Deplinoa, n.s. **33-34**: 13-29. 1991-1992

Preliminary data on cellular localization and effects of chromium and mercury on the growth and ultrastructure in the protonemata from *Funaria hygrometrica* Hedw.

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Riassunto

La tossicità di due metalli pesanti, cromo (Cr^{+3}) e mercurio (Hg_2^{+2}), sul comune muschio acrocarpo *Funaria hygrometrica* è stata studiata su campioni coltivati in condizioni controllate *in vitro*, mediante microscopia ottica a contrasto di fase, a fluorescenza, elettronica convenzionale e microanalisi a raggi X a trasmissione. Le spore del muschio sono state coltivate per 30 giorni in presenza di diverse concentrazioni (da 10^{-3} a 10^{-7} M) di mercurio o cromo forniti come sali inorganici. Inibizione della crescita del 50 % si è verificata a concentrazioni di 10^{-7} M per il mercurio e 10^{-4} M per il cromo. Concentrazioni subletali di questi metalli hanno causato alterazioni nell'ultrastruttura delle cellule protonemali e nel processo morfogenetico che hanno mostrato di essere almeno in parte metallo-specifiche. Pur essendo meno tossico a dosi subletali, ma anche a basse concentrazioni, il cromo induce alterazioni morfogenetiche più evidenti e persistenti come il disturbo della crescita apicale e la formazione di cellule più corte ed in numero ridotto rispetto al controllo. Inoltre, è evidente in tutti gli esemplari la formazione di numerose brood cell e la presenza di cellule altamente vacuolate. Il mercurio causa invece una elevata inibizione dose-dipendente, con riduzione marcata nella percentuale di germinazione perfino a concentrazioni decisamente basse, e la morte degli esemplari a partire da una concentrazione di 10^{-5} M. Nelle colture trattate con mercurio a concentrazioni sub-letali, dopo un'alterazione iniziale nel modello di crescita (7 giorni), il protonema assume un aspetto simile a quello del controllo.

Il differente comportamento degli esemplari trattati con i due metalli è considerato in relazione alla differente organizzazione dei microtubuli citoplasmatici.

Per quanto riguarda gli effetti ultrastrutturali, il mercurio e il cromo hanno alterato soprattutto i cloroplasti che hanno mostrato un'elevata presenza di granuli di amido e plastoglobuli.

Con il supporto della microanalisi a raggi X al microscopio elettronico a trasmissione, è stata determinata la localizzazione cellulare dei due cationi. E' stato suggerito che il cromo viene trattenuto in vacuoli e nelle

Key words: Cellular localization, Chromium, *Funaria hygrometrica*, Morphogenetic alteration, Ultrastructural damages.

pareti cellulari e che il mercurio è di preferenza localizzato nelle pareti cellulari come pure a livello delle membrane e degli organelli cellulari.

INTRODUCTION

Protonemal development in *Funaria hygrometrica* has been extensively studied with regard to morphogenesis (SLIEVERS & SCHNEPF, 1981; SCHNEPF, 1982; DUCKETT *et al.*, 1993). The protonemal system has been shown to be a particularly flexible and useful model for studying the effects of lead on morphogenesis. In a previous work (BASILE *et al.*, 1995) we showed that inorganic lead causes significant alterations to the protonemal growth pattern of *Funaria hygrometrica* obtained both from spores and from shoots. Such alterations depended at least partly on the disorganization induced by the metals upon the microtubules.

This work aims to study the effect of Hg and Cr upon the protonemal system of *Funaria hygrometrica*. In particular, for both cations some criteria were used to characterize moss responses to heavy metal exposure: growth of protonema, aberration of habitus, damage to cytoskeletal organization, changes in fine structure of protonemal cells and cellular localization of metals.

