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The bianchetto truffle (*Tuber borchii*) a lead-resistant ectomycorrhizal fungus increases *Quercus cerris* phytoremediation potential

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Running title: *Tuber borchii-Quercus cerris* Pb tolerance

Originality-significance statement

This work firstly demonstrates the high tolerance of *Tuber borchii* mycelium and of *Quercus cerris* seedlings mycorrhized with *T. borchii* to high Pb concentrations.

Tuber borchii mycorrhization as well as Pb treatment influence the uptake and translocation of Pb and other elements within the host plant.

The results suggest that *T. borchii* - *Q. cerris* mycorrhized plants could have great potential for practical application in bioremediation.

Abstract

Tuber borchii is a European edible truffle which forms ectomycorrhizas with several soft- and hardwood plants. In this paper the effects of high level of Pb on the in vitro growth of five *T. borchii* strains and the molecular mechanisms involved in Pb tolerance were studied. Moreover, the effects of the Pb treatment on *T. borchii* ectomycorrhizas and on the growth, element uptake and distribution in different organs of *Quercus cerris* seedlings were investigated. The results showed an extraordinary tolerance of *T. borchii* mycelium to Pb: all the tested strains were able to grow at Pb concentration over 4000 mg L^{-1}. The mechanisms of tolerance seem related to Pb sequestration in the vacuole and its immobilization as crystal of Pb oxalate outside the hyphae rather than detoxification processes, considering the low expression of glutaredoxin and thioredoxin genes.

T. borchii - *Q. cerris* mycorrhizas tolerate a soil concentration of Pb from 1,869 to 4,030 mg kg^{-1} although at these Pb concentrations *T. borchii* showed a reduced ability to colonize roots. *T. borchii*

mycorrhization increased the uptake of Pb by *Q. cerris*. Mycorrhization and Pb treatment also significantly influenced the uptake and translocation in the plant of other elements.

Key words: lead nitrate, mycelium, detoxification mechanisms, ectomycorrhizas, bioconcentration factor, translocation factor, element uptake

Introduction

Contamination of groundwater and soil with heavy metals is now one of the major problems of pollution of natural environments and pose risks to human health as they enter the food chain via agricultural products or contaminated drinking water (Cerbasi and Yetis, 2001; Zhang, 2020). Acidification of forest soils in highly industrialized areas accompanied by high concentrations of heavy metals determine the impoverishment of the fungal community and, a strong reduction of biodiversity in different ecosystems (Fellner and Pešková, 1995; Lenart-Boroń and Boroń, 2014; Passarini *et al.*, 2022). Among soil organisms, fungi are highly resistant to heavy metal pollution and play important roles in element cycling, mineral transformation, plant stress tolerance and soil bioremediation (Massaccesi *et al.*, 2002; Gadd, 2007; Văcar *et al*., 2021). In particular, mycorrhizal fungi have a high tolerance to heavy metals and are able to colonize areas that have high concentrations of Pb^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Ni^{2+} (Hachani *et al.,* 2020; Yu *et al.,* 2020). The biochemical and genetic mechanisms underlying heavy metal uptake and translocation in the plant by mycorrhizal fungi are not completely known at this time, and their understanding is the basis for developing new strategies for myco-remediation (Akhtar and Mannanab, 2020; Robinson *et al.,* 2021). To fill this knowledge gap, specific *in vitro* and in greenhouse studies on the tolerance and the accumulation capacities of mycorrhizal fungi to heavy metals are powerful strategies.

Among heavy metals, lead is one of the most harmful to plant and animal health. In the plants lead toxicity causes disturbs in plant water and nutritional relations and causes oxidative damages; as consequence, it inhibits the plant growth from seed germination (Zulfiqar *et al.,* 2019). The oxidative damages caused by lead exposure have also detrimental effects on animals and humans causing a range of cardiovascular, renal, neurologic, and hematologic dysfunctions with serious health problems which might be permanent and lead to fatality (Assi *et al.,* 2016).

The diffusion of lead into the environment is primarily due to disposal of metals, the use of paints based on Pb and the use of pesticides and fertilizers. Lead is also the main pollutant produced by the combustion of leaded petrol (Page and Gange, 1970), and it was accumulated in intense traffic areas for a long time (Wang and Zhang, 2018).

