

Autophagy Induced by Heavy Metal and Starvation Stress in Microalgae

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ABSTRACT

Several genera and species of unicellular algae proved to be resistant, at different level of effectiveness, to heavy metals pollution. Some cells may arrest the metals at the wall level, by excluding the entrance into the cytoplasm; others may concentrate and precipitate the metal in a vacuole. Besides these methods, some cells appear to increase their cellular turnover by increasing autophagic activity in order to replace organelles that get damaged. Some preliminary results in *Dictyosphaerium* sp. may even suggest that some members of the population accumulate faster the heavy metals than the others, eventually dying and thus subtracting part of the metal from the medium. If confirmed, this activity would represent a Programmed Cell Death (PCD) case, representing the sacrifice of some cells in unicellular organisms in order to accomplish a specific task of general interest for the population. PCD, in this case, would be a method to reduce the metal availability in the medium by precipitating it in a vacuole of a dead cell.

A survey of the ultrastructural morphology of effects of heavy metal-induced stress on cell organelles in unicellular algae is presented. The observed autophagic feature involved both accumulation of metal in the vacuole and formation of multilamellar bodies in consequence of organelle recycling. These two aspects of vacuolar response appear to be two different responses to toxic metal excess in the medium.

The capability of some strains of species of unicellular or colonial algae to resist to high concentration of heavy metals in the environment is noteworthy, since the mechanism of resistance may provide clue about how to modify other strains known for production of useful compounds. Modified strains of species of industrial relevance could be grown even in polluted cultures and/or suggest methods to remove heavy metals from polluted water (phytoremediation).

The starvation-induced autophagy in *Heterosigma* was preliminary to a type of PCD showing nuclear fragmentation. This specific feature is more typical of animals than algae and is probably related to the isolated phylogenetic position of *Heterosigma* with respect to the other microalgae here investigated, rather than to the type of PCD inducer.

Key words: autophagy, green algae, heavy metals, programmed cell death, stress, ultrastructure

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Introduction

Various genera and species of microalgae and particularly of unicellular green algae have proved to be resistant, at different levels, to heavy metals pollution (Kumar et al., 2015). For instance, species of genera *Chlorella* (Kumar et al., 2015), *Dictyosphaerium* (Pereira et al., 2014) and *Micrasterias* (Lütz-Meindl, 2016) showed different level of resistance, depending on the strain and populations. Interestingly, even the aluminium accumulation in *Hydra* L. (Hydrozoa) in symbiosis with green algae, showed that aluminium depositions were concentrated in the tissue-the gastrodermal cellular layer- containing the symbiotic alga, the latter also occasionally containing the metal, suggesting that

also *Hydra* capability to resist to heavy metal may be related to the symbiosis itself (Kovacevic et al., 2006, 2009).

The presence of heavy metals in water can be faced by these organisms in very different ways with respect to the metal involved and to the strategy carried out by the cell itself. Some cells may arrest the metals at the wall level by excluding the entrance into the cytoplasm; others may concentrate and precipitate the metal in a vacuole (Lütz-Meindl, 2016), also in angiosperms growing hydroponically (Colzi et al., 2015).

Autophagy is a very general phenomenon in eukaryotes: eukaryotic cells dismantle part of themselves

by segregating these parts in spaces enclosed by membranes and by lysing the content of this so-formed space by enzymes. The main known ways of performing autophagy are the following (van Doorn & Papini, 2013; Klionsky et al., 2016). 1) Chaperon-mediated autophagy, where a protein is targeted to another protein acting as chaperone; this last will eventually lead the transport of the so formed complex to lysosomes. This type of autophagy is known in mammalian cells. 2) Microautophagy, the uptake of cellular components by a double lysosome/vacuole membrane that surrounds a portion of the cytoplasm. The result is a portion of the cytoplasm surrounded by a piece of lysosomal/vacuolar membrane, inside the lysosome/vacuole. 3) Macroautophagy, which occurs through a portion of cytoplasm sequestering in a double-membrane-bound organelle. Firstly described in yeast, similar processes were also found in photosynthetic eukaryotes, or more exactly in plants, performed by autolysosomes (Papini et al., 2014). These last organelles are different from the autophagosomes of yeasts and animal cells, since in plants these organelles contain hydrolases from the onset of their formation. Usually in plants, also mega-autophagy or mega-autolysis may occur, consisting in the extensive degradation of the cell cytoplasm at the end of one type of programmed cell death (PCD).

