micología, Dpto. de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur. San Juan 670, (8000) Bahía Blanca, ARGENTINA.
E-mail: michetti@uns.edu.ar
Received on February 13, 2006. Accepted on July 6, 2006.

et al., 2000). APases exhibit broad and overlapping substrate specificities (Gellatly *et al*., 1994). There are two different categories of plant APases that are differentiated from each other according to their relative substrate selectivities. One of such categories includes specialized enzymes such as 3-P glycerate (3-Pga) phosphatase and phosphoenolpiruvate (PEP) phosphatase which display a clear though non-absolute substrate specificity. The second category includes those truly nonspecific enzymes that show either low or no substrate specificity (Duff *et al*., 1994). These nonspecific enzymes are the focus of attention of the present research. APases are responsible for extra- and intracellular phosphate cleavage and they are apparently ubiquitous in nature, occurring in many animal tissues, plants, and microorganisms (Törnqvist, 1989; Duff *et al*., 1994; González *et al*., 1994; Ferreira *et al*., 2000). Intracellular forms occur in all plant tissues in vacuolar as well as cytoplasmic locations (Gilbert *et al*., 1999; Turner and Plaxton, 2001). APases might be of relevance mainly in habitats with low concentrations of inorganic phosphorus as they may enable cells to hydrolyze extracellular phosphate esters, increasing the amount of available orthophosphate (Beißner and Römer, 1999).

Ultrastructural localization of APases has been reported in plant tissues (Sexton and Hall, 1991) and, to a lesser extent, in algae (DuBois *et al*., 1984; Törnqvist, 1989; Tsekos and Schnepf, 1991; Yamaguchi and Anderson, 1994; Zhou and Fritz, 1994; Holland *et al*., 1997).

The genus *Stigeoclonium* Kütz includes attached, branched, uniseriate, filamentous green algae. The thallus is heterotrichous and is composed of a prostrate and an erect system of filaments. The terminal cells of the erect filaments may produce multicellular, hyaline hairs particularly under nutrient deficiency conditions (Whitton and Harding, 1978).

There have been few studies of APases localization on freshwater algae. Thus far, there have been no studies on filamentous algae. The aim of the current investigation, therefore, was to detect the occurrence and location of APases in a *Stigeoclonium* species which is very common in the periphyton community all over the world in a wide range of habitats in eu- and hypertrophic aquatic environments (de Vries, 1986) and to ascertain whether hyaline hairs were related with nutrient capture.

To our knowledge, this is the first report on the fine structural distribution of APase activity in the green alga *Stigeoclonium tenue* (C. Agardh) Kütz.

