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Effects of lead on the regeneration of the moss *Pleurochaete squarrosa* (Brid.) Lindb.

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Riassunto

Gli effetti del piombo sulla rigenerazione del muschio *Pleurochaete squarrosa* sono stati studiati in condizioni controllate *in vitro*. Foglie distaccate del muschio sono state coltivate per 30 giorni in presenza di differenti concentrazioni (da 10^{-3} M a 10^{-7} M) di piombo fornito come sale inorganico. L'inibizione della crescita del 50 % (EC_{50}) si è verificata alla concentrazione di 10^{-5} M. Concentrazioni sub-letali di questo metallo hanno causato alterazioni morfogenetiche durante la crescita del protonema. Le più frequenti alterazioni osservate sono state l'emergenza di nuovi protonemi dalla parte basale delle foglie, a differenza di ciò che accade nel controllo dove essi originano a partire dall'apice fogliare, e la formazione di apici biforcuti. E' stato osservato inoltre una inibizione dose-dipendente nello sviluppo del filamento.

INTRODUCTION

The effects of heavy metals have been described in detail on moss protonemata obtained from spores and shoots (COOMBS & LEPP, 1974; BASILE *et al.*, 1995; BASILE *et al.*, in press), while very few data are available on the effects of heavy metals on protonemata derived from other tissues, such as moss leaves (BASILE *et al.*, 1990 b).

The inhibition of sexual reproduction under polluted or stressed conditions seems to be common to bryophytes. Therefore, in these conditions, vegetative reproduction represents a very important means for the persistence of a species in a disturbed site (RAO, 1982).

The pottiales hold pride of place amongst the orders of mosses for the number of genera and species showing a high vegetative capacity, producing rhizoidal and protonemal gemmae (see

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reviews by RISSE, 1987; WHITEHOUSE, 1987 and subsequent articles by ARTS, 1987a-b; 1988, 1989).

The data on the effects of heavy metals on the regeneration of the pottiales stands in contrast with the wealth of information on their vegetative reproduction. Although spore germination has been described in some genera (NEHIRA, 1983) and a handful of studies have investigated the hormonal stimulation of bud formation (GORTON & EAKIN, 1957; CHOPRA & RASHID, 1969; CHOPRA & REKHI, 1979; RAHBAR & CHOPRA, 1982), there are no reports on the effects of heavy metals on the morphogenesis and regeneration of species belonging to this order.

This work concerns the effects of lead on the regeneration of new filaments in *Pleurochaete squarrosa*. *Pleurochaete squarrosa* is a xerophilous, sub-Mediterranean species growing prevalently on dry, open, sandy soil often near the sea and sometimes locally abundant. Critical studies on the reproductive ecology of *Pleurochaete squarrosa* are scarce but it is well documented that sporophytes are rarely produced in North America (QUARTERMAN, 1956) and Northen Europe (SMITH, 1978), and also in Mediterranean regions (CASARES-GIL, 1932), where the species becomes important in plant successions. However, it was demonstrated that the ability to regenerate from detached leaves is the most important means of reproduction for this species, which otherwise lacks specialized structures for vegetative reproduction (GIORDANO *et al.*, in press a).

Thus, the aim of this work is to describe the effects of lead on the morphogenesis of the protonemal apparatus and bud formation from detached leaves of *Pleurochaete squarrosa in vitro* and compare them with the effects of the same metal on the morphogenesis of the protonemal apparatus produced by spore germination or shoot regeneration in other mosses.

MATERIALS AND METHODS

Plant material

Shoots of *Pleurochaete squarrosa* were collected from sandy soil at the Castelvolturno Nature Reserve (CE - Southern Italy), in a Mediterranean "macchia" site. The plants were washed in a solution of Triton X 100 (0.8%) and rinsed several times in distilled water. The leaves were stripped from the stem, avoiding the very old and very young ones.