MATERIAL AND METHODS

Plant material

Field-grown *Funaria hygrometrica*. was gathered in the Reserve Castelvolturno (Province of Caserta, Southern Italy). Mature capsules of *F. hygrometrica* were surface sterilized in ethanol 70% (2 min.) and in 2% NaClO with the addition of a few drops of Triton X-100 (5 min.). Subsequently, they were washed (10 min.) with distilled sterile water and the contents of 10 capsules were suspended in 10 ml of distilled sterile water. Aliquots (200 ml) of this sporal suspension were inoculated in modified Mohr medium (KRUPA, 1964), pH 7.5 (KNO_3 100 mg, $CaCl_2$.$4H_2O$ 10 mg, $MgSO_4$ 10mg, KH_2PO_4 136 mg, $FeSO_4$ 0.4 mg and 1 ml of BMM solution (NICHOLS, 1973) to 100 ml distilled water) used as control and in the same medium with the addition of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} M Hg_2SO_4 or $CrCl_3$.

The sporal cultures were kept in a climatic room with a temperature ranging from 13 to 20 °C (night/day temperature within the chamber), 70% constant relative humidity, and 16 hrs light (24 to 60 µEm-2s-1)/8 hrs dark photoperiod.

Visualization of MTs by IFT

For visualization of MTs by IFT, *Funaria hygrometrica* protonemata were grown on a sterile cellophane foil covering on nutrient agar, with Hg_2SO_4 10^{-6} M or $CrCl_3$ 10^{-4} M added, for 10-14 days and subsequently transferred to fresh culture dishes with mercury or chromium added as above. The protocol followed is reported in a previous work (BASILE *et al.*, 1995). Photographs were taken using a Leitz Aristoplan microscope equipped with differential interference contrast optics (Nomarski), and with a filter combination BP 450-490, RKP 510, LP 520. Photographs were taken on Ilford PANF 50 ASA and Ilford HP5 400 ASA films.

Electron microscopy.

For the observation of ultrastructural damage and cellular localization of metals, use was made of protonemata cultured with Hg 10^{-7} M or Cr 10^{-4} M (EC_{50} values) for 30 days, and subsequently transferred to fresh culture for 24 h. After mercury or chromium treatment, plants were thoroughly washed in distilled water for 15 min with several changes to eliminate unbound cations, fixed in 2% glutaraldehyde in phosphate buffer (0.065 M pH 7.2-7.4) for 2h at room temperature and dehydrated with ethanol and propylene oxide. Ultra-thin (50 nm), unstained sections of osmium-free samples were embedded in Spurr's epoxy medium, mounted on 100-mesh nylon grids and placed in a PHILIPS CM12 microscope (STEM) fitted with an energy dispersive X-ray spectrometer. The accelerating voltage was 40 KV and the specimen probe size was 50 nm in diameter. The mean count rate was 800-1000 counts sec^{-1} and the take off angle 35 °C. X-ray counts were made over a counting time of 100 sec. A beryllium splint was used to avoid covering the peak of chromium. The X-ray microscope standards, mounted in resin, were supplied by Microanalysis Consultants Ltd, Cambridge (SHUMAN *et al.*, 1976). About

300 specimens were observed and analysed by microanalysis.

RESULTS

During all the experiments, as regards the study of the morphogenetic alterations, we considered the number and length of newly formed filaments, the type of filaments, the shape and polarity of cells and the presence of brood cells. The organization of the cytoskeleton was investigated only in the samples at the concentration corresponding to the EC_{50}, with evident morphological alterations associated with a reduced development (Hg 10^{-7} M and Cr 10^{-4} M).

The observations were carried out 3, 7, 14, and 30 days after culturing.

Abbreviations

EC_{50} = effective cation concentration causing 50% inhibition of protonemal growth. IFT = immunofluorescence technique. MTs = microtubules.

Control

In *Funaria hygrometrica* the formation of a protonemal apparatus from spores has been extensively studied (KNOOP, 1984; SCHNEPF, 1982; SEBASTIO & MACCHIA, 1979) and some data are available regarding the effects induced by lead (KRUPINSKA, 1976; KARDASH & DEMKIV, 1991; BASILE *et al.*, 1995). In our experimental conditions, in the control samples, 90-95% of spores germinated after 3 culturing days, forming a chloronema with 2-3 cells and sometimes a rhizonema (Plate I, Fig. 1). As for cytoskeletal organization, in control samples the tip cells showed MTs which mainly formed orderly lines along the main axis with a more evident MTs presence in the apical zone (Plate II. Fig. 7). The ultrastructural organization is elsewere discussed (SCHNEPF, 1982) and the plastids usually did not show starch accumulation (Plate II. Fig. 10). The development of protonemal apparatus of both control and treated samples was reported in Fig. 17.

