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Tissue localization of experimentally-supplied zinc in the moss *Funaria hygrometrica* Hedw.

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Riassunto

L'accumulo dello zinco nel muschio *Funaria hygrometrica* è stato studiato *in vitro* usando soluzioni a differenti concentrazioni (10^{-2}-10^{-6} M) e per periodi da 1 a 30 giorni. L'accumulo di zinco è stato qualitativamente analizzato dalla microanalisi a raggi X a scansione in differenti tessuti. Lo zinco è di preferenza accumulato in alcuni tessuti (idroidi del gametofito, idroidi dello sporofito a livello del piede e transfer cells) e raggiunge la parte superiore della seta solo quando usato alle concentrazioni più alte e per un periodo di 30 giorni. Lo zinco, comunque, non raggiunge mai la capsula.

I nostri esperimenti dimostrano che *F. hygrometrica* mostra una evidente capacità di sequestrare lo zinco in particolari tessuti, come già dimostrato per il piombo (BASILE *et al.*, 1994), ma in questo caso il sequestro di zinco a livello della placenta è meno efficace se confrontato con quello del piombo, raggiungendo la parte superiore della seta quando usato alla concentrazione di 10^{-2} M. Comunque nelle condizioni utilizzate nel nostro esperimento non raggiunge mai la capsula, confermando che il massivo accumulo di metalli pesanti nei tessuti del gametofito e a livello della placenta gioca un ruolo importante nei mecccanismi di detossificazione. Inoltre, le differenze tra i dati qui presentati e i risultati riportati in precedenti lavoro ci consentono di ipotizzare che il blocco operato dal gametofito e dalla placenta sono dovuti principalmente alla saturazione progressiva dei siti di legame presenti nelle pareti.

Key words: *Funaria hygrometrica*, Heavy metals, X-ray SEM microanalysis, Zinc accumulation.

INTRODUCTION

A number of heavy metals, including zinc, are required as micronutrients in biological systems to act as cofactors and/or as part of prosthetic groups of enzymes in a wide variety of metabolic and developmental pathways. At high concentrations however, most heavy metals are toxic. Toxic levels of some of these metals can occur in some natural soils, or as the results of environmental pollution from mining, smelting, manufacturing, agricultural or waste disposal technologies (FOY *et al.,* 1978). Bryophytes are important constituents in the vegetation of many natural and man-made ecosystems in the world. They are essentially ectohydric, which means that they absorb water and also heavy metals over their entire surface. Hence the bryophytes are extensively used as bioindicators of environmental pollution (BROWN, 1982; RAO, 1982; BROWN, 1984; RUHLING & TYLER, 1984; TYLER, 1990). Little is known about the processes involved in heavy metal immobilization or the localization of heavy metals at tissue level. In previous works, using X-ray SEM and TEM microanalysis (BASILE *et al.*, 1994) and atomic spectroscopy (BASILE *et al.*, 1993 b) we determined tissue and cell localization of lead in plants of *Funaria hygrometrica* with sporophytes at different stages of development. Lead does not reach the upper part of the seta or the sporogenous tissue in the capsule but is sequestered especially in gametophyte hydroids and placental transfer cells. This blockage protects the reproductive sites from the toxic action of this metal. To ascertain whether the blockage of metal was dependent on its affinity for cell wall components and therefore on the capacity of the gametophore and placenta to become progressively saturated, we decided to test the same moss in the same experimental conditions employed for lead to test its ability to accumulate zinc.

In this work we investigated zinc accumulation, using experimentally supplied zinc at different concentrations and for times from 1 to 30 d, in the gametophyte, foot and sporophyte of the moss *Funaria hygrometrica.* The accumulation in different tissues was qualitatively analysed using X-ray SEM microanalysis. The data obtained were compared with the results reported for lead (BASILE *et al.*, 1994).

MATERIALS AND METHODS

Plant material.

Field-grown *Funaria hygrometrica* Hedw. was gathered in the MAF reforestation Reserve Castelvolturno (CE, Southern Italy). This species has optimum growth on soils with high concentrations of soluble salts and a basic pH, and occupies a relatively peculiar ecological niche circumscribed in space and time (BROWN, 1982). *Funaria hygrometrica* is an acrocarpous, terricolous moss which forms short turfs. The MAF reforestation Reserve at Castelvolturno consists of a flat coastal strip covered with low Mediterranean "maquis". In this site *F. hygrometrica* grows extensively both due to the type of substrate (dune sand) and the frequent presence of burned areas. It is in fact a cosmopolitan ephemeral species (DURING, 1979), playing a very important role in post-fire recovery. *Funaria hygrometrica* has optimum growth on soils with high concentrations of soluble salts and a basic pH, (conditions present in the post-fire soils).