In plants also an apparently specific autophagic activity by plastids, still to be further investigated has been described (Papini et al., 2014). Sometimes plastids appear to engulf even organelles as peroxisomes (Papini & van Doorn, 2015).

Autophagy is a process considered specific of eukaryotes, due to the need of independent membranes performing specific tasks. An open question is hence related to the evolution of autophagy and if a similar process may be present in prokaryotes, where some cytoplasm component recycling is however an important task of the cell as shown for instance by Hayek and Walsby (1984) in cyanobacteria.

This article deals with the question about how the presence of stress, such as excess of heavy metals or starvation can induce autophagy in some microalgae and how autophagy may assume different forms in microalgae that are not phylogenetically closely related.

All these processes must be studied using Transmission Electron Microscope, since they are related to complex disposing and movements of

membranes and hence require specific techniques to reduce membranes damage (Papini, 2018).

Some cases of the ultrastructural morphology of effects of heavy metal-induced stress on cell organelles in unicellular green algae are here presented together with the effect of starvation in *Heterosigma hakashiwo*, a species phylogenetically belonging to heterokonts.

Material and Methods

The algal strains were maintained as follows: *Dictyosphaerium* sp. (green algae) and a strain of *Arthrospira platensis* (Cyanobacteria) in Zarrouk medium with natural illumination in 25 ml vials, exposed to 45 µg/l of Cr(III), as CrCl₃·6H₂O; *Chlorella vulgaris* in SAG medium (<http://sagdb.uni-goettingen.de/>) with a supplement of 20 µg Zn/l (as ZnCl₂); *Heterosigma hakashiwo* (Raphidophyceae) in natural sterile seawater (Mediterranean Sea water) amended with nutrients, metals and vitamins, according to the f/2 medium concentrations after Guillard (1975), at 19 °C and under a 12:12 light-dark period at about 25 µE m⁻² sec⁻¹ by cool white lamps. *H. hakashiwo* was collected after 2 months of growth in the same medium.

Algal cells in growth medium (15 ml) were harvested, centrifuged and fixed overnight in 1.25 % glutaraldehyde at 4° C in 0.1 M phosphate buffer (pH 6.8), then post-fixed in 1% OsO₄ in the same buffer for 1 hr. After dehydration in an ethanol series and a propylene oxide 100% last step, the samples were embedded in Spurr's epoxy resin. At each step the cells were centrifuged at 1500 g for 5 minutes and only the pellet was used for the following step, in order to safely substitute various solvents with micropipettes without losing cells.

Transverse sections approximately 80 nm thick were cut with a diamond knife and a Reichert-Jung ULTRACUT ultramicrotome. The sections were then stained with uranyl acetate, lead citrate, and then examined with a Philips EM300 TEM at 80 kV. The images on *Chlorella* were recorded with a Talos L120C by FEI (Eindhoven, The Netherlands). About five Cryostat sections 20 µm thick (Cryo-Cut American Optical Corporation) were stained with toluidine blue and with Lugol's solution and observed with a Leica microscope DM RB Fluo with immersion oil.

Results and Discussion

Even if the presence of autophagy is considered specific of eukaryotes, prokaryotes are also able to recycle proteins and cellular components, see for instance Hayes

and Walsby (1984). Even if by definition prokaryotes should not own membranes autonomous from the plasma membrane, at least in *Arthrospira platensis* (cyanobacteria) some cytoplasm portions containing granular material and surrounded by membranes are present (Fig. 1). If the membranes surrounding this space are autonomous from the plasma membrane or not is still to be ascertained, also because in cyanobacteria the cytoplasm contains a very high amount of membranes, mainly thylakoids (Fig. 1), and hence it is difficult to distinguish the end of a membrane layer from another, even with serial 3D reconstruction.