Protonema cultures

Detached leaves of *P. squarrosa* were surface sterilized in ethanol 70% (10 seconds) and in 2% NaClO with the addition of a few drops of Triton X-100 (10 seconds). Subsequently, they were washed (10 minutes) with distilled sterile water and kept 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 BBM 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 $Pb(NO_3)$. The cultures were kept in a climatic room with a temperature ranging from 13 °C at night to 20 °C in day, 70% constant relative humidity, and 16 hrs light (45 $\mu Em^{-2}s^{-1}$)/8 hrs dark photoperiod. All the experiments were conducted in triplicate and repeated at least three times. The observations represent averages observed on all the experiments at the different times. The observations and photographs were taken using a Leitz Aristoplan microscope equipped with differential interference contrast optics (Nomarski)

Statistical analysis

Statistical analysis was performed using standard deviation and Student's *t* test and significance of difference was accepted at $p<0.05$. Data are presented as averages from three tests in triplicate.

OBSERVATIONS

Control

The leaves of *Pleurochaete squarrosa* after one week in culture produce filaments of several kinds originating firstly from the apical zone of the leaves (Plate I. Fig. 1). The filaments emerge only from the transitional cells between the midrib and the lamina. The percentage of leaves that produced filaments was 65 %. Each leaf shows 12-13 protonemata emerging from the leaf tips (Plate I. Fig. 2). The filaments look like caulonemata; they consist of 4-6 cells and show the typical cytological organization: the apical cell, in the main filament and in its ramifications, has a characteristic cytoplasmic exclusion zone where the organelles are absent; in the basal zone of this cell there is a large vacuole and the spherical central nucleus is surrounded by small plastids (Plate I. Fig. 3). At this stage of

TAB. I Development of *Pleurochaete squarros*

	protonemal system after 7 days				
	control	**10-7 M**	**10-6 M**	**10-5 M Pb**	**10-4 M Pb**
n. of cells of the main filament	5,3	5.2	4.9	3.4	0
kind of filaments	ca	ca	ca	ca	-
order of SB	I	I	I	-	-
n. of cells from apex for emergence of SB	2	2	2	-	-
n. of cells of I order SB	1	1	1	-	-
n. of cells of II order SB	-	-	-	-	-
	protonemal system after 14 days				
	control	**10-7 M**	**10-6 M**	**10-5 M Pb**	**10-4 M Pb**
n. of cells of the main filament	8.9	8.5	8.6	5.4	2.5
kind of filaments	ca, ch	ca, ch	ca, ch	ca	ca
order of SB	I and II	I and II	I and II	I	-
n. of cells from apex for emergence of SB	5.3	4.9	5	4	-
n. of cells of I order SB	6	5	5	1.4	-
n. of cells of II order SB	1.4	1.6	1.4		-

	protonemal system after 21 days				
	control	**10-7 M**	**10-6 M**	**10-5 M Pb**	**10-4 M Pb**
n. of cells of the main filament	16.2	16	16	9.5	4.4
kind of filaments	ca, ch	ca, ch	ca, ch	ca, ch	ca
order of SB	I and II	I and II	I and II	I and II	I
n. of cells from apex for emergence of SB	5.3	4.9	5	4	4.3
n. of cells of I order SB	9.3	8.9	8.7	6.3	2.4
n. of cells of II order SB	5.2	5.3	4.9	1	-

	protonemal system after 30 days				
	control	**10-7 M**	**10-6 M**	**10-5 M Pb**	**10-4 M Pb**
n. of cells of the main filament	25.3	24.4	24.3	13.4	7.2
kind of filaments	ca, ch	ca, ch	ca, ch	ca, ch	ca
order of SB	I, II and II	I, II and III	I, II and III	I and II	I
n. of cells from apex for emergence of SB	5.3	4.9	5	4	4.3
n. of cells of I order SB	16.3	15.8	15	9.4	4.3
n. of cells of II order SB	5.2	5.3	4.9	3	-

development there are no ramifications. 2-3 buds per leaf emerge at the leaf tips (Plate I. Fig. 2).

At 10 culturing days, the caulonemata have developed short primary branches consisting of chloronemata (transverse septs and numerous chloroplasts). Both in the main filament and in the secondary ones, the nucleus occupies the intermediate position in the cell, the basal zone is occupied by a large vacuole and the apex has the same section as the filament. There are 23-25 caulonemeta per leaf, each consisting of 7-8 cells, while the I order side branches consist of 3-4 cells.