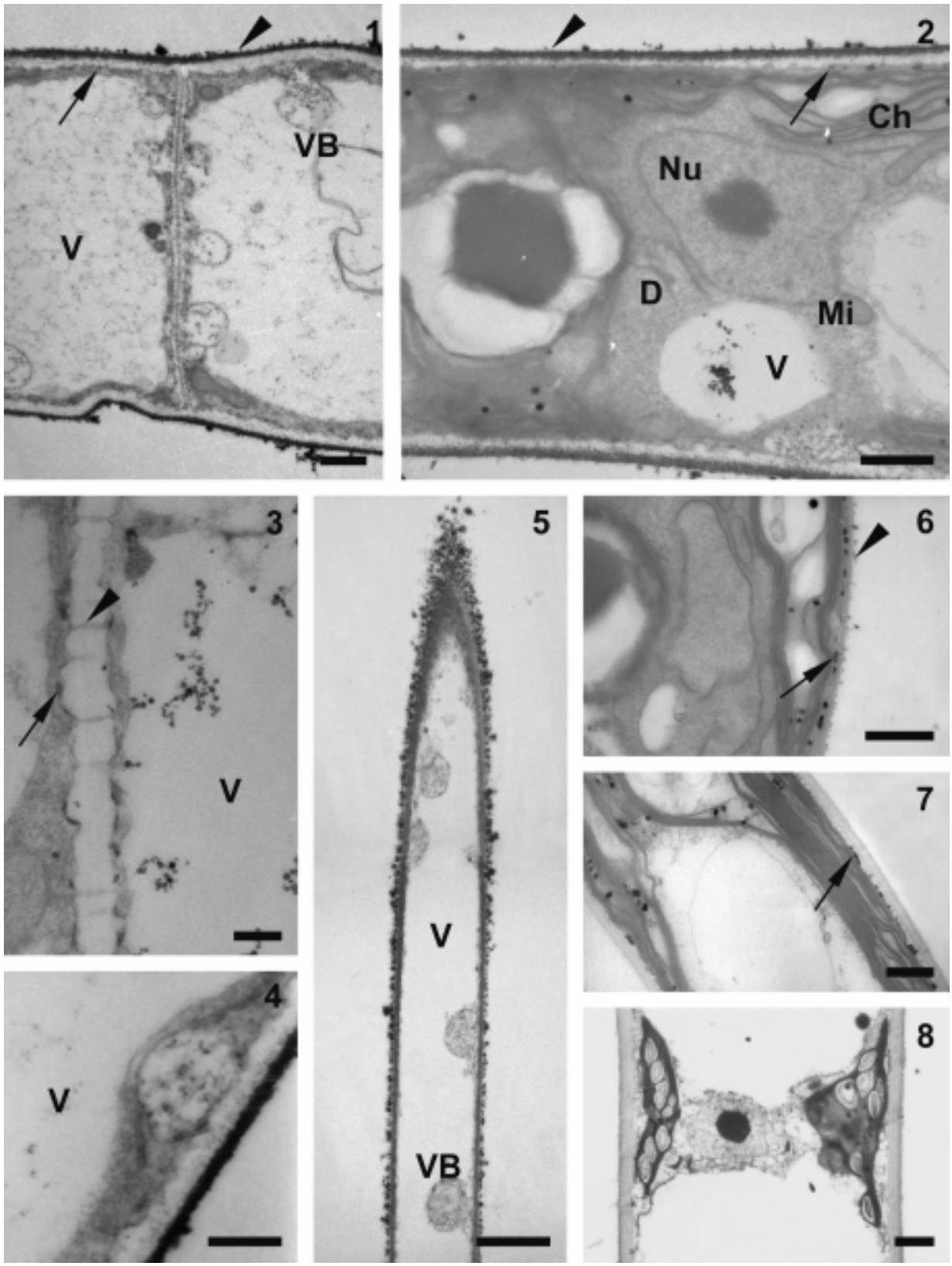
Material and Methods

The alga *Stigeoclonium tenue* was collected from Paso de las Piedras dam (Buenos Aires province, 38º 22' S, 62º 12' O). Cultures were grown in Bold's Basal Medium-BBM (Stein, 1973) with both normal phosphate content (7.5 g/l K_2HPO_4 and 17.5 g/l KH_2PO_4)

FIGURES 1-7. Ultrastructural localization of acid phosphatase in *Stigeoclonium tenue* cells. Figs. 1-5. Thalli from Paso de las Piedras dam.
Fig. 1. Overview of cells showing accumulation of reaction product in the longitudinal cell wall (arrowhead), plasmalemma (arrow) and vacuoles. Scale bar: 1 μm.
Fig. 2. Positive reaction in the longitudinal cell wall (arrowhead), plasmalemma (arrow) and vacuole. Note the absence of the reaction product in the chloroplast, dictyosomes, mitochondria, and nucleus. Scale bar: 1 μm.
Fig. 3. Portion of a cell showing precipitates in the plasmalemma (arrow) and in the lumen of the vacuole. Plasmodesmata shows no reaction product (arrowhead). Scale bar: 1 μm.
Fig. 4. Labeled cytoplasmic vesicle in contact with the plasmalemma. Scale bar: 0.5 μm.
Fig. 5. Hyaline hair. Positive reaction in the cell wall and intravacuolar vesicular bodies. Scale bar: 0.5 μm.
Fig. 6. Thalli in BBM. Precipitates in the plasmalemma (arrow) and cell wall (arrowhead). Scale bar: 1 μm.
Fig. 7. Thalli in P-plus BBM. Precipitates in the plasmalemma (arrow). Scale bar: 1 μm.

FIGURE 8. Control. Thalli incubated without substrate. Note the absence of reaction product. Scale bar: 1 μm.

Ch: chloroplast, D: dictyosomes, Mi: mitochondria, N: nucleus, V: vacuole, VB: vesicular body.