Truffles are hypogeous ectomycorrhizal ascomycetes belonging to the order of Pezizales. Some species in the genus *Tuber* are highly appreciated for the organoleptic properties (i.e., taste and flavor) of their edible ascomata (Mello *et al. ,* 2006). *Tuber borchii* Vittad. is one of the most common truffles in Europe being found from Finland to Sicily and from Spain to Iran, in different soil conditions (Hall *et al.,* 2007; Puliga *et al.*, 2021) and, in contrast to other *Tuber* species, also tolerates acidic soils (Gardin, 2005; Lancellotti and Franceschini, 2013) where heavy metals are more available (Sintorini *et al.,* 2021). It can form mycorrhizas with many soft- and hard-wood plants (Hall *et al.,* 2007). It is also the unique *Tuber* species which mycelium can growth easily in vitro and, for this reason, it is considered a model species for studying the truffle biology and genetics (Leonardi *et al.,* 2019). Previous *in vitro* studies carried out on agarized substrate have shown that *T. borchii* increases the tolerance of *Cistus creticus* L. to zinc, lead and chromium (Sabella *et al.,* 2016) but the molecular mechanisms involved in the tolerance to heavy metals of a truffle species have never been investigated so far. Among the favorite hosts, *Quercus cerris* L.

is a pollution-tolerant tree native to southern Europe and Asia Minor which dominates in the mixed forests of the Mediterranean basin (Najib *et al.,* 2021). This tree is often used as host plant in truffle cultivation being able to form ectomycorrhizas with all the European edible *Tuber* species (Hall *et al.,* 2007).

In this paper the effects of high level of Pb on *T. borchii* mycelium and the molecular mechanisms related to its tolerance have been investigated by *in vitro* growth tests and gene expression analyses. Moreover, the effects of Pb on *T. borchii* root colonization as well as the growth and nutrient uptake of *Q. cerris* and the distribution of Pb and other elements in the different plant organs (roots, stems and leaves) have been also tested. The potential of *Q. cerris* mycorrhized plants for phytoremediation was also discussed.

Results

In vitro assessment of Pb on T. borchii *mycelial growth*

All *T. borchii* strains analyzed showed a dry weight not significantly dependent from Pb concentration ($p=0.60$), whilst strains showed a different overall mean weight ($p<0.001$) (Fig. 1 and Tables S3). The oldest strain (1BO in culture from 1987) had the lowest dry weight at all the tested Pb concentration and in the control (Fig. 1). However, all strains mostly responded to the presence of Pb by increasing their diameter growth, in particular at the highest Pb concentrations (Table S4, Fig. S1). Light microscope images of the Tbo5118 mycelium showed that the Pb absorbed was sequestered into the vacuoles of mycelia grown at concentrations up to 2.56 mM (Fig. 2a) while at higher Pb concentrations (up to 20.48 mM), a large number of needle- and star-shaped crystals were formed on the cell wall surface of hyphae (Fig. 2b).

Gene expression

Changes in gene expression of mycelia treated or untreated with lead was verified by analyzing the following five genes: 1) thioredoxin (TbThio), 2) glutaredoxin (TbGlut), 3) a putative Na(+)/Li(+)-exporting P-type ATPase (TbNL), 4) TbRhoGdi, 5) TbCdc42. TbThio and TbGlut were used to evaluate the possible activation of cell detoxification mechanisms, TbRhoGdi and TbCdc42 are related to hyphal apical growth (Menotta et al., 2007; Picceri et al., 2018) and TbNL was selected as possible gene involved in metal transport.

The expression of the genes Tb*Cdc42*, Tb*RhoGdi* and Tb*NL* was significantly higher in pure culture of the strain Tbo5118 grown in presence of lead with respect to the controls (Fig. 3). Instead, the expression analysis of Tb*Glu* and Tb*Thio*, genes did not reveal significant differences.

Effects of Pb on plant growth and on mycorrhizal infection

Both mycorrhizal colonization with *T. borchii* and Pb treatment did not influence the height of the plants one year after inoculation and eight months after the first Pb treatment, whereas the collar diameter was only significantly higher in mycorrhizal plants (Table S5). No foliar symptoms were observed in any plant treated with Pb (mycorrhized and non-mycorrhized: 1mic-1Pb and 0mic-1Pb). At the second check of mycorrhizal infection two plants, one treated with Pb (1mic-1Pb) and one not treated with Pb (1mic-0Pb), were found damaged and therefore were not considered in the subsequent analyses. No contaminations with other fungal species were found. The plants increased their mycorrhization degree with *T. borchii* 8 months after the first Pb treatment. However, the mycorrhizas increased significantly more ($p<0.05$) in the plants not treated with Pb (+ 30% for 1mic-0Pb and + 5% for 1mic-1Pb, Fig. 4a). Microscope analyses of ectomycorrhizas

showed the presence of Pb in vacuoles of the extraradical mycelium (Fig. 4c) and needle-like Pb crystals on the surface on some hyphae and cystidia (Fig. 4d, f). Inside the cross and longitudinal sections of the mycorrhizas, lead was found to accumulate in the inner part of the mantle and in the Hartig net (Fig. 4e,g).