Chromium

Morphological alterations

Chromium caused a dose-dependent inhibition of all the parameters considered, with EC_{50} at 10^{-4} M. Alterations in the protonemal growth pattern are evident also at lower concentrations and are persistent even after a month of culturing. In particular, the septs in all the filament orders are essentially transverse, making it impossible to distinguish caulonemata from chloronemata even after 28 days and in the samples treated at the lowest concentrations. The side branches often show a disordered growth, and the cells are shorter and wider than the control. The growth tip is often swollen. The most evident alteration is extensive vacuolization (Plate I. Fig. 3). Moreover, many cells both in the main filament and in the secondary ones are particularly poor in chloroplasts. The general protonemal pattern is curled (Plate I. Fig. 3) or zig-zag, and shows out-growth and knees (Plate I. Fig. 2) along its course. After 14 culturing days, numerous brood cells may already be noted which form both along the main filament and along the secondary ones (Plate I. Fig. 2).

Cytoskeletal organization

In Cr 10^{-4} M samples it was very difficult to evidence the presence of cytoskeletal structures. In fact, in chromium-treated samples we observed a diffuse fluorescence indicating MTs disgregation (Plate II. Fig. 9).

Ultrastructural damage

Some chloroplasts presented a poorly developed lamellar system with a few badly compartmentalized grana and a considerable presence of plastoglobules (Plate II. Fig.12). Chloroplasts with enormous starch grains were found. The cells show several vacuoles with a large amount of electron-dense material (Plate II. Fig. 15).

Cellular localization

Observation of specimens with X-ray microanalysis shows that the accumulation of Cr is essentially localized in the cell walls (Plate II. Fig. 13) and in the vacuoles as electron-dense material (Plate II. Fig. 15).

Mercury

Morphological alterations

Hg10^{-7} M. The samples showed after 7 days only a few protonemata. The germination percentage was 20-30%. Protonemal length and the number of cells per protonema were low. The most evident alteration was the high number of hyalin filaments that look like rhizoids (Plate I. Fig. 5). Moreover, the differentiation of each type of filament, chloronema, rhizonema and caulonema was not so clear and the tips were swollen (Plate I. Fig. 6). At Hg 10^{-6} M concentration the germination percentage was markedly reduced to 8-10 % and we observed the most evident alterations. In fact, germination from spores gives rise to very abnormal forms: swollen tips, knees, lateral out-growths and altered orientation of the septa (Plate I. Fig. 4). The cellular alterations induced by 10^{-7} M Hg concentration recovered a normal morphology after 7 days, whilst those induced by Hg 10^{-6} M began to weaken only after 7 days, recovering after 14 days a morphology similar to the control at 14 days. In particular, the orientation of the septs recovers its typical appearance (transverse in the chloronema and oblique in the caulonema), the apical cell is tapered and growth straightens once again. However, the decrease in the germination percentage and inhibition in the development of the protonemal system persisted in both samples. The concentrations from 10^{-5} to 10^{-3} M determined the death of the samples.

Cytoskeletal organization

In Hg 10^{-7} M treated samples, after 7 days, it is difficult to observe the microtubular network, whereas after 14 days there are already no evident alterations in the arrangement of the microtubules compared with the control, nor is the accumulation of Mts in the tip zone less extensive (Plate II. Fig. 8).

Ultrastructural damage

After culturing moss in a concentration of 10^{-7} M mercury, the chloroplasts showed the most prominent changes: besides the chloroplasts organized like those of the controls, 50% or more modified ones were found. In this case, a variation is present in the lamellar system with only a few grana (Plate II. Fig. 11). The most significant chloroplast variation concerned

the considerably increased starch granules (Plate II. Fig. 14) and plastoglobules (Plate II. Fig. 11). The nucleus and cell walls appeared to be normally organized.

