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# Effects and localization of lead in *Nicotiana tabacum* L. (Solanaceae)

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*Riassunto*

È stato condotto uno studio *in vitro* sugli effetti del piombo sulla crescita, la morfogenesi e la localizzazione a livello di organi e tessuti in *Nicotiana tabacum*. Per gli esperimenti sia piante intere che talee sono state coltivate per 15 giorni in presenza di piombo alle concentrazioni $10^{-3}$ e $10^{-4}$ M. In entrambi i test la concentrazione di $10^{-4}$ M di piombo determina una forte inibizione della produzione e dell' allungamento di nuove radici, mentre la concentrazione $10^{-3}$ M di piombo causa anche alterazioni della morfologia fogliare. La localizzazione del piombo è stata studiata sulle piantine esposte *in vitro* a concentrazioni di $10^{-3}$ M. L'accumulo di piombo è stato analizzato dal punto di vista qualitativo utilizzando la microanalisi X collegata al microscopio elettronico a scansione nei differnti organi e tessuti dei campioni considerati. Il piombo è risultato presente in tutti gli organi e tessuti analizzati.

## INTRODUCTION

While the harmful health effects of carbon monoxide, nicotine, tar, irritants and other noxious gases that are present in tobacco smoke are well known, those due to heavy metals and other toxic mineral elements in tobacco smoke are not sufficiently emphasized. Cigarette smoking may be, in fact, a substantial source of intake of such elements not only for smokers but also, through passive smoking, for non-smokers (CHIBA & MASIRONI, 1992).

The average lead concentration in filter-tipped cigarettes is 2.4 mg/g (MUSSALO-RAUHAMAA, 1986). Smokers and former smokers have higher blood Pb levels than non-smokers (KROMHOUT, 1985). Cigarette consumption depresses the activity of the enzyme 5-

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aminolevulinic acid dehydratase (the most sensitive indicator of the lead burden in the body) in erythrocytes (VIVOLI, 1974). Passive smoking plays an important role in the exposure of children to lead.

Lead can cause damage in the brain and other tissues (STASIK *et al.*, 1969; STURROCK, 1979; CHANG, 1980). Organic lead compounds are also mutagenic (AHLBERG *et al.*, 1972) by interacting with the process of chromosome separation during mitosis and meiosis.

Toxic levels of lead can occur as the results of environmental pollution from mining, smelting, manufacturing, agricultural or waste disposal technologies, but especially as a derivative of the antiknock motor fuel additive triethyl lead (FOY *et al.*, 1978, ZIMMERMANN *et al.*, 1988).

Tobacco plants are able to live and grow on soils near main roads and they undergo the effects of high lead quantities. Little is known about the morphogenetic effects of lead and the processes involved in its immobilization or localization at tissue level (SHARPE & DENNY, 1976).

This work aims to study the effects on growth and morphogenesis of tobacco plantlets after transplanting whole plants or cuttings in lead containing medium as well as the localization of lead at organ and tissue level by X-ray SEM microanalysis.

## MATERIAL AND METHODS

### *Plant culture*

Plants of *Nicotiana tabacum* var. Burley 23 (selectionaded by Sez. Genetica - Ist. Sper. per il Tabacco), were aseptically germinated in solidified MURASHIGE & SKOOG (1962) medium (MS) without hormones at pH 5.7 and cultured at 26°C under 360 $\mu Em^{-2}\ s^{-1}$, 16/8 hours light/dark cycle. After four weeks, when the plants had six leaves, they were used for two different biological assays, namely a) whole plant test and b) cutting test. The whole plants and cuttings (obtained by resecting at collar level) were aseptically transferred into the following media (pH 5.7):

1) MS
2) MS + $Na_2EDTA$ $10^{-4}$ M
3) MS + $Na_2EDTA$ $10^{-3}$ M
4) MS + Lead $10^{-4}$ M
5) MS + Lead $10^{-3}$ M

Lead was added as equimolecular $Pb(NO_3)_2$ complexed with $Na_2EDTA$. In all media containing lead the $NO_3^-$ mole content was kept unchanged by reducing $KNO_3$ and $NH_4NO_3$ in equal amounts. For both assays plants were cultured in the same temperature and light photoperiod previously used.