Phytoextraction efficiency

At the end of the experiment, the mean soil Pb concentration was 2,006 and 3,594 mg kg^{-1} in 1mic-1Pb and 0mic-1Pb pots, respectively and reached 4,000 mg kg^{-1} in two 0mic-1Pb pots. It was significantly higher in the 0mic-1Pb pots than in the 1mic-1Pb pots (Fig. 5b). The mean Pb concentration of untreated soils was 12.98 and 22.95 mg kg^{-1} in 1mic0Pb and 0mic-0Pb pots, respectively and no significant differences were found between them. The presence of *T. borchii* mycorrhizas as well as the Pb treatment significantly increase the BFC (Fig. 5a). The Pb was significantly more concentrated in roots and stem than in the leaves (Table 1). The Pb quantity was significantly higher in all the organs of treated plants, whereas *T. borchii* mycorrhization significantly increases the Pb content only in the stems (Table 1).

The treatment with Pb and the mycorrhization with *T. borchii* influenced also the concentration of several elements in plant organs. In particular, the leaf concentration of K and Zn significantly increases in 1Pb and 1mic. The Pb treatment also increases the leaf concentration of Ca, S, Li, Ni, Sr in the leaf, whereas decreases the P concentration. In contrast, the mycorrhization with *T. borchii* increased the P, Cu and Na concentration in the leaf and reduced the Se and Mn concentration.

153 The concentration of K, Cu, P and Zn was also significantly higher in the stem of both 1mic and
154 1Pb plants. In contrast the Ba concentration was significantly lower in both 1mic and 1Pb plants.
155 The Pb treatment significantly increased Mo concentration in the stem and decreased the B content.
156 *Tuber borchii* mycorrhization decreased Ca concentration in the stem.
157 In the roots Ca, K, P and Sr concentration was significantly higher of both 1mic and 1Pb plants.
158 The Pb treatment also significantly increased the Na, S, Ba, Cu and Se concentration in the roots.
159
160 The translocation factors (TFs and TFl) reported in the Fig. 6 show how the elements are
161 translocated from the roots into the other organs. Most of heavy metals such as Pb, Mo, Cu, Cr, Fe
162 and Al are scarcely translocated in the leaf and in the stem. In contrast Ca, Mn and Ba are
163 translocated from the root to the stem and the leaf. P, Mg, Li, B, Na, S and K are principally
164 translocated in the leaf. There was no obvious effect of Pb treatment and mycorrhization on TFs
165 and TFl.
166
167 **Discussion**
168 In the last 25 years, *T. borchii* has been frequently used as a model species within the genus *Tuber*
169 for transcriptomic and functional studies and to shed light on issues of the truffle life cycle
170 (Zambonelli *et al.,* 2021). The responses of its mycelium and/or mycorrhizas to different stress
171 conditions such as high temperatures, nitrogen starvation or heavy metals have been also
172 investigated (Montanini *et al.,* 2006; Leonardi *et al.,* 2017; Sabella *et al.,* 2016).
173 In this work we adopted a multidisciplinary approach to analyses the effect of lead on mycelium
174 and mycorrhizas of *T. borchii* and the detoxification strategies implemented by the fungus as well
175 as the effect on plant nutrition.

In our study, *T. borchii* showed a very high tolerance to Pb concentration in the substrate. All the tested strains tolerate a Pb concentration up to 4,200 mg L^{-1} although they were isolated in non-contaminated soils. This Pb concentration is higher than those previously tested for most of the other mycorrhizal fungi (Table S6) .Only the mycelia of fungal species isolated from contaminated soils showed a similar tolerance to Pb (Liquat *et al.,* 2020). The mycelial biomass was little affected by high Pb concentration in the substrate whereas the colony expansion was even slightly stimulated by high Pb concentration in the substrate. In this regard, we evaluated the expression of genes related to apical growth in fungi, such as Tb*Cdc42* and Tb*RhoGdi* in the tested fungal strains. The greater expression of these two genes in mycelia grown in the presence of lead compared to the controls, demonstrated that *T. borchii* is stimulated in apical growth in the presence of Pb. This behavior, in our opinion, can be interpreted as a defensive strategy adopted by the fungus to colonize new parts of the substrate in search of unpolluted soil.