Cellular localization

X-ray TEM microanalysis of the cultures at the concentration of 10^{-7} M Hg highlights the presence of accumulations especially at the wall level which shows homogeneous and diffuse punctation (Plate II. Fig. 16). Also the nucleus, plastidial membranes and the other systems of cellular membranes allow peaks to be observed for mercury. By contrast, the cytoplasm did not emit for the metal in question.

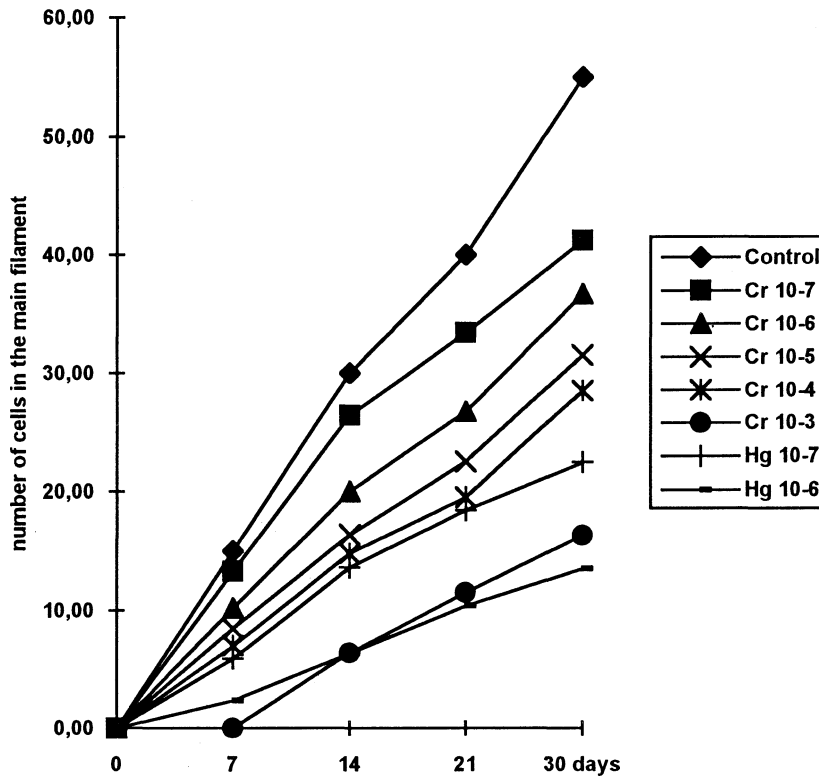

Fig. 17 - Growth of protonema in *Funaria hygrometrica* during 30 culturing days. SD ranges between ± 1.2 and ± 2.6.