After 14 days, protonemal formation is also observed from the middle part. I and II order branches develop, consisting of 6 and 1-2 cells, respectively.

After 21 days, protonemata develop from the basal part of the leaf, although the greatest growth still occurs in the apical part. The main filaments, consisting of 16 cells, form I and II order side branches of 9 and 5 cells respectively. 12-15 buds are observed per leaf, originating either from the transition cells between the midrib and the lamina or from the first cells of the phyllonemata.

A month after the start of culturing, the protonema number per leaf is so high as to make it difficult to measure the parameters. The filaments which have formed in the apical zone are responsible for the greatest growth. In particular, the caulonemata consist on average of 25.3 cells with chloronemal II order side branches. The filaments look like caulonemata and and chloronemata, having both first and second order side branches of similar diameter but narrower than the main axis. The cross-walls are oblique in the main axis, transverse in second order side branches and oblique and/or transverse in the first order side branches (for details, see GIORDANO *et al.*, in press a). The caulonemata in the proximate zone to the leaflet appear browned. There are 5-6 buds per leaf, which originate directly from the transition cells between midrib and lamina and from the basal (first or second) protonemal cells. The buds have two distinct poles, an upper one with imbricating leaf primordia and a basal one with two or three small elongated cells similar to rhizoids.

The development of the protonemal apparatus in control and lead treated samples is reported in detail in the table 1 and Figs. 7-9.

Lead.

Development is observed at all the concentrations used except for that of 10^{-3} M, but whereas at 10^{-6} and 10^{-7} M concentrations no alterations are noted in the growth pattern compared with the control, at 10^{-5} and 10^{-4} M there is a

reduction in the number of cells per filament and the dose-dependent number of the filaments themselves (Tab.I). EC 50 is 10-5 M.

10-5 M lead concentration.

In the samples treated with a 10-5 M concentration at 7 days from culturing, 25 % of the leaves have developed. Each leaf has, on average, 4.5 filaments with 3-5 cells. No side branches are observed. Unlike in the control, in the samples cultured in the presence of lead, protonemata originate from the middle part of the leaf.

After 14 culturing days the percentage of leaves in which regeneration occurs, rises to 65% and there are 5-7 cells per filament with short I order side branches.

After 21 culturing days there are 8-10 cells per filament, with the presence of I and II order side branches which have the typical arrangement of chloronemata. At this development phase, growth still occurs exclusively at the middle and basal parts of the leaves (Plate I. Fig. 4).

After a month of culturing, 90 % of the phyllonemata have produced protonemata especially from the middle and basal zones of the leaves. At the above lead concentration, there are no alterations in the morphogenetic growth pattern of the protonemal filament. From 27-28 culturing days, 1 or 2 buds per leaf are observed, which originate in a normal position and develop with a similar morphology to that of the control.

10-4 M lead concentration.

The growth of the samples at a lead concentration of 10-4 M appears further retarded compared with the control. Protonemata begin to form from the 15th culturing day in 5-15% of the leaves. The filaments consist of 2-3 cells and originate, also in this case, exclusively from the middle and basal zones of the leaf. After 21 culturing days, I order branches begin to develop (2-3 cells).

Also after a month of culturing, there is very little development of the protonemal apparatus, with a low percentage (20-25%) of protonemal filaments formed per leaf, a fairly small number of cells per filament (7-8) and the presence of only I order side branches. Morphogenetic alterations are observed such as forked tips (Plate I. Figs.5-6) and the absence of filaments at the leaf tip.