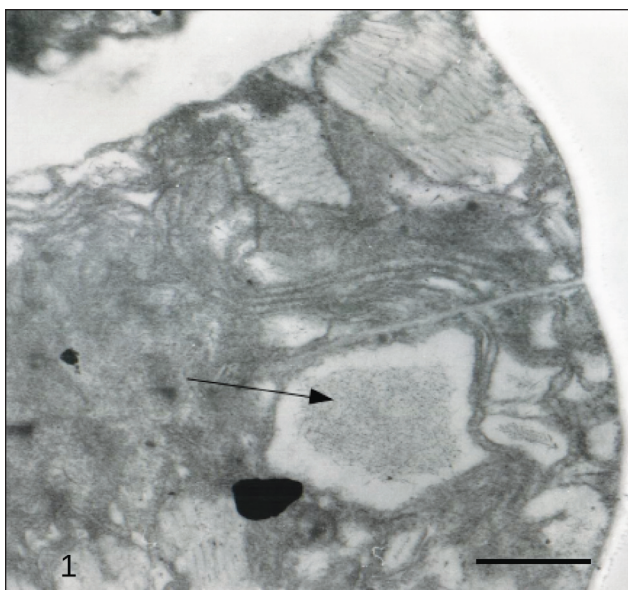


Figure 1. *Arthrospira platensis*. The arrow indicates an area surrounded by membranes containing granular material in apparent degeneration. Bar = 250 nm.

In microalgae, specific features of autophagy are considered related to stress or to other more specific morpho-physiological constraints, such as the presence of a cell wall and of a huge vacuole in green algae and land plants. The observed differences in autophagic features may be instead related to the phylogenetic distance, which is particularly high between the main three groups of eukaryotic algae (Fig. 2). Most studies, however, are concentrated on green algae, for instance the model organisms *Chlamydomonas* and *Micrasterias*, besides land plants.

In *Dictyosphaerium* sp., in condition of high concentration of Chromium III in the medium, some large inclusions enclosed by membranes were observed to contain cytoplasm residual and even organelles remnants (Fig. 3). Some cells appeared to be more affected than