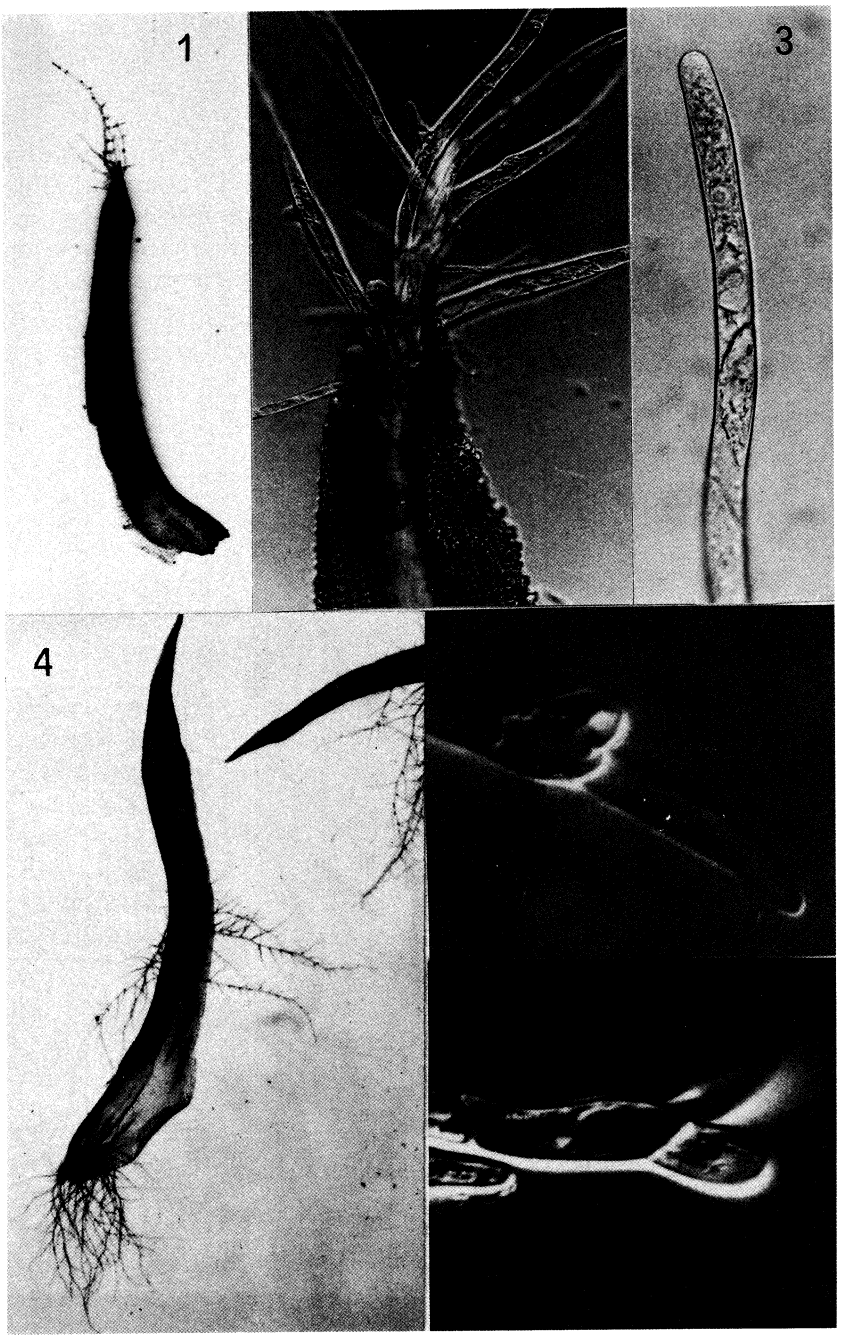
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Plate I - (1-6) Phase contrast micrographs. (1) Control sample of *Funaria hygrometrica* after 7 culturing days. x 220. (2) Chromium-treated sample after 14 culturing days. The presence of brood cells in the main filament and in the side branches is evident. Also the presence of scattered plastids and an abnormal course of filament with contractions and swellings is shown. x 300. (3) Chromium 10^{-6} M sample showing the high vacuolization of protonemal cells. x 330. (4) Mercury-treated sample (10^{-6} M) after 7 culturing days. The abnormal germinating pattern, vacuolization and loss of polarity of the sample is evident. x 330. (5) Mercury-treated sample (10^{-7} M) after 14 culturing days. The large number of hyaline filaments originating from normal chloronemal filaments is shown. x 180. (6) Swollen apical cell of mercury-treated sample. x 240.