Observations were carried out at 7 and 15 days after culturing. All the experiments were conducted in triplicate and repeated at least three times.

*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 experiments conducted in triplicate.

*X-ray SEM microanalysis.*

After lead treatment, plants were thoroughly washed in distilled water for 15 min with several changes to eliminate unbound Pb, 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 30 specimens were observed and analysed by microanalysis.

OBSERVATIONS

*Development and morphological observations*

*Control from whole plants*

After 7 culturing days, the plantlets sprout new roots (10 elements) from the collar, each root being 2 to 3 cm long. The plantlets appear generally vigorous, with new 3-4 leaves per sample. After 15 culturing days, there are over 20 new root elements, which often appear intertwined. The new roots attain a length of 5 cm (Plate 1. Fig.1a).

*Control from cutting*

After 7 culturing days, 100% of the plantlets have rooted, producing a tuft of 8 elements from 1.5 to 2.5 cm long. The plants are green and vigorous, with two new leaves being formed per sample. After 15 culturing days, we observed numerous tufts consisting on average of 20 elements 2-4 cm long. The leaves already present appear enlarged and the emergence of other two new leaflets is noted (Plate 1. Fig. 2a).

*Control from whole plants and cutting with EDTA $10^{-4}$ M*

The general appearance of the plants and their growth pattern is not significantly different from those of the control (see above) (Plate 1. Fig. 1a and 2a).

*Control from whole plant with EDTA $10^{-3}$ M*

After 7 culturing days the growth of the plantlets is slightly lower than those of the control, with fewer (6 elements) and shorter (2 cm) roots being formed. The two new leaves have a slightly chlorotic appearance although no malformations were observed in the morphology of the new leaflets. After 15 culturing days, growth is still retarded compared with the control, with fewer new roots (8 elements) and only one leaflets being formed (Plate 1. Fig. 1a and 2a).

*Control from cutting with EDTA $10^{-3}$ M*

Plantlet growth is also, in this case, slightly lower with regard to the control, with the formation of 7-8 new roots on average 1.8 cm long. An average of 2 new leaflets is produced, which are also chlorotic but without malformations. After 15 culturing days, growth is still retarded compared with the

control, with fewer new roots (8 elements) and only one new leaflets being formed (Plate 1. Fig. 2a).

*Lead $10^{-4}$ M from whole plants*

After 7 days, the samples which developed in the presence of lead show only slightly less shoot development than the control, while both the production of new roots (2-3 elements) and their elongation (0.8 cm) are strongly inhibited. After 15 culturing days the severe root inhibition observed after 7 days persists, without the formation of new roots and with no growth in that already present, whereas there is only slightly less shoot growth than in the control. Only slight decoloration was observed in the basal leaves, whose morphology appears nonetheless normal (Plate 1. Fig. 1b).

*Lead $10^{-4}$ M from cutting*

After 7 days, 100% of the plantlets showed roots with a tuft consisting of 4 to 9 elements on average 0.5 cm long. Shoot growth is only slightly less than in the control. After 15 culturing days, also in this case the shoot shows no significant differences compared with the control except for slight less growth and a bland yellowing of the lower leaves. By contrast, root growth is completely inhibited (Plate 1. Fig. 2b).

*Lead $10^{-3}$ M from whole plants*

After 7 culturing days, both root and shoot growth are strongly inhibited. Plantlet growth is much lower than in the control (also that with EDTA): new roots (1-2 mm) are rarely produced and the leaves already present do not develop; they do not expand and on average one new leaf is formed which appears morphologically abnormal in that the length-width ratio is anomalous (narrow leaf). The basal leaves show evident patches of decoloration. After 15 culturing days, the aerial part suffers from advanced decoloration of the basal leaves. In almost 100% of the plantlets, the first leaf to develop after culturing is narrow, while subsequently formed leaves are morphologically normal, albeit smaller than the control. The emerging new roots do not grow (Plate 1. Fig. 1c).