1
VB
V
2
Ch
Nu
D
Mi
V
3
V
4
V
5
V
VB
6
7
8

and containing a 3-fold increase in phosphates (22.5 g/l K_2HPO_4 and 52.5 g/l KH_2PO_4) at 10ºC in a 12-12 light-dark regime provided by cool-white fluorescent lamps. *S. tenue* was grown in P-plus BBM during 15 days. Nonspecific APase detection was carried out according to Robinson and Karnovsky (1983) using sodium-ß glycerophosphate as substrate and cerium as capture reagent. Portions of thalli were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4ºC for 1 h and then washed briefly in the same buffer four times at 4ºC. The material was incubated in medium containing 1mM sodium-ß-glycerophosphate (Sigma Chemical Co) and 2 mM $CeCl_3$ (Alfa Products) in 0.1 M acetate buffer (pH 5) and Triton X-100 0.0001%. Incubation was then carried out at 37ºC for 60 min with a medium change after 30 min, the medium was filtered through a 0.45 µm pore size Millipore filter just prior to use. Control specimens were incubated in a similar medium without substrate. After incubation, the material was washed twice in 0.1 M acetate buffer at room temperature and twice in 0.1 M cacodylate buffer at 4ºC. Samples were subsequently refixed in 3% glutaraldehyde in 0.1 M cacodylate buffer for 1 h at 4ºC and rinsed over night in the same buffer at 4ºC and postfixed in 2% OsO_4 in cacodylate buffer for 1 h at room temperature. Samples were dehydrated in a graded acetone series (50%, 70%, 90%, 96% and 100%), infiltrated and embedded in Spurr's (1969) resin. Samples were sectioned with a diamond knife, collected on Formvar-coated cooper grids and examined in a Jeol 100 CX-II electron microscope without further staining.

Results

The cytochemical experiments carried out for the localization of APases in vegetative *Stigeoclonium tenue* cells demonstrated a very fine electron-opaque reaction product which corresponds to cerium phosphate.

Thalli from Paso de las Piedras dam showed a high and uniform density of precipitates along the longitudinal cell wall (Figs. 1-2, arrowheads). The reaction product was irregularly distributed in the plasmalemma (Figs. 1-3, arrows) although no reaction product was observed in the plasmodesmata (Fig. 3, arrowhead). Labeled vesicles were observed in contact with the plasmalemma in the outer region of the cytoplasm (Fig. 4). Fine and coarse precipitates were found in the lumen of vacuoles and associated with vesicular bodies present within the vacuoles; the tonoplast showed no reaction product (Figs. 1-5). Likewise, no reaction product was observed in the dictyosomes, nuclei, mitochondria or chloroplasts (Fig. 2). Hyaline hairs showed a strong APase activity at the cell wall and associated with intravacuolar vesicular bodies next to the tonoplast (Fig. 5).

Thalli grown in BBM showed abundant precipitates in the plasmalemma (Fig. 6, arrow). Precipitates were scarce in the cell wall (Fig. 6, arrowhead). In P-plus BBM, filaments exhibited the reaction product only in the plasmalemma (Fig. 7). Controls from the natural medium as well as from BBM and enriched BBM showed no precipitates (Fig. 8).

Discussion

The method that is traditionally used for localization of APases employs lead as a capture agent of the orthophosphate released in the enzymatic site as an insoluble precipitate phosphate (Gomori, 1952). This method has been criticized by several researchers because it may yield false positive results, partially due to that some enzymes are sensitive to lead. However, it is still used to date for the histochemistry of plants and animals (Tsekos and Schnepf, 1991; Yamaguchi and Anderson, 1994; Weber *et al*., 2001; Vori√ek and Kalachová, 2003). On the other hand, it has been demonstrated that cerium-based methods are more specific (Robinson and Karnovsky, 1983). The latter have been widely used for ultrastructural localization of several phosphatases and ATPases in animal tissues while they have been used with minor frequency for localization of such enzymes in plant cells (Olmos and Hellín, 1997). The cerium-based localization method was found to be appropriate for the present research as a result of the apparent absence of nonspecific reaction product deposits.

Our study reports the first lines of evidence of activity of nonspecific APases in vegetative cells of *Stigeoclonium tenue*. *S. tenue* from its natural environment has been found to evidence an intense enzymatic activity in the longitudinal cell walls. This suggests that *S. tenue* is able to hydrolyze organic P compounds available in the environment on the cell surface to obtain the necessary orthophosphate.