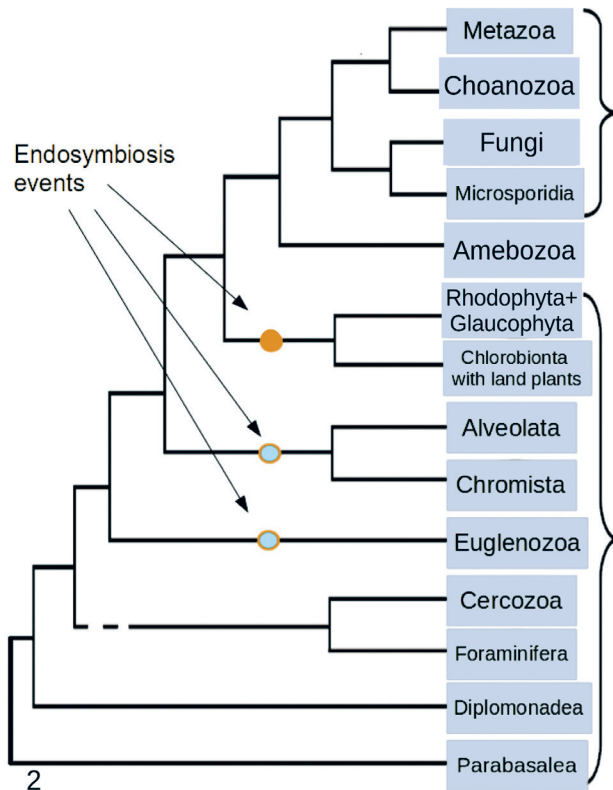
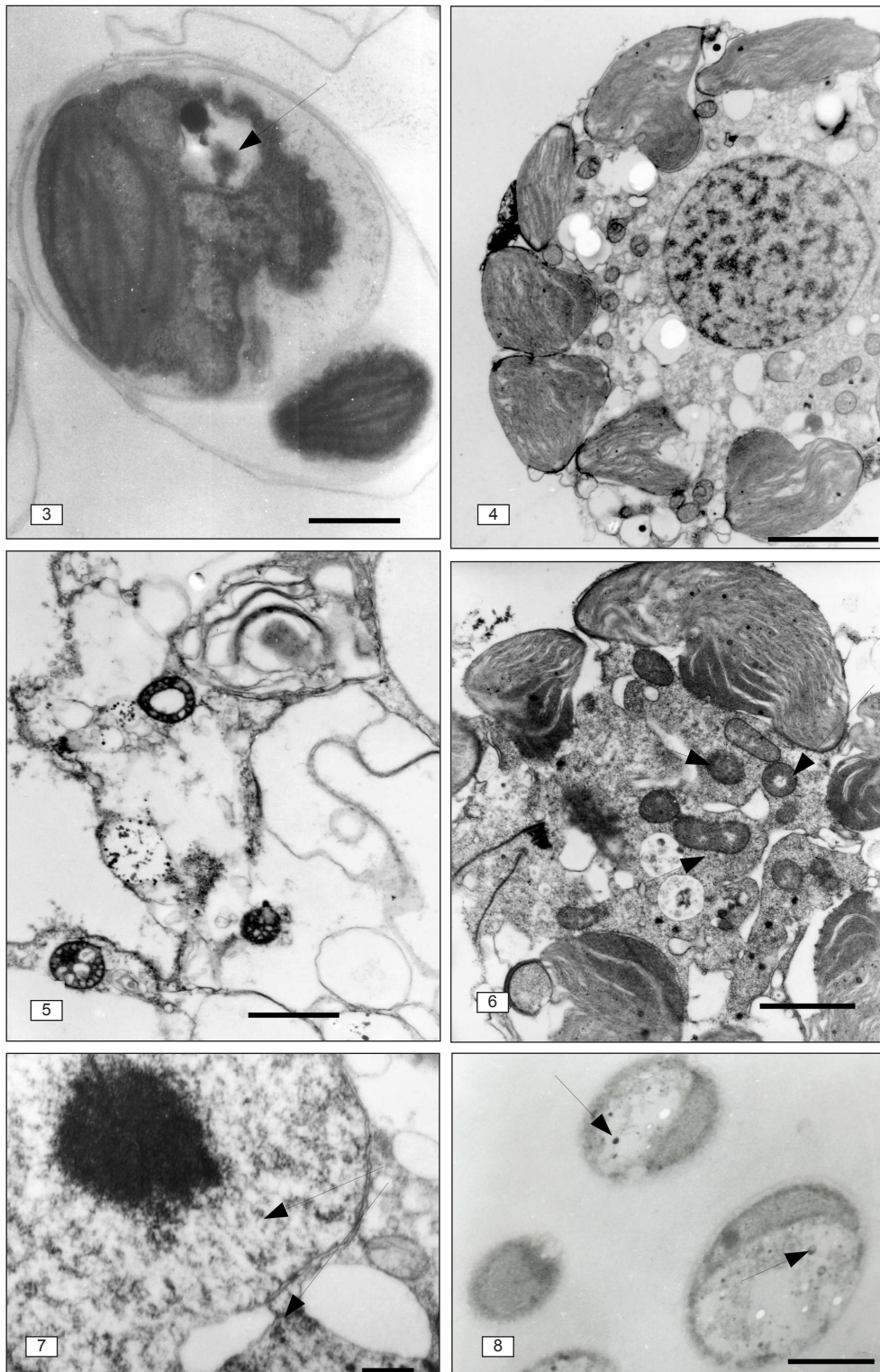


Figure 2. Phylogeny of eukaryotes. Redrawn from Schlegel (2003). Most relevant clades containing photosynthetic clades are indicated by arrows.

others. In *Heterosigma hakashiwo*, the change from fresh growth medium (Fig. 4) to a starvation situation with very low nitrogen supply, leads to a strong increase in the number and dimension of vacuoles (Fig. 5), together with an increase in mitochondria (Fig. 6) and, in some cells, of nuclear fragmentation (Fig. 7). We can conclude that *Dictyosphaerium* sp., a green alga, behaves similarly to the other photosynthetic organisms previously investigated and belonging either to green algae or land plants, while *Heterosigma*, belonging to heterokonts and without a cell wall, shows a type of autophagy apparently more energy consuming (hence the amount of mitochondria) and with some traits typical of programmed cell death, such as the fragmentation of the nucleus in metazoans (Papini et al., 1999). Programmed cell death in *Heterosigma* may be related to the following processes of “cannibalism” acted by other cells of the population on the dying ones, possibly in order to recover nitrogen supply in a condition of starvation (Papini et al., 2017).