The microscopic analyses showed that the fungus is able to perform two different active mechanisms in order to avoid the cellular toxic effects of the lead. As shown by the specific coloration with sodium rhodizonate (Tung and Temple, 1996), Pb at low concentration is sequestered in the vacuole of the fungal cell. This type of behavior would allow the fungus to continue to perform its vital functions without suffering the toxic element. In fact, it has been reported that ECM fungi are able to chelate metal by glutathione and to compartment in the vacuole the metal-glutathione complex (Smith and Read, 2010).

At higher concentrations of Pb, up to 20.48 mM, we also noted the presence of a large number of crystals of Pb on the hyphal cell wall. This led us to speculate that beyond a certain threshold concentration, the Pb is inactivated, in crystalline form, and accumulated outside the fungal cells. In this way the fungus would prevent the intracellular accumulation of potentially lethal amounts

of Pb. The formation of crystals was also observed on the mycorrhizal mantle as well as in the cystidia and the extra-radical mycelium.

The precipitation of extracellular Pb could be enhanced by an active secretion of fungal exudates in the wall, such as oxalic acid which was found to contribute to the 'outer defense line' to resist Pb (Tian *et al.,* 2019). In fact, oxalic acid can react with Pb cations to form lead oxalate crystals (Ceci *et al.,* 2015) which were easily recognized under microscopy due to their characteristic shape and red color after staining with sodium rhodizonate.

These data are strictly correlated to the evidence highlighted by gene expression analyses, that showed a higher expression of the Tb*NL* gene in the presence of lead in the growth substrate. We can assume that this gene may be involved in the compartmentalization in the vacuoles of Pb, favoring an outward transport, to compensate for the low level of protective detoxification enzymes. In fact, the lack of upregulation of thioredoxin and glutaredoxin genes in mycelia grown in substrate added with 5.12 mM $Pb(NO_3)_2$ indicates that the cellular mechanisms of detoxification were not activated.

The treatments with $Pb(NO_3)_2$ in the pots where mycorrhized and non mycorrhized plants were grown allowed to reach a final Pb concentration in the soil variable from 1869 to 4030 mg/kg. These concentrations are similar to those found in the highly polluted soil around mining areas in China (Shi *et al.,* 2021). At so high Pb concentration no symptoms were observed in neither control nor mycorrhized plants treated with lead. The plant development was also not affected by Pb treatment. *Tuber borchii* ectomycorrhizas were not visibly damaged by the presence of Pb but the total degree of mycorrhization at the end of the experiment increased significantly more in non-treated plants. The lower level of root colonization in plants treated with Pb could be interpreted as conflicting with the results obtained from mycelial growth tests. However, the presence of Pb

222 in soil may interfere with the symbiosis establishment, hindering the plant-fungus recognition or
223 slowing down the root colonization. The negative effect of Pb and other heavy metals on
224 abundance of ectomycorrhizas was already demonstrated by different authors (Chappelka *et al.,*
225 1991, Bojarczuk and Kieliszewska-Rokicka, 2010; Ouatiki *et al.,* 2021).
226 Nevertheless, the presence of *T. borchii* mycorrhizas significantly increase the uptake of Pb from
227 soil and its accumulation in plant tissues. These results are in contrast with the experiment carried
228 out on poplar microcuttings mycorrhized with *Paxillus involutus* where the presence of
229 ectomycorrhizas significantly reduced the bioaccumulation factor of Pb (Szuba *et al.*, 2021) but it
230 is coherent with Sun et al. (2022) who found that *Suillus luteus* enhanced Cd accumulation in oak
231 seedlings.
232 After absorption, Pb concentration in stems and roots of mycorrhized plants was, respectively, 13
233 and 15 times higher than leaves while this gap was reduced to about 4.5 times in plants without *T.*
234 *borchii* ectomycorrhizas. Our result is consistent with the reports of many similar studies that
235 demonstrate that Pb is mainly accumulated in the roots (Xiong, 1997; Escobar and Dussan, 2016
236 Szuba *et al.,* 2021) and partially translocated in the shoots. The leaf showed a low concentration
237 of Pb that was the probable reason why no foliar symptoms were detected during the four months
238 of experiments. As reported in literature foliar tissues are the final frontier for metal(oid) uptake
239 via the roots; thus, part of a plant´s plan is to limit the entrance or translocation of metallic elements
240 into photosynthetic tissues (Clemens and Ma, 2016; Angulo-Bejarano, 2021).
241 The uptake and translocation of the elements in the plant was differentially affected by Pb
242 treatment and/or the symbiosis with *T. borchii*. Only 6 out of 21 analyzed elements (Fe, Al, Cr,
243 Mg, Sb, V) did not significantly changed their concentration in roots, stems or leaves regardless
244 the treatment. The effect of mycorrhizas was limited to 10 elements of which 6 increased and 3