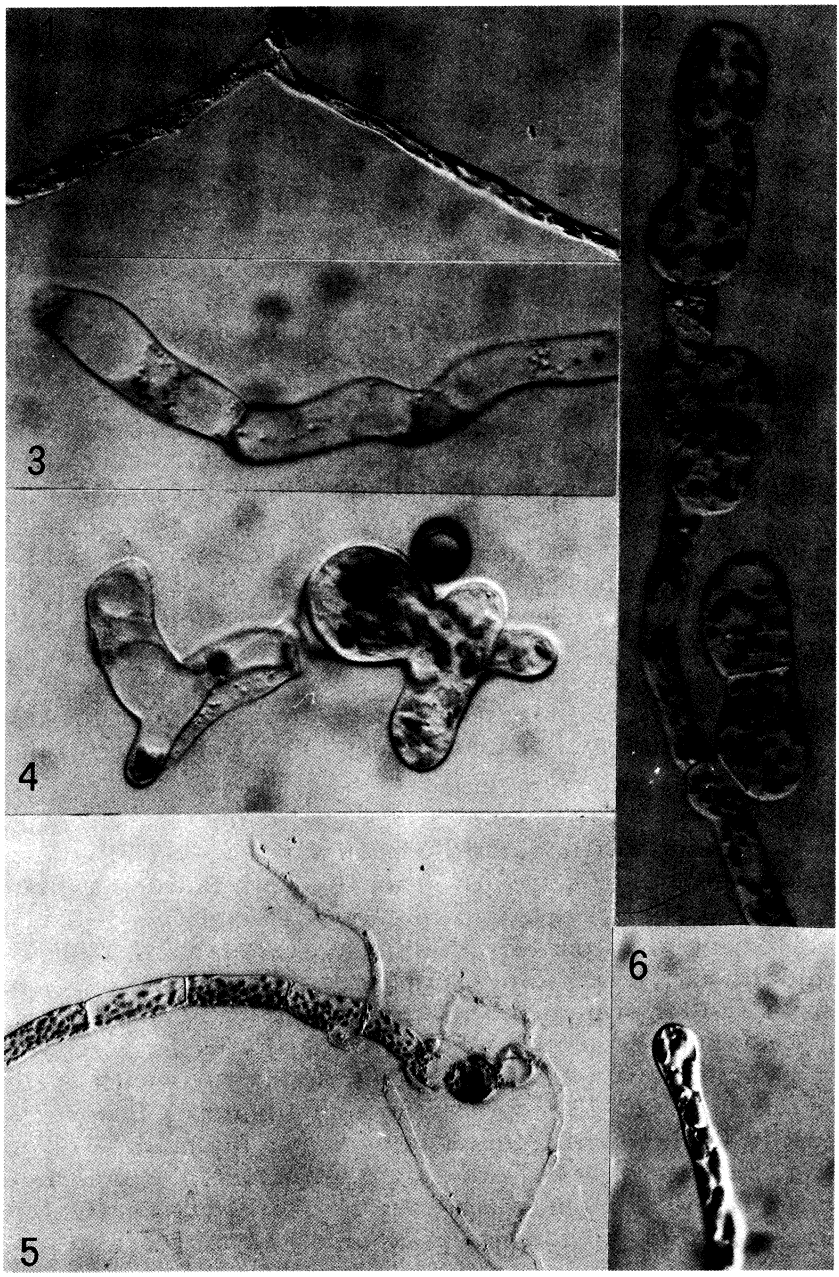
3
4
5
6

Plate I - (1) Detached leaf of *Pleurochaete squarrosa* after 4 days in control medium, showing some protonemata originating from the tip (32x). (2) Detached leaf of *Pleurochaete squarrosa* after 7 days in control medium. It is evident that leaf germination only affects the tip. Moreover, the protonemal apparatus shows I order side branches and some buds (12x). (3) Apical cell of caulonema. Note the apical exclusion zone, the nucleus in the middle and the large vacuole at the base (130x). (4) Detached leaf of *Pleurochaete squarrosa* after 21 days in lead (10^{-5} M) treated samples. It is evident that the protonemal apparatus originates only from the middle and the basal part of the leaf (32x). (5-6) Tip of main filament showing alterations. The forked tip is evident in both cases (230x).