A significant accumulation of reaction product was observed in hyaline hairs, particularly in the longitudinal walls and in the vesicular bodies next to the tonoplast. *S. tenue* does not form hairs under regular BBM culture conditions while it does under conditions of N and P deficiency in both culture and the natural environment (Abbas and Godward, 1963). In *S. tenue*, the

hairs formed under P deficiency are more numerous and longer than those formed under N deficiency (Vanaja *et al*., 1999). Several researchers agree that the hair formation mechanism involves a marked increase in the area-volume relation of thalli which contributes to increasing the capture efficiency of certain nutrients under limiting conditions (Tupa, 1974; Whitton and Harding, 1978; Francke, 1982; Gibson and Whitton, 1987a,b; Vanaja *et al*., 1999). Our results validate the role of hairs in the absorption of nutrients.

A positive correlation between alcaline phosphatase activity and hair formation has been observed in *Stigeoclonium*, *Chaetophora* Schrank, and *Draparnaldia* Bory (Gibson and Whitton, 1987a,b). Gibson and Whitton (1987a) observed, at light microscope level, alcaline phosphatase activity in hairs of *Chaetophora* and *Draparnaldia* and in basal filaments of *Stigeoclonium*, while at ultrastructural level they observed the product reaction in the cell wall of a hair of *Chaetophora*.

In filaments of *S. tenue* growing in BBM, low precipitates were registered in the cell wall while there were no precipitates in P-plus BBM. The regulatory action of orthophosphate has been observed in unicellular Chlorophyta, Euglenophyta, bacteria, fungi and higher plants (Price, 1962; Knutsen, 1968; Moraes, 1991; Lubián *et al*., 1992; Duff *et al*., 1994; Wasaki *et al*., 1999; Ferreira *et al*., 2000). Our results suggest, therefore, that in *S. tenue* either the synthesis or the activation of APases might be induced by P deficiency. Worthy of note is that phosphates concentration registered in the natural environment was much lower (ranging from 0.077 to 0.089 mg/l) than that under culture conditions which might explain the intense reaction observed in cell walls of thalli growing in its natural environment. Phosphatase activity has been used as an indicator of P deficiency in algal populations from freshwater habitats (Jansson *et al*., 1988).

APases were registered in the plasmalemma under the three above-mentioned assayed conditions, suggesting that, at this level, they are not affected by concentration of phosphates in the medium. In contrast, these enzymes may participate in the active transportation of nutrients as it has been demonstrated in some higher plants (Mogensen and Rossiter, 1982; Mogensen, 1985).

Taking into account the hydrolytic activity of vacuoles in any metabolic state of the cell, APases are expected to be found in these organelles in *S. tenue* in accordance with previous observations in other algal species (Noguchi, 1994; Zhou and Fritz, 1994; Yamaguchi and Anderson, 1994). In *S. tenue,* the precipitate was found mainly in the membranes of vesicular bodies. These results are consistent with observations in the unicellular green alga *Asteromonas gracilis* Artari (Swanson and Floyd, 1979). In higher plants, digestion processes of these membranes might be related to its subsequent label (Weber, 2002).

The presence of APases in cytoplasmic vesicles next to the plasmalemma suggests enzyme transport towards the cell wall where they will perform their hydrolytic function at the extracellular level. A similar effect was observed in the mycelium of the Ascomycete *Claviceps purpurea* Tul., in which the reaction product was accumulated in the cell wall (Vori√ek and Kalachová, 2003).

Under the three assayed experimental conditions, no precipitate was found in the cisterns of dictyosomes. The absence of precipitates in the dictyosomes of other organisms has been interpreted either as a different sensitivity of organelles to fixatives and substrates (Lin *et al*., 1977) or as an indicator of the metabolic state of the organism (Cooper *et al*., 1974).

In conclusion, APases in *S. tenue* might be -in accordance with observations in higher plants by Plaxton (1999)-, part of an extracellular system responsible for generating P_i from phosphate esters. Our study reveals the first lines of evidence regarding the localization of APases in *S. tenue*. It represent, therefore, the starting point for further studies about the dynamics of the activity of these enzymes at ultrastructural level in relation to the level of nutrients in the medium, interesting and useful information from an environmental point of view.

Acknowledgements

Funds have been provided by CONICET (*Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina*, Grants PEI 6024 to PIL and PIP 0949/98 to EJC), and by *Universidad Nacional del Sur* (Grants PGI SGCyT 1462/99 and 2119/00 to EJC). We are grateful to Dr. José María Guerrero who provided us with phosphate data from Paso de las Piedras dam.