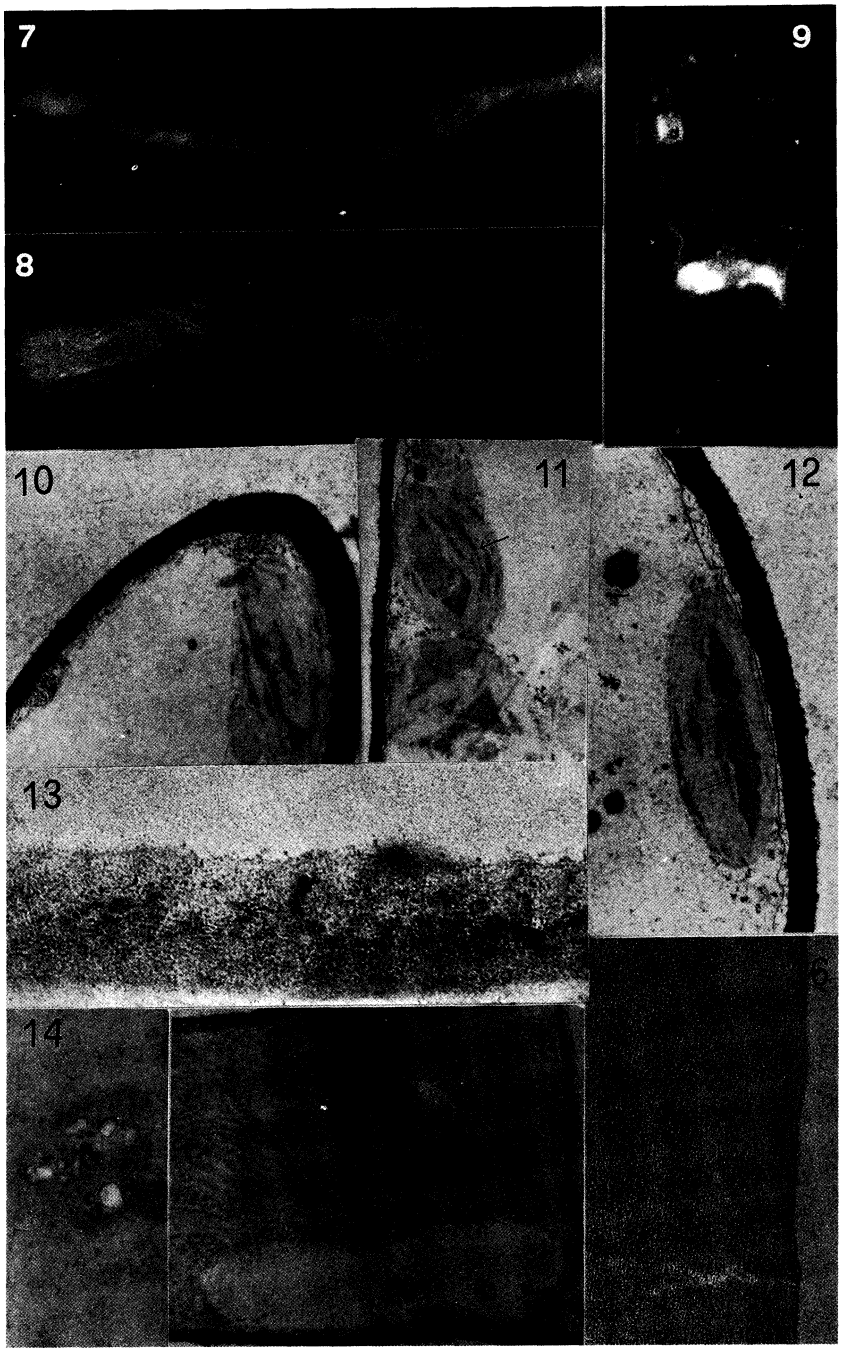
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Plate II - (7-9) IFT stained micrographs, (10-16) TEM micrographs. (7) Cytoskeletal organization in a chloronemal apical cell in control sample. x 280. (8) IFT image of cytoskeletal organization in a chloronemal apical cell of a mercury-treated sample after 14 culturing days that appears similar to the control. x 300. (9) Chromium-treated sample after 21 culturing days. The continuous MT network, observed in the control, is absent. x 400. (10) Protonemal cell from control samples showing a chloroplast without stark accumulation. Untreated with osmium 10000x. (11) Protonemal cell from a mercury-treated sample showing the presence of a chloroplast with a large number of plastoglobules and a alteration of thylachoids (see arrow). Untreated with osmium and stained with uramil acetate and lead citrate 10000x. (12) TEM micrograph of chromium-treated samples showing a chloroplast with numerous plastoglobules (see arrow). Untreated with osmium and stained with uramil acetate and lead citrate 11000x. (13) TEM micrograph of cell wall in chromium-treated sample unstained. Granulation is due to chromium deposits 12400x. (14) Micrographs of mercury treated sample shows a chloroplast with numerous grana starks (see arrow). Untreated with osmium and stained with uramil acetate and lead citrate 9400x. (15) Chromium-trated sample unstained showing an elettron dense material in the vacuoles (see arrow) 5600x. (16) TEM micrograph of cell wall in mercury-treated sample unstained. Homogeneous granulation is due to mercury deposits. 13000x.