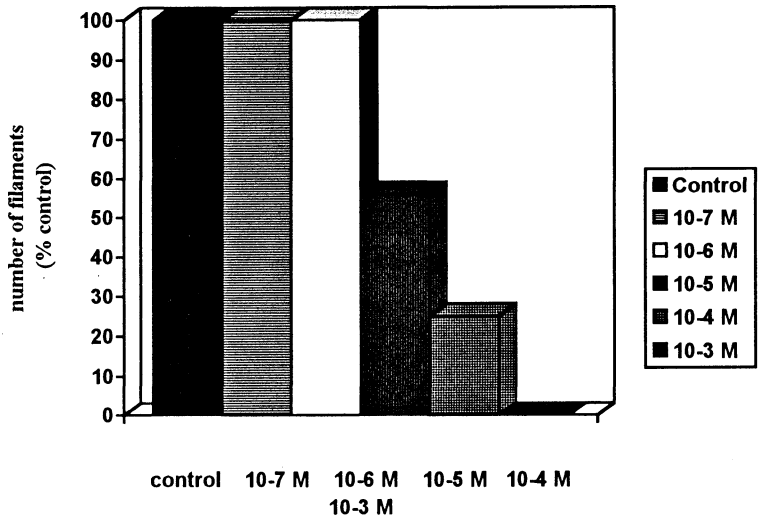

Fig. 7 - Number of filaments per leaf of *P. squarrosa* expressed as % of control after 21 days in culture.

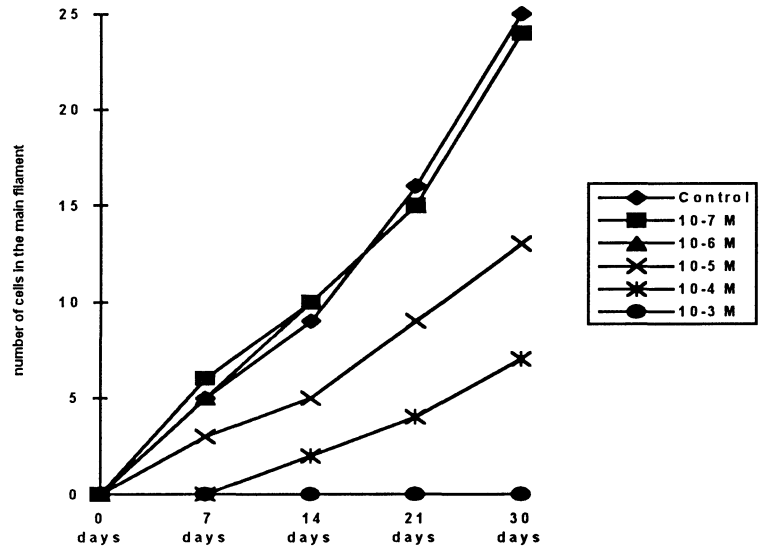

Fig. 8 - Growth of protonema in *Pleurochaete squarrosa* during 30 culturing days. The SD is comprised between ±1 and ±2.5. The most evident effect due to inhibitory fractions is a prolonged lag phase in the emergence of the main filament.

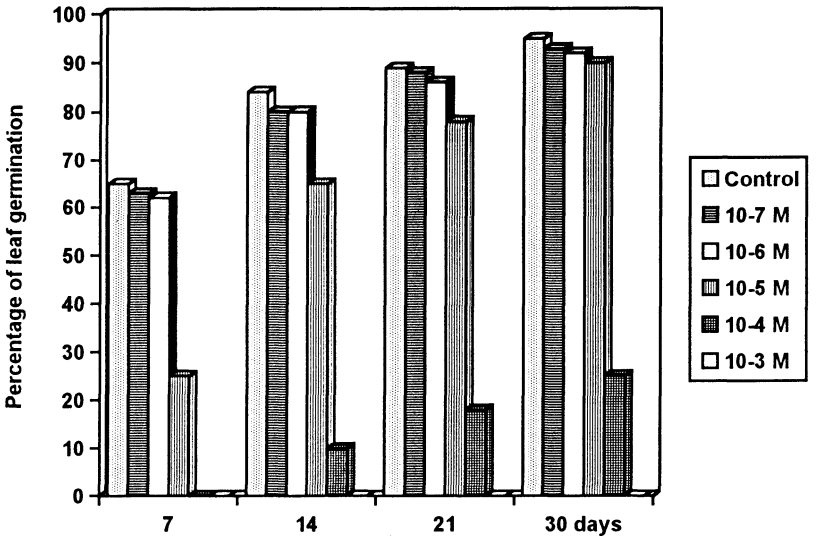

Fig. 9 - Effect of lead on the growth of the main filament expressed as number of cells after 21 culturing days.