Plants of *F. hygrometrica* were gathered at different stages of sporophyte development, corresponding to the three developmental stages described by NEIDHART (1975) and WIENCKE & SCHULZ (1977): stage I (growth of seta), stage II (growth and differentiation of capsule), stage III (capsule ripening and sporogenesis). They were used the same day or the day after gathering. Single gametophytes were thoroughly washed with deionized water and put in plastic weighing boats each with 30 small holes. Each hole supported a single plant with its base (about 1 mm) immersed in an experimental solution. The plastic boats were floated on 200 ml water (control) or $Zn(NO_3)_2$ solution, respectively, the latter at concentrations of 10^{-2}, 10^{-4} and 10^{-6} M for 24 h, 7, 15 and 30 d, and maintained in a controlled-environment room at 20° C, 70% relative humidity with a 16 h light (2000/5000 lux)/8h dark cycle. Since the zinc concentration changes during the exposure period (due to carbonate formation with atmospheric CO_2 and H_2O evaporation), every 2 days the $Zn(NO_3)_2$ solutions were replaced by fresh ones. The treatments were duplicated and repeated several times.

X-ray SEM microanalysis.

After zinc treatment, plants were thoroughly washed in distilled water for 15 min with several changes to eliminate unbound Zn, fixed in 2% glutaraldehyde in phosphate buffer (0.065 M pH 7.2-7.4) for 2 h at room temperature and dehydrated with ethanol. Tissue pieces untreated with osmium were critical point dried and mounted on carbon stubs, covered with 15 nm carbon film and observed with a Cambridge 250 Mark 3 scanning electron microscope. Analysis was performed using an energy-dispersive detection system spectrometer and an analyser computer system Link AN 10000. Spectra were collected over 50 sec live time using a 0.5 mm diameter circular probe (spot size); the accelerating voltage was 20 kv and the probe current 400 mA. The mean count rate was 1000-1500 counts sec^{-1}. and the take-off angle 35^{o} (GODFRIED & SHELBURNE, 1983). About 300 specimens were observed and analysed by microanalysis.

OBSERVATIONS

Zinc localization in different organs and tissues of *Funaria hygrometrica* was qualitatively assessed employing SEM X-ray microanalysis on the specimens treated with $Zn(NO_3)_2$ 10^{-2}, 10^{-4} and 10^{-6} M solutions for periods from 1 to 30 days. The presence of zinc in the specimens was always assessed by X-ray microanalysis.

After 24 h the specimen treated with the lowest concentration shows the presence of zinc only at the level of the gametophytes in the rhizoids and in the hydroids. The other tissues of the moss gametophytes (parenchyma and epidermis) contain no zinc. By contrast, at a concentration of 10^{-4} M, after 24 h zinc was found in all gametophyte tissues and also at the level of vaginula. At the highest concentration zinc is found after 24 h also at placenta level in both gametophytic and sporophytic sites. In particular, Zinc reached its highest levels in the gametophytic hydroids, in the hydroids present in the sporophytic portion of the foot and in the transfer cells. Zinc levels drastically decreased in the vaginula and in the hydroids at the base of seta, 2-3 mm above the foot, after the placental barrier, and the metal was always absent from the upper part of the seta, the meristem generating the capsule (phase I) and from the ripening capsule (phases II and III).

After 7 d in 10^{-6} M Zn solution, the metal also reached the foot and was strongly accumulated by the sporophyte hydroids (in the foot) and by the transfer cells. At this concentration Zinc was never found at the base of the seta (2-3 mm above the foot). At 10^{-4} M, zinc accumulation increased in the foot hydroids, while the transfer cells became the preferential site of accumulation. Zinc was detected by X-ray SEM microanalysis even in the upper part of the seta (1-2 mm under the capsule) in the specimen treated with 10^{-2} M Zn.

After 30 days of treatment, zinc, used at 10^{-6} M , reaches the hydroids at the base of the seta and the basal portion of the parenchyma of the sporophyte. The specimens treated with 10^{-4} M zinc solution, show the presence of the metal in the middle and upper part of the seta (1-2 mm under the capsule), but never in the capsule nor in the spores, even after 30 d of treatment at the highest concentration.

DISCUSSION

Typically in the Bryidae the sporophyte foot is highly elongated, conical in shape and penetrates the gametophyte stem tissue. It is partially or completely surrounded by the vaginula, a multilayered parenchymatous sheath derived from the proliferation of archegonial cells and underlying stem tissue (ROTH, 1969). The foot of the Bryidae has the same histological organization as the seta. When the seta contains a central strand of conducting tissue (HEBANT, 1977), this is also present in the foot. The lower part of the foot, however, differs from the seta in that it lacks a peripheral sterome and has highly specialized epidermal cells. Epidermal cells in the foot and the adjoining layer of gametophyte cells show transfer cells. They exhibit labyrinthine walls that are present on both sides of the placenta (LIGRONE & GAMBARDELLA, 1988). In *Funaria hygrometrica* the wall labyrinths develop at a very early stage of sporophyte development, reaching their maximum extension well before capsule differentiation (WIENKE & SCHULZ, 1977; BROWNING & GUNNING, 1979).