DISCUSSION

The protonema is a tip growing system which shows a polar arrangement of organelles. It is used as a model system in order to study cell polarity because protonemata can be obtained from different sources: spore, shoots, detached leaves; and cultivated in a simple growth medium, and grow quickly.

In a previous work we reported that protonemata of *Funaria hygrometrica,* grown at different lead concentrations, show a reduction in growth rate, a disturbance of oriented polar growth, a change in the arrangement of the chloroplasts, an alteration of the nucleus position, and a different orientation of septa, compared with the control. Morphological effects were dose-dependent. In addition, a variety of aberrant forms were present: knees, swelling tips, out-growths and high vacuolization. Using the IFT technique we demonstrated a correlation between the lead concentration and alteration of the cytoskeletal organization (BASILE *et al.*, 1995).

In the present work, the morphogenetic alterations induced by the two cations have features in common but also present different characteristics both from each other and from those induced by lead in the same protonemal system (BASILE *et al.*, 1995). Indeed, at the level of light microscopy, a marked qualitative difference in the effect of cations was observed. Under the influence of Hg, there were growth anomalies analogous to those encountered in the presence of lead, but only after 7 days did the protonema grow linearly and exhibit the typical form of an apical cell, like the untreated controls. On the other hand, protonemata treated with chromium showed, above all, high vacuolization in all the cultures but also showed drastic swelling of the tips with a zig-zag pattern. Such effects first occurred at 10^{-7} M and were more pronounced at higher concentrations.

The above findings may partly be justified if we consider the different behaviour of the two cations in the cell environment related to the different chemical characteristics of the heavy metals in question. Despite showing generally higher toxicity, mercury allows rapid recovery of normal conditions. Both phenomena are probably caused by the high reactivity of the cation: it binds to the many cell structures which are able to react with it or participates in oxidoreductive cell phenomena and, because of its high potential, is quickly removed in its active form from the cell environment. The high reactivity of

mercury and its capacity to interfere with all oxidoreductive systems would, on the one hand, explain the higher toxicity of the metal but also the possibility shown by the damaged cells to temporarily recover the normal morphogenetic growth pattern more rapidly. Also, the MTs organization evidenced in the Hg treatments would appear to be a consequence of such chemical behaviour: the cytoskeletal structures of the protonemal cells regain, in a short period of time, a similar pattern to the control. On the other hand, the morphogenetic phenomena which depend on MTs integrity, such as apical growth, zoning of the organelles in the apical cell and form of the cells (SCHNEPF, 1982; WACKER *et al.*, 1986), recover similar characteristics to those of the control at a speed related to the dose of mercury used.

Chromium, despite having a less toxic effect upon germination and protonemal development, shows more marked, persistent morphogenetic effects which may be correlated to the considerable effect on the MTs caused by treatment with this metal. Tip growth is particularly modified, as shown by the swollen tips and as well as cell size and shape. Also the arrangement of the septs, linked to MTs integrity, is modified.

Ultrastructural damage also appears to have both common and distinct features according to the metal used. The increase in starch granules and the formation of numerous plastoglobules is common to both the chromium and mercury treated cultures: it probably allows the protonemal filament, which has to face a situation of severe stress from heavy metals, to accumulate nutritional reserves to overcome the "dangerous" situation in question.