*Lead $10^{-3}$ M from cutting*

After 7 culturing days, only 50% of the plantlets rooted (5-10 elements), producing a tuft of 1-2 mm-long. The shoot is also

inhibited with regard to the control, with fewer leaflets being formed and the presence of less laminar expansion. Decoloration also occurs in the basal leaves. After 15 culturing days, all the samples show root elements which, however, are short compared with the control. The shoot is less developed than the control and shows decoloration of the basal leaves. Also on the same plant, the leaves have two types of malformations: narrowness (lamina restriction) and curling of the leaf tip towards the inside (spoon-shaped lamina). Such malformations affect the first and the second leaf formed after culturing (Plate 1. Fig. 2c).

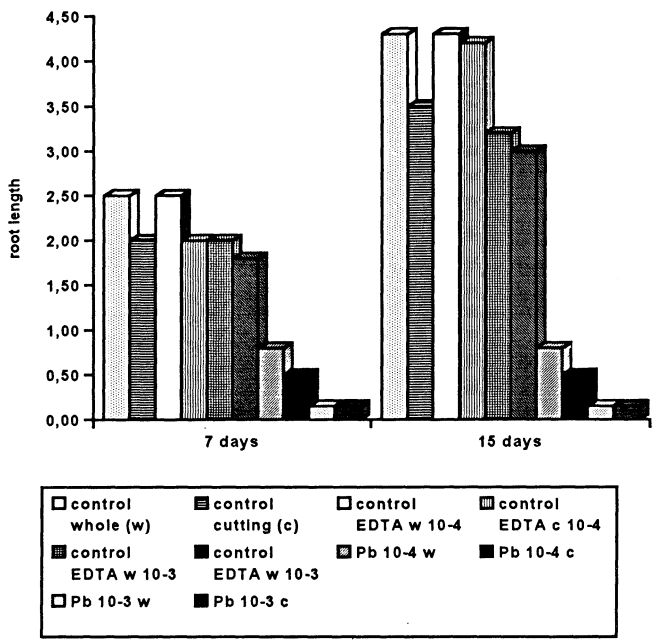


Fig. 7. Effects of lead on root growth expressed in cm. The data represent means calculated from three tests conducted in triplicate for each kind of sample at different times. Statistical analysis was performed using Student's *t* test ($p>0.05$). SE was not more than ± 0.2.

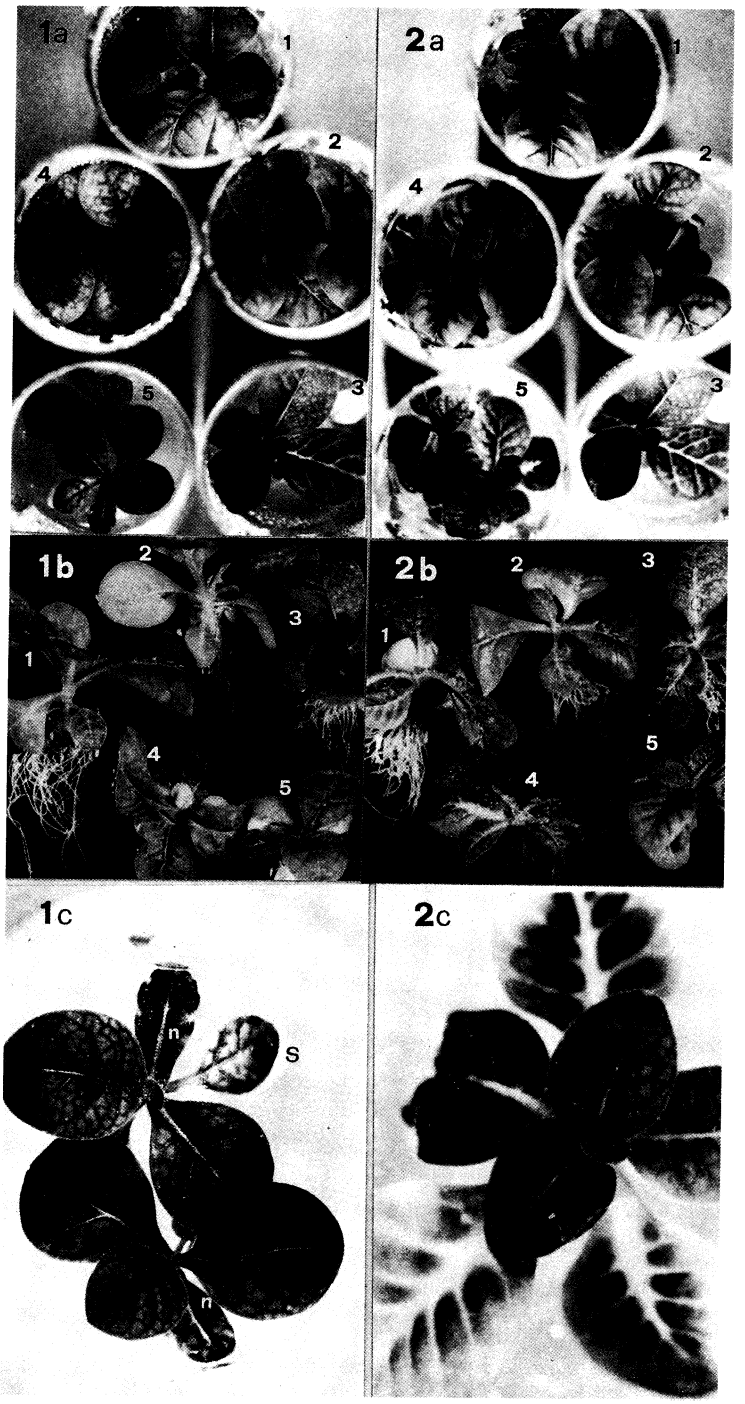
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Plate I - Fig. 1a, 1b, 1c: whole plant test