DISCUSSION

Until recently, studies on the effects of heavy metals on bryophyte development were restricted to the study of the effects on morphogenesis in toxin-tolerant species (COOMBS & LEPP, 1974; BASILE *et al.*, 1995; BASILE *et al.*, submitted a and b). It has been demonstrated that in these species the reproductive structures are protected from damage on the part of heavy metals by anatomical structures or particular tissue characteristics (BASILE *et al.*, 1990a, 1993, 1994; BASILE *et al.*, submitted b). *Pleurochaete squarrosa* is a moss whose sensitivity to pollutants is not well known. Moreover, the reproduction of the moss in question, both in vitro and in the wild, is not entrusted to specialised structures but occurs thanks to regeneration from detached leaves.

The general effect induced by lead in *Pleurochaete squarrosa* is a reduction and delay in growth compared to the control. Indeed, a reduction is observed in all growth parameters,

including the germination percentage of leaves, number of cells per filament, filament length and their relative side branches. This generalized inhibition may be due to an aspecific toxic effect of the metal upon the cytoplasmatic function, which may occur both through binding to enzymatic proteins (TOMSETT & THURMAN, 1988) and through general damage to the membranes, altering their permeability, exchange of ions and enzymatic activities (HAGER *et al.*, 1987). The damage to such functions causes damage to the numerous cellular activities and to the organelles. In fact, organic lead is known to damage the ultrastructure of chloroplasts, causing alterations to the grana (HEUMANN, 1987). Moreover, it can interfere with the accumulation of ions inside the vacuoles with a consequent loss of turgor. Overall, such effects probably cause general growth inhibition and the reduction in the number of germinating leaves.

Alterations concerning the shape of the growing tip, that frequently appears forked, may be due to the effects of heavy metals on the cytoskeleton (BASILE *et al.*, 1995; HAGER *et al.*, 1987). Also the smaller number of cells formed and of protonemata per leaf, besides a generalized toxic effect, could be due to the direct action of the metals on the microtubules as already shown for lead (WIERZBICKA, 1988) with disassembly of the spindle and altered segregation of the chromosomes during nuclear division.

The most evident effect due to inhibitory fractions is a prolonged lag phase in the emergence of the main filament (Figs. 8-9). Inhibition, in fact, is expressed both as a decreased or lack of ability to produce new filaments (an event that involves first dedifferentiation and later redifferentiation at the cell level) and as an effect which slows down cellular multiplication. In any case, the effect observed is a long delay before filament formation. The lag-phase may also correspond to the time employed by the moss to inactivate the lead down to a critical concentration at which it loses its inhibiting power so that growth can start. After this period, development recovers without alterations. Indeed, also the anomalies in the morphogenetic process disappear, such as forked tips or the absence of apical growth. Such a recovery in the normal growth pattern has also been demonstrated for other species (BASILE *et al.*, 1995 and in press).

In the field *Pleurochaete squarrosa* grows extensively in the gaps of Mediterranean *maquis* and pine-wood vegetation.

Detached leaves of *Pleurochaete* were constantly present in all the samples, but the presence of filaments and buds on the leaves was more frequent on those from gaps in mature Mediterranean vegetation and clearings in the pinewoods, than from recently burned areas (GIORDANO *et al.*, in press a).

The ability of detached leaves to give rise to new plants by regeneration involves dedifferentiation and redifferentiation (BOPP, 1983; LONGTON & SCHUSTER, 1983; CHOPRA & KUMRA, 1988). In *Pleurochaete squarrosa* regeneration occurs mainly at the leaf tip.