References

Abbas A, Godward MBE (1963). Effects of experimental culture in *Stigeoclonium*. Br Phycol Bull. 2: 281-282.

Beißner L, Römer W (1999). Ermittlung kinetischer Parameter der sauren Phosphatasen intakter Zuckerrübenwurzeln bei variierter Phosphaternährung. J Plant Nutr Soil Sci. 162: 561-569.

Cooper RA, Bowen ID, Lloyd D (1974). The properties and subcellular localization of acid phosphatases in the colourless alga, *Polytomella caeca*. J Cell Sci. 15: 605-618.

de Vries PJR (1986). Bioassays on water quality using the attached filamentous alga *Stigeoclonium* Kütz. Free University Press, Amsterdam, pp 1-159.

DuBois JD, Roberts KR, Kapustka LA (1984). Polyphosphate body and acid phosphatase localization in *Nostoc* sp. Can J Microbiol. 30: 8-15.

Duff SMG, Sarath G, Plaxton WC (1994). The role of acid phosphatases in plant phosphorus metabolism. Physiologia Plantarum 90: 791-800.

Ferreira CV, Taga EM, Aoyama H (2000). Inhibition of acid phosphatase isoforms purified from mature soybean (*Glycine max*) seeds. J Enzyme Inhibition. 15: 403-410.

Francke JA (1982). Morphological plasticity and ecological range in three *Stigeoclonium* species (Chlorophyceae, Chaetophorales). Br Phycol J. 17: 117-133.

Gellatly KS, Moorhead GBG, Duff SMG, Lefebvre DD, Plaxton WC (1994). Purification and characterization of a potato tuber acid phosphatase having significant phosphotyrosine phosphatase activity. Plant Physiol. 106: 223-232.

Gibson MT, Whitton BA (1987a). Hairs, phosphatase activity and environmental chemistry in *Stigeoclonium, Chaetophora* and *Draparnaldia* (Chaetophorales). Br Phycol J. 22: 11-22.

Gibson MT, Whitton BA (1987b). Influence of phosphorus on morphology and physiology of freshwater *Chaetophora*, *Draparnaldia* and *Stigeoclonium* (Chaetophorales, Chlorophyta). Phycologia 26: 59-69.

Gilbert GA, Knight JD, Vance CP, Allan DL (1999). Acid phosphatase activity in phosphorus-deficient white lupin roots. Plant Cell and Environment 22: 801-810.

Gomori G (1952). Microsocopic Histochemistry: Principles and Practice. University of Chicago Press, Chicago, pp. 1-273.

González F, Fárez-Vidal ME, Arias JM, Montoya E (1994). Partial purification and biochemical properties of acid and alkaline phosphatases from *Myxococcus coralloides* D. J Appl Bacteriol 77: 567-573.

Holland RD, Pitt D, Moore MN, Brownlee C (1997). Characterization of the egg vesicular components in the seaweed, *Fucus serratus* L. (Fucales, Phaeophyta), using enzyme histochemistry and vital staining: the search for a lysosome-like body. Histochem J. 29: 239-248.

Jansson M, Olsson H, Pettersson K (1988). Phosphatases: origin, characteristics and function in lakes. Hydrobiologia 170: 157-175.

Knutsen G (1968). Repressed and derepressed synthesis of phosphatases during synchronous growth of *Chlorella pyrenoidosa.* Biochim Biophys Acta 161: 205-214.

Lee TM, Tsai CC, Shih MC (1999). Induction of phosphorus deficiency and phosphatase activity by salinity (NaCl) stress in *Gracilaria tenuistipitata* (Gigartinales, Rhodophyta). Phycologia 38: 428-433.

Lin J, Uwate WJ, Stallman V (1977). Ultrastructural localization of acid phosphatase in the pollen tube of *Prunus avium* L. (Sweet Cherry). Planta 135: 183-190.

Lubián LM, Blasco J, Establier R (1992). A comparative study of acid and alkaline phosphatase activities in several strains of *Nannochloris* (Chlorophyceae) and *Nannochloropsis* (Eustigmatophyceae). Br Phycol J. 27: 119-130.

Mogensen HL (1985). Ultracytochemical localization of plasma membrane-associated phosphatase activity in developing tobacco seeds. Amer J Bot. 72: 741-754.