Fig. 1a - Shoot development of lead treated and untreated plantlets after 15 culturing days (1. MS; 2 MS + EDTA $10^{-4}$ M; 3 MS + EDTA $10^{-3}$ M; 4 MS + lead $10^{-4}$ M; 5 MS + lead $10^{-3}$ M).

Fig. 1b - Shoot and root development of lead treated and untreated plantlets after 15 culturing days (1. MS; 2 MS + EDTA $10^{-4}$ M; 3 MS + EDTA $10^{-3}$ M; 4 MS + lead $10^{-4}$ M; 5 MS + lead $10^{-3}$ M).

Fig. 1c - $10^{-3}$ M lead treated plantlets after 15 culturing days. Details of the two types of malformations: narrow lamina (n) and spoon shaped lamina (s).

Plate I - Fig. 2a, 2b, 2c: cutting test

Fig. 2a - Shoot development of lead treated and untreated cuttings after 15 culturing days (1. MS; 2 MS + EDTA $10^{-4}$ M; 3 MS + EDTA $10^{-3}$ M; 4 MS + lead $10^{-4}$ M; 5 MS + lead $10^{-3}$ M).

Fig. 2b - Shoot and root development of lead treated and untreated cuttings after 15 culturing days (1. MS; 2 MS + EDTA $10^{-4}$ M; 3 MS + EDTA $10^{-3}$ M; 4 MS + lead $10^{-4}$ M; 5 MS + lead $10^{-3}$ M).

Fig. 2c - $10^{-3}$ M lead treated cuttings after 15 culturing days. Details of leaf cost and lamina decoloration.

*Tissue localization.*

Lead localization in different organs and tissues of *Nicotiana tabacum* was qualitatively assessed employing SEM X-ray microanalysis on the specimens treated with $Pb(NO_3)_2$ $10^{-3}$ M solutions. The specimens show the presence of lead both in root and shoot part. The localization appears to affect all the tissues in question.

## DISCUSSION

The general effect induced by lead in *Nicotiana tabacum,* var Burley 23, is a reduction and delay in growth compared to the control. Both kinds of samples (whole plants and cuttings) show a similar behaviour with regards to lead treatment; however, cuttings appear to be more sensitive. In particular, the parameter mainly affected by lead is root elongation. The main target of lead would seem to be the root tip. It is well-known that lead is strongly bound by the cell walls which reduce the quantity of the metal able to penetrate the cytoplasm. It is reasonable to suppose that meristematic cells with their thinner cell walls have less protection in relation to the entry of lead in the cytoplasm and are thus more affected by its toxicity. On the other hand, lead which penetrates the cytoplasm may induce an effect on the microtubular cytoskeleton (BASILE *et al.*, 1995), disassembly of the spindle and altered segregation of the chromosomes during nuclear division (WIERZBICKA, 1988), thereby causing damage to the phenomenon of cellular division. However, root growth is reported to be so highly sensitive to metals that it has been used for a number of years as a parameter for assessing the metal tolerance of plants (WILKINS, 1978).