In *Pleurochaete squarrosa*, as also in *Aloina* (GOODE *et al.*, 1994) the regeneration site is consituted by the cells of the apex and those between the lamina and midrib. These cells appear to be the only ones capable of dedifferentiation and give rise to the initial cells of protonemata and buds (GIORDANO *et al.*, in press a).

This kind of cell is the most sensitive to the effects of lead. In fact, in lead-treated samples they regenerate until 21-30 days after culturing. The reproductive capacity is the task of the cells at the basal or middle site of the leaf. The fact that the apical zone is the one most affected may be due to the fact that lead interferes with enzymatic systems involved in the complex mechanism of dedifferentiation and redifferentiation. In other bryophyte species, however, the zones involved in reproduction are those least damaged by the heavy metal in question (BASILE *et al.* 1990 a and *submitted* b). Such different behaviour may be explained by the fact that this species has a prefential accumulation of lead in different zones from those involved in reproduction. The greater sensitivity of the reproductive zones in *P. squarrosa* may partly account for the lower resistance shown by this moss compared with more toxin-tolerant species.

Indeed, if we compare the EC_{50} values obtained for the above species with those obtained in the same experimental conditions for other species, we note that the sensitivity to lead of *P. squarrosa* is higher than that shown by the protonemata of *Funaria hygrometrica* (BASILE *et al.*, 1995), *Bryum dunense* and *Tortula muralis* (BASILE *et al.*, in press and submitted a); moreover the capacity to form new buds on the part of liverworts such as *Lunularia cruciata* and *Metzgeria furcata* (BASILE *et al.*, submitted b), compared with which *P. squarrosa* let us to hypotesize that *P. squarrosa* tolerates lead at ten times lower concentrations (this finding is probably also due to the absence of an anatomical barrier which somehow avoids or

reduces the amount of lead reaching the reproductive sites). Moreover, such species rely on specialized structures for their reproduction and possess tissue sequestration mechanisms for heavy metals which enhance their resistance. In *Tortula laevipila* var. *laevipiliformis* sensitivity to lead is very high insofar as it is completely inhibited by concentrations which are ten times lower than those which lead to complete inhibition in *P. squarrosa* (BASILE *et al.*, 1990b). In *T. Laevipila*, reproduction in highly polluted conditions is assigned to the production buds consisting of small apical leaves which form long protonemata originating new gametophytes. The two regenerative systems may thus be compared as they both originate from leaf tissue structures and lack anatomical protection.

In natural ecosystems detached leaves (or their fragments) of *Pleurochaete squarrosa* appear to have a particularly marked capacity for regeneration, as also happens in mosses that usually remain sterile (CHOPRA & KUMRA, 1988), and they certainly represent the major propagative shuttle for dispersal and colonization (GIORDANO *et al.*, in press a). Even if the leaves of *Pleurochaete* are not caducous, they became separated from the plant especially during the summer when the entire plants are fragmented in the desiccated state.

The relative resistance of detached leaves of *P. squarrosa* to the pollutant in question is probably a feature of more generalized tolerance to various environmental factors such as competition with other organisms (the lichen *Cladonia foliacea* as reported in GIORDANO *et al.*, in press b) and $Gram^+$ and $Gram^-$ bacteria (unpublished data). The above tolerance together with resistance to drought and salinity conditions which are typical of the environment where the species is found, is most probably the key factor influencing the distribution and survival of *Pleurochaete squarrosa*.

Abastract

The effects of lead on the regeneration of the moss *Pleurochaete squarrosa* were studied under laboratory conditions. Detached leaves of the moss were cultivated for 30 days in the presence of different concentrations (from 10^{-3}M to 10^{-7}M) of lead used as inorganic salt. Fifty percent growth inhibition (EC_{50}) occurred with a concentration of 10^{-5} M. Sublethal concentrations of this metal caused morphogenetic alterations during protonemal development. The most frequent alterations observed were the emergence of new protonemata from the basal part of the leaves, unlike in control specimens, a generalized delay in filament growth and the formation of forked tips.

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