The possible pattern of nutrient transport in the sporophyte-gametophyte junction is probably based upon the standing gradient osmotic hypothesis. On the gametophytic side of the placenta, the plasmalemma of the transfer cells actively pumps solutes from the cytoplasm into the lumen of the wall ingrowths. Conversely, the plasma membrane in sporophyte transfer cells pumps solutes from the lumen of wall ingrowths into the cytoplasm. In the steady state, active solute secretion by gametophyte transfer cells and active absorption by sporophyte transfer cells (possibly coupled with proton export/import) maintain a standing concentration gradient across which the solute diffuses. The same mechanism maintains a standing concentration gradient, with the lowest gradient potential at the extremities of wall ingrowths in the gametophyte transfer cells and the highest at the extremities of wall ingrowths in the sporophyte transfer cells. This causes water loss by gametophyte cells and water uptake by sporophyte cells, resulting in a mass flow of water from gametophyte to sporophyte through the placental space (GUNNING & PATE, 1974, LIGRONE & GAMBARDELLA, 1988). In the light of such findings, the massive accumulation of heavy metals in the gametophyte and their scarce presence or absence in the sporophyte may be due to two different mechanisms: a progressive saturation of cell wall sites along the way of the solutions (mechanism based on the high chemical affinity of lead for the negative sites of wall components) or damage to the protein sites responsible for mantaining the osmotic gradient (mechanism independent of lead affinity for wall binding sites). It is possible that the sequestration of heavy metals is correlated to both kind of transport mechanisms. Lead and zinc may damage the protein sites responsible for mantaining the osmotic gradient (inhibiting the diffusion of solutions through the plasma membrane) and may bind to cell walls.

Bryophyte tissues are excellent cation exchangers. For heavy metals the retention efficiency rate is Cu, Pb>Ni>Co>Zn, Mn, as demonstrated by RUHLING & TYLER (1970) for *Hylocomium splendens*. This order has proved valid for a large range of concentrations. In addition, some authors observed that dead parts of mosses show higher cation exchange capacity (CEC) values than living tissues, probably because cell membrane rupture allows cations to link to newly exposed protein sites which are not normally available (BUCK & BROWN, 1978). Unlike lead, zinc has a very low binding affinity for cell walls and is in fact at the other end of the affinity scale.

Under the conditions employed in our experiments, plants of *F. hygrometrica* show a marked capability to absorb zinc in particular tissues. The gametophore is the first site in which the metal is accumulated and the zinc distribution, as also shown by lead, reflects the pathway along which solutions are absorbed by internal conduction (HEBANT, 1977). In addition, the metal is present especially in the hydroids and in the transfer cells. Besides the gametophyte, zinc was accumulated in the foot especially at the level of transfer cells and in the hydroids. At the highest concentration and with the longest time, zinc overcomes the placental blockage and reaches the upper part of the seta (2-3 mm under the capsule). This behaviour is very different from that obtained with lead which never reaches the upper part of the sporophyte.

We suppose that the different behaviour of the metals is due to their opposite affinities for wall binding sites. Hence the two metals employed at the same concentrations and for the same times are sequestred with different effectiveness by moss tissue. We can therefore hypothesize that the mechanism mainly responsible for metal sequestration at placenta level is represented by the saturation of wall binding sites encountered by solutions along their path.

Most studies indicate the importance of heavy metal binding by cell walls. For instance, walls of the aquatic moss *Fontinalis antipyretica* contained 80-90% of the accumulated zinc (BURTON & PETERSON, 1979), while in *Jungermannia vulcanicola* mercury-sulphur binding seemed to be responsible for the very high concentration of mercury, with most present in the walls (SATAKE & MIYASAKA, 1984; SATAKE *et al.*, 1983). A recent study with the aquatic liverwort *Scapania undulata* (SATAKE *et al.*, 1989) indicates that Pb is combined with organic (sulphur) compounds present in the wall. Cell wall labyrinths typical of transfer cells are the sites which are mainly affected by lead accumulation (BASILE *et al.*, 1994). At this level, lead is present in the form of bigger granules close to the middle lamella and smaller, scattered granules close to the primary wall.

Also the accumulation of zinc in the hydroids of the gametophore is probably due to its link to wall sites. In these cells, in fact, in which the enzymatic degradation of walls and cytoplasm is in progress, massive zinc accumulation may depend on the higher availability of binding sites due to enzymatic degradation (BASILE *et al.*, 1994).