The primary toxic effect of mercury seems to be membrane damage whereas chromium essentially causes an increase in vacuolization. Such findings may be easily interpreted by considering the different localization encountered for the two cations: from microanalysis, mercury is found, in addition to the cell wall, especially in the systems of the cell membrane and organelles. At this level, probably binding to structural or enzymatic electron donor groups or exerting its high oxidation power, the metal shows its toxic power. By contrast, chromium is compartmentalized in cytoplasmatic vacuoles. The presence of chromium may induce high vacuolization in the moss, as has already been reported for *Tortula muralis* and *Bryum capillare* (BASILE *et al.*, in press and submitted), and hence triggers a mechanism which ultimately leads to compartmentalization at

the vacuole level (VAZQUEZ *et al.*, 1987). Such a detoxification mechanism probably allows growth, albeit with severely modified morphology, even at fairly high Cr concentrations. Indeed, chromium is much less active than mercury or lead (BASILE *et al.*, 1995; BASILE *et al.*, submitted; BASILE *et al.*, in press) in inhibiting germination and protonemal growth, with an EC_{50} of about 10^{-4} M, while mercury is the cation that inhibits protonemal growth much more effectively. This observation is in agreement with studies on the toxicity of various heavy metals (for a review, see RAO, 1982; BROWN, 1984; TYLER, 1990).

In the presence of both cations, a high production of brood cells occurs. The latter are spherical or ovoid cells which are produced in nature or in control cultures in the case of senescence or in stress conditions, such as desiccation or lack of nutrients in general and calcium ions in particular, and are structures of resistance due to the presence of a thick wall and a large number of lipid drops. They have been interpreted as diaspores with the task of propagating not only in adverse nutritional conditions (GOODE *et al.*, 1993; GOODE *et al.*, 1994) such as desiccation or nutritional deficiency, but also in conditions determined by the toxic effect of heavy metals (BASILE *et al.*, 1995). In fact, it was hypothesized that brood cell formation is a generalized resistance mechanism which occurs in response to the presence of toxic cations and which has the significance of transforming the filamentous protonema, with its thin and highly permeable wall as regards toxic ions, into many structures of propagation and resistance with thick walls and an abundant nutritional reserve (BASILE *et al.*, submitted).

To summarize, the results presented in this study demonstrate that the two metals considered determined morphogenetic alterations and damage to cell structures and to organization of the cytoskeleton in the moss *Funaria hygrometrica*. The alterations found here may be sufficient to eventually cause the death of the moss. Further investigations are necessary in order to find out whether the laboratory studies can be extrapolated to conditions in nature where the situation is much more complicated.

ACKNOWLEDGMENTS

The authors wish to thank Mark Walters for reviewing the English. This work was supported by MURST and CNR grants.

Abstract

The toxicity of two heavy metals, chromium (Cr^{+3}) and mercury (Hg^{+2}), on the common acrocarpus moss *Funaria hygrometrica* was studied, under laboratory conditions, by light, fluorescence, conventional electron microscopy and X-ray TEM microanalysis. Spores of moss were cultivated for 30 days in the presence of various concentrations (from 10^{-3} M to 10^{-7} M) of mercury or chromium used as inorganic salts.

Fifty percent growth inhibition occurred with concentrations of 10^{-7} M mercury and 10^{-4} M chromium. Sublethal concentrations of these metals caused alterations in the fine structure of protonemal cells and in morphogenetic processes which proved at least to be partly metal specific. Despite being less toxic at sublethal doses but also at fairly low concentrations, chromium induces more evident and persistent morphogenetic alterations such as disturbance of apical growth and cellular straining with a decrease in both cell size and cell number. In addition, the formation of numerous brood cells and the presence of highly vacuolated cells is evident in all samples.

By contrast, mercury causes high inhibition in particular, with a marked reduction in the germination percentage even at decidedly low concentrations and the death of the samples from a concentration of 10^{-5} M. In the cultures treated with mercury at sublethal concentrations, after an initial alteration in the growth pattern (7 days) the protonema assumes a pattern similar to the control.

The different behaviour of the samples treated with the two metals is considered in relation to the different organization of the cytoplasmatic microtubules encountered.

As for ultrastructural effects, mercury and chromium affected the chloroplasts which mostly showed considerably increased grana starches and plastoglobules.

With the aid of X-ray TEM microanalysis, cellular localization of the two cations is determined. It is suggested that chromium is retained in vacuoles and cell walls and that mercury is preferentially localized in the cell walls as well as at the level of the cellular membranes and organelles.

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Finito di stampare nel marzo 1996.