Some organisms may deter toxic metal effects at the wall level by excluding the entrance into the cytoplasm; others may concentrate and precipitate the



Figures 3-8. (3) *Dictyosphaerium* sp., in condition of high concentration of Chromium III in the medium. Some large cytoplasm portions enclosed by membranes contain cytoplasm residuals and organelles remnants (arrow). Bar = 1 μ m. (4) *Heterosigma hakashiwo*. In fresh growth medium. Bar = 1 μ m. (5) *Heterosigma hakashiwo* in depleted growth medium. A strong increase in the number and dimension of vacuoles can be observed. Bar = 500 nm. (6) *Heterosigma hakashiwo* in depleted growth medium. An increase in mitochondria (arrows) number can be observed. Bar = 500 nm. (7) *Heterosigma hakashiwo* in depleted growth medium. Nuclear fragmentation can be observed (arrows). Bar = 250 nm. Fig. 8. *Dictyosphaerium* sp. Autophagic vacuoles in part of the cells of the culture occupy almost the whole cell and contain electron dense accumulations (arrow). Bar = 600 nm.

metal in a vacuole (Colzi et al., 2015). Besides these methods, some cells appear to accelerate their turnover by increasing autophagic activity in order to replace organelles that get damaged by the heavy metal toxicity. Preliminary results in *Dictyosphaerium* sp. show that about 10% of the cells are almost completely filled by a vacuole containing granular electron dense material (Fig. 8), an observation which may even suggest that some members of the populations accumulate faster the heavy metals than the others, eventually dying and precipitating, in order to subtract part of the metal from the medium.

If confirmed, this activity would represent a Programmed Cell Death case, resembling the sacrifice of some cells in multicellular organisms in order to accomplish a specific task of general interest for the entire organisms. Such cases are quite rare or of difficult interpretation in unicellular organisms. Also in *Chlorella vulgaris* the presence of Zinc causes an increase of the autophagic activity and an accumulation of electron dense material in the vacuoles (Fig. 9). Multilamellar bodies can be found in the vacuoles (Fig. 10), as a probable result of organelle degradation, as suggested by van Doorn and Papini (2016).

The strategy with which unicellular (or colonial) green algae are able to resist to heavy metals in the

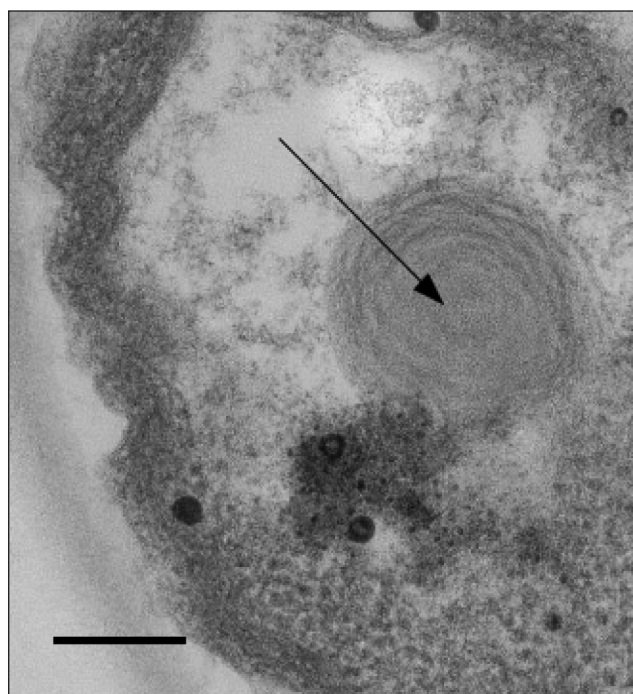
environments is object of interest, since reducing the adverse biological effects may allow to use these strains for industrial purposes even in polluted waters and/or their exploitation for heavy metals removal (Phycoremediation).

Conclusions

Programmed cell death, combined with autophagy, is capable of reducing the metal availability by precipitating it in a vacuole of a dead cell. This kind of resistance mechanism to metal is common in multicellular while it is quite rare, overlooked or of difficult interpretation in unicellular organisms.

The increase in autophagic activity in some groups of unicellular algae is linked to the need to increase the speed of organelle turnover, while metal segregation in vacuoles linked to PCD may eventually lead to precipitation of the dead cells containing metal, removing it from the medium. This possible explanation of the observed autophagic features may pave the way to possible applications in phytoremediation.