Besides the cell wall polysaccharides, which seem to be the most suitable sites for the absorption of heavy metals, other molecules display negative charges (*e. g.* S-proteins) and could be binding sites (SOMA *et al.*, 1988). A recent biochemical study showed the presence of low molecular weight, soluble ligands (polypeptides with general structure (g-glutamycysteinil)$_n$-glycine (g-EC)$_n$G, where n=2-11) binding zinc, but also copper and cadmium in the moss *Rhynchostegium riparioides* (JACKSON *et al.*, 1991).

Funaria hygrometrica is a moss which is particularly resistant to environmental pollution and frequently colonizes mining sites where soil is particularly rich in Pb, Zn, Cu and other metals. In addition, it is a bryotherophyte and is one of the few species that can form numerous sporophytes in these conditions (SHAW, 1987). Moreover, this species is resistant to high concentrations of SO_2 (more than 170 mg m-3) (GILBERT, 1970) and it is well known that *F. hygrometrica* protonemata stressed by high concentrations of Cu and Zn develop capsule cells or brood cells, morphological changes that render the species more tolerant to such pollutants (COOMBES & LEPP, 1974). Furthermore, MILES & LONGTON (1990) highlight the high percentage of protonemata surviving under water stress.

It is likely that tolerance is the result of several physiological abilities, rather than a single mechanism. The high resistance shown by *Funaria hygrometrica* to various unfavourable conditions may be determined by many concurring characteristics, among which the capacity of binding toxic ions before they can reach meristematic or reproductive sites is very important. This capacity, together with the high resistance shown by reproductive structures and the high capability of recovering the normal development once favourable environmental conditions are restored (BASILE *et al.*, 1993 a, 1995), may also determine its success in prohibitive habitats for other species.

The success of some species in habitats with a high degree of pollution lies in their high regenerating power and rapidity of spore germination and protonemal development. *F. hygrometrica* has a high reproductive potential, short life cycle (short time of spore germination and protonemal growth), reaching in ten months high values of biomass, rapid growth and a high density of sporophytes (gametophyte biomass : sporophyte ratio = 6.4 : 1) and high number of spores per capsule, about 535,000 (LONGTON, 1976).

Also at the cell level, it is likely that the tolerance is the result of several detoxification mechanisms such as binding by walls or sequestration in cell compartments such as vacuoles or vesicles. Indeed, THURNER & MARSHALL (1971, 1972) suggested that zinc was bound to the cell walls in roots of Zn-tolerant *Agrostis capillaris*. Studies have also revealed the presence of other mechanisms which act at the cell level and which combine to determine the set of detoxification mechanisms. Other authors (THURMAN & RANKHIN, 1982; THURMAN & COLLINS, 1983) have proposed that a cellular compartmentalization mechanism is involved: the metal may become bound to organic acids such as citrates or oxylates or mustard oil glucosides within vacuoles. More recently VAN STEVENICK *et al.* (1987) showed that Zn is complexed with phytate in small vacuoles in root cortical cells. It has been proposed that vacuolar compartmentalization is a mechanism that reduces the toxic effects at the cytoplasm level, and it has been demonstrated that Zn induces vacuolization in root meristematic cells of cereals (DAVIES *et al.* 1992)

Therefore, zinc blockage may be effected through mechanisms and in cellular districts which are different from those typical of lead.

Finally, it is striking that zinc never reached the capsule of *F. hygrometrica.* This blockage took place at each stage of sporophyte maturation, preventing the sporogenous tissue and spores from damage. The massive accumulation of zinc at the placental level found in *F. hygrometrica* confirms the proposed special role of this tissue in detoxification mechanisms (BASILE *et al.*, 1993 b, 1994).

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Abstract

Zinc accumulation in the moss *Funaria hygrometrica* was investigated using experimentally-supplied solutions at different concentrations (10^{-2}-10^{-6} M) and for periods from 1 to 30 d. Zinc accumulation was qualitatively analysed by X-ray SEM microanalysis in different tissues. Zinc is preferentially accumulated in some tissues (gametophyte hydroids, sporophyte hydroids at foot level, and transfer cells) and it reaches the upper part of the seta only when used at the highest concentrations and for the period of 30 days. Zinc, however, never reaches the capsule.

Our experiments demonstrate that *F. hygrometrica* shows a marked capability to sequester zinc in particular tissues, as also demonstrated for lead (BASILE *et al.*, 1994) but in this case the sequestration of zinc at the level of the placenta is less effective if compared to lead, reaching the upper part of the seta when used at 10^{-2} M. However, in the conditions used here, it never reaches the capsule, confirming that the massive accumulation of heavy metals in the gametophyte tissue and at the placenta level plays an important role in detoxification. Moreover, the differences between the data presented here and the results reported in a previous work lead us to hypothesize that the blockage operated by the gametophyte and placenta mainly occurs due to progressive saturation of binding sites present in the walls.

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