Mogensen HL, Rossiter PP (1982). Biochemical determination of Mg^{++}, K^{+}-dependent diethylstilbestrol-inhibited phosphatase activity in the ovules of *Saintpaulia ionantha* before fertilization. Amer J Bot. 69: 1322-1325.

Moraes G (1991). Phosphate uptake by *Ankistrodesmus densus* under batch culture and phosphate limited conditions. Rev Brasil Biol. 51: 847-851.

Noguchi T (1994). Formation and decomposition of vacuoles in *Botryococcus* in relation to the trans-Golgi network. Protoplasma 180: 29-38.

Olmos E, Hellín E (1997). Cytochemical localization of ATPase plasma membrane and acid phosphatase by cerium-based method in a salt-adapted cell line of *Pisum sativum*. J Exp Bot. 48: 1529-1535.

Plaxton WC (1999). Metabolic aspects of phosphate starvation in plants. In: Phosphorus in plant biology: regulatory roles in molecular, cellular, organismic and ecosystem processes, J.P Lynch and J. Deikman, Eds. American Society of Plant Physiologists, Rockville, pp. 229-241.

Price CA (1962). Repression of acid phosphatase synthesis in *Euglena gracilis*. Science 135: 46.

Robinson JM, Karnovsky MJ (1983). Ultrastructural localization of several phosphatases with cerium. J Histochem Cytochem. 31: 1197-1208.

Sexton R, Hall JL (1991). Enzyme cytochemistry. In: Electron microscopy of plant cells. J.L. Hall and C. Hawes, Eds. Academic Press, London, pp. 105-180.

Spurr AM (1969). A low-viscosity epoxy embedding medium for electron microscopy. J Ultrastruct Res. 26: 31-43.

Stein JR (1973). Handbook of phycological methods. Culture methods and growth measurements. Cambridge University Press, London, pp. 1-448.

Swanson J, Floyd GL (1979). Acid phosphatase in *Asteromonas gracilis* (Chlorophyceae, Volvocales): a biochemical and cytochemical characterization. Phycologia 18: 362-368.

Törnqvist L (1989). Ultrastructural changes and altered localization of acid phosphatases in *Monoraphidium* and *Stichococcus* cells (Chlorophyceae) influenced by aluminum. Env Exp Bot. 29: 457-465.

Tsekos I, Schnepf E (1991). Acid phosphatase activity during spore differentiation of the red algae *Gigartina teedii* and *Chondria tenuissima*. Pl Syst Evol. 176: 35-51.

Tupa DD (1974). An investigation of certain Chaetophoralean algae. Nova Hedwigia. 46: 1-155.

Turner WL, Plaxton WC (2001). Purification and characterization of banana fruit acid phosphatase. Planta 214: 243-249.

Vanaja M, Charyulu NVN, Rao KVN (1999). Effect of cadmium toxicity and nitrogen and phosphorus deficiency on the growth and morphology of *Stigeoclonium tenue* Kütz. J Phytol Res. 12: 25-29.

Vori√ek J, Kalachová L (2003). Secretion of acid phosphatase in *Claviceps purpurea*-an ultracytochemical study. Folia Microbiol. 48: 767-770.

Wasaki J, Omura M, Ando M, Shinano T, Osaki M, Tadano T (1999). Secreting portion of acid phosphatase in roots of Lupin (*Lupinus albus* L.) and a key signal for the secretion form the roots. Soil Sci Plant Nutr. 45: 937-945.

Weber RWS, Wakley GE, Thines E, Talbot NJ (2001). The vacuole as central element of the lytic system and sink for lipid droplets in maturing appressoria of *Magnaporthe grisea*. Protoplasma 216: 101-112.

Weber RWS (2002). Vacuoles and the fungal lifestyle. Mycologist 16:10-20.

Whitton BA, Harding JPC (1978). Influence of nutrient deficiency on hair formation in *Stigeoclonium*. Br Phycol J. 13: 65-68.

Yamaguchi T, Anderson R (1994). Fine structure of laboratory cultured *Distigma proteus* and cytochemical localization of acid phosphatase. J Morphol. 219: 89-99.

Zhou J, Fritz L (1994) The pas/accumulation bodies in *Prorocentrun lima* and *Prorocentrum maculosum* (Dinophyceae) are dinoflagellate lysosomes. J Phycol. 30: 39-44.