Starvation is a known inducer of autophagy in eukaryotes (van Doorn & Papini, 2013) that in *Heterosigma* was apparently preliminary to PCD with features similar to PCD in animals, such as nuclear fragmentation. This specific feature appears to be



Figures 9-10. (9) *Chlorella vulgaris* in presence of Zinc. The autophagic activity is present in almost the whole cytoplasm and accumulation of electron dense material can be observed in the vacuoles (arrows). Bar = 1.5 μ m. (10) *Chlorella vulgaris* in presence of Zinc. Multilamellar bodies are present in some vacuoles (arrow). Bar = 500 nm.

more related to the isolated phylogenetic position of *Heterosigma* with respect to the other microalgae here investigated, rather than to starvation.

References

- Colzi, I., Pignattelli S., Giorni, E., Papini, A. & Gonnelli, C. 2015. Linking root traits to copper exclusion mechanisms in *Silene paradoxa* L. (Caryophyllaceae). *Plant and Soil*, 390: 1-15.
- Guillard, R.R.L. 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L. & Chanley, M.H. (eds) *Culture of Marine Invertebrate Animals*: 26-60, Plenum Press, New York, USA.
- Hayes, P.K., & Walsby, A.E. 1984. An investigation into the recycling of gas vesicle protein derived from collapsed gas vesicle. *Microbiology*, 130: 1591-1596.
- Klionsky, D. et al. 2016. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edn). *Autophagy*, 12: 1-222.
- Kovacevic, G., Kalafatic, M. & Horvatin, K. 2006. Detection of aluminium depositions in green and brown *Hydra*. *Symbiosis*, 42: 175-176.
- Kovacevic, G., Kalafatic, M. & Horvatin K. 2009. Aluminium Deposition in Hydras. *Folia Biologica (Kraków)*, 57: 139-142.
- Kumar, K.S., Dahms, H.U., Won, E.J., Lee, J.S. & Shina, K.-H. 2015. Microalgae—A promising tool for heavy metal remediation. *Ecotoxicology and Environmental Safety*, 113: 329-352.
- Lütz-Meindl, U. 2016. *Micrasterias* as a model system in plant cell biology. *Front. Plant Science* <https://doi.org/10.3389/fpls.2016.00999>
- Papini, A., Fani, F., Belli, M., Niccolai, C., Tani, C., Di Falco, P., Nuccio, C. & Lazzara, L. 2017. Structural and ultrastructure changes show an increase of amoeboid forms in *Heterosigma akashiwo* (Raphidophyceae), during recovery after nutrient depletion. *Plant Biosystems*, 151: 965-973.
- Papini, A., Mosti, S. & van Doorn, W. 2014. Classical macroautophagy in *Lobivia rauschii* (Cactaceae) and possible plastidial autophagy in *Tillandsia albida* (Bromeliaceae) tapetum cells. *Protoplasma*, 251: 719-725.
- Papini, A. & Van Doorn W. 2015. Crystalloids in apparent autophagic plastids: remnants of plastids or peroxisomes? *Journal of Plant Physiology*, 174: 36-40.
- Papini, A. 2018. The investigation of morphological features of autophagy during plant Programmed Cell Death (PCD). In *Plant Programmed Cell Death: Methods and Protocols*. De gara and V. Locato (eds.). Springer Verlag.
- Papini, A., Mosti, S. & Brighigna, L. 1999. Programmed cell death events in the tapetum development of Angiosperms. *Protoplasma*, 207: 213-221.
- Pereira, M., Bartolomé, C.M. & Sánchez-Fortún, S. 2014. Photosynthetic activity and protein overexpression found in Cr (III)-tolerant cells of the green algae *Dictyosphaerium chlorelloides*. *Chemosphere*, 108: 274-280.
- Schlegel, M. 2003. Phylogeny of eukaryotes recovered with molecular data: highlights and pitfalls. *European Journal of Protistology*, 39: 113-122.
- van Doorn, W. & Papini, A. 2016. Plastid degeneration in *Tillandsia albida* (Bromeliaceae) and *Lobivia rauschii* (Cactaceae) provides evidence about the origin and destiny of multilamellar bodies in plants. *Phytomorphology*, 66: 103-112.
- van Doorn, W.G. & Papini, A. 2013. Ultrastructure of autophagy in plant cells: a review. *Autophagy*, 9: 1922-1936.