Besides the effect of lead on microtubules, growth inhibition may be due to a general toxic effect of lead on enzymatic proteins (TOMSETT & THURMAN, 1988; HAGER *et al.*, 1987).

The root apparatus would thus appear to be the structure which is most sensitive to the effects of metal insofar as it is already strongly inhibited at a $10^{-4}$ M concentration. On the contrary, there is no observable lead intoxication on the shoot at $10^{-4}$ M and concentrations 10 times greater need to be reached for there to be observable damage in leaf morphology. At a $10^{-3}$ M concentration, besides less development in the leaf area, malformations occur (narrow and spoon leaves). Such report may

also be related to the effect of lead on microtubules and therefore on the correct orientation of the division planes and hence for normal morphological development. It is worth pointing out that such malformations occur especially on the first leaves produced after culturing, while subsequent leaves show normal morphology. Subsequent morphologically normal leaf production may be due to the lowering of the lead concentration below a given "threshold" which is necessary for a malformation to occur. These observations are in agreement with the results of a previous work on *Funaria hygrometrica* (BASILE *et al.*, 1994), where the authors reported that there is a recovery of the normal growth pattern after a certain time from the beginning of culturing of samples in the presence of lead. This has been interpreted as the result of the removal of free lead from the culture, because of its bonding with wall sites or with other structures able to interact with it. Moreover, it is reported that lead toxicity in tobacco resembles an early symptom of frenching, that is a physiological disease in tobacco characterized by a narrower leaf (DAVID *et al.*, 1955).

Observations with x-ray SEM microanalysis show that lead is present in all the plant tissues and its accumulation thus also affects the shoot. The viability and growth, although reduced in our specimens, in which a large lead uptake occurred, is surprising in the light of evidence that lead concentrations as low as 1 p.p.m have a profound effect on events associated with photosynthesis and respiration. Furthermore, it was hypothesized that large amounts of lead may be taken up by plant roots, immobilized by dictyosome vesicles and deposited in cell walls (ZIMDAHAL 1977). It was reported that a large amount of Pb ions links to negative charges of pectic compounds (galacturonic acids) of the cell wall (BROWN & BATES, 1972; BURTON & PETERSON, 1979; SATAKE *et al.*, 1983; SATAKE & MIASAKA, 1984; BROWN, 1984; BROWN & BUCK, 1985; SATAKE *et al.*, 1989; BROWN & SIDHU, 1992; BASILE *et al.*, 1994). Such lead compartmentalizations remove it from the cytoplasma where it may have a toxic effect, which may explain the absence of lethal damage in the presence of large quantities of heavy metal.

X-ray SEM microanalysis experiments are under way to ascertain whether there is prefential accumulation in some tissues in additional to a conventional and X-ray TEM microanalysis for a study of the phenomenon at the cell level.

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*Abstract*

A study was carried out under laboratory conditions on the effects of lead on growth and morphogenesis as well as lead localization at the tissue and organ level in *Nicotiana tabacum*.

For the experiments whole plants or cuttings were cultivated for 15 days in the presence of various concentrations ($10^{-3}$ M and $10^{-4}$ M) of lead used as nitrate. In both kinds of tests lead concentrations of $10^{-4}$ M determined significative reduction in root formation and, in particular, elongation, while concentrations of lead $10^{-3}$ M also caused alterations in leaf morphology . Lead localization was investigated using experimentally-supplied solutions at concentrations of $10^{-3}$ M. Lead accumulation was qualitatively analyzed by X-ray SEM microanalysis in different tissues and organs. It is present in all the organs (roots, leaves and stems) and in all the tissues under consideration.

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