decreased their concentration in one or more plant tissues while Ca was differentially translocated in stems and roots. Pb treatment significantly increased the concentration of 13 different elements in at least one of the plant tissues and reduced the concentration of only three elements in leaves (P) or stems (B and Ba). In general, our results are in contrast with those reported by other authors who report that Pb can disrupt the assimilation of some fertilization elements such as Ca, Fe, and Mg and limit their absorption and/or transport to leaves (Houda *et al.*, 2016). In our work, the Pb treatment increased the quantity of Ca as well as of other metals such as K, Li, Zn, Ni and Sr in the leaves. On the other hand, the treatment with Pb increases the concentration of heavy metals (such as Cu and Sr) in the roots of the plants treated with Pb especially in the mycorrhized plants. As reported in literature, most of the Pb is translocated to the shoots after its absorption into the roots using passive mechanisms that rely on H+/ATPase systems (Angulo-Bejarano, 2021). The increased expression of Tb*NL* in *T. borchii* mycelium in presence of Pb (this work) as well in truffle mycorrhizas (Martin *et al.,* 2010) would explain the greater Pb and other metal uptake by the mycorrhizal structure and their transport into the plant body. In fact, the protein encoded by this gene has a homology with Na(+)/Li(+)-exporting P-type ATPase, a P-type sodium transporter involved in several processes, including cellular response to glucose starvation, hyperosmotic response and metal ion transport. Moreover, it has also a lower homology with a metal transporter ATPases (OSNPB_060665800 Smith *et al.,* 2014, Amari *et al.,* 2017).

On the other hand, the high presence of Pb could have also induced changes in the activity of the plant plasma membrane H+-ATPase and increased their transport into the host plant.

The Pb absorbed by *T. borchii* hyphae seems not to have accumulated in the mantle as suggested by some works (Chot and Reddy, 2022) but transferred towards the Hartig net where Pb was found more visible by staining with sodium rhodizonate.

Another hypothesis to explain the increasing Pb uptake in mycorrhizal plants is that the Pb uptake of roots follows water flow, which is facilitated by the aquaporins as suggested by Zhang *et al.* (2021) in plants of *Medicago truncatula* colonized with the arbuscular mycorrhizal fungus *Rhizophagus irregularis*.

P concentration in the leaf was remarkably reduced in the plants treated with Pb. This could be attributed to Pb phytotoxicity on major metabolic processes such as reducing water and nutrients uptake and transport, chlorophyll formation with induced oxidative stress via production of reactive oxygen species (Zulfiquar *et al.,* 2019). *Tuber borchii* mycorrhization promoted P uptake and translocation compared to non-inoculated plants in the soil contaminated with Pb, similarly to those observed with soybean plants inoculated with arbuscular mycorrhizal fungi (Adeyemi *et al.,* 2021). Similarly, a previous study on the heavy metal Cd showed that the mycorrhization of *Quercus acutissima* seedlings even doubled the P content in the roots although only at low Cd concentrations (0.1 mg/kg) (Sun *et al.,* 2022). It is supposed that soil exploration type (Agerer 2001) of the ectomycorrhizal fungi is one of the most important factor in P uptake. ECM fungal species with long exploration type via abundant extraradical mycelium may have a competitive advantage over species with contact exploration in the search for water and P (Köhler *et al.,* 2018). Even though the mycorrhizas formed by *Tuber* spp. are considered of contact exploration type (Agerer, 2006), they were able to significantly increase P uptake. On the other hand, *T. borchii* as other ectomycorrhizal fungi seems to secrete organic acids and probably phosphatases which improve the availability of soil P, or increase mycelium inputs to facilitate plant acquisition of P. In the acidification process, organic acids secreted by *T. borchii* could reduce pH in the rhizosphere soil and increase the exchange capacity of soluble and exchangeable cations (e.g., K^+, and Ca^{2+}),

thereby liberating these mineral elements increasing their uptake (Liu *et al.,* 2020) as shown by our results.

The discovery of an ectomycorrhizal fungal species (*T. borchii*) highly tolerant to Pb and its ability to increase Pb uptake by the host plant is of fundamental importance for phytoremediation. *Quercus cerris* plants mycorrhized with *T. borchii* could have great potential for practical application in phytoremediation due to the high biomass of its aerial parts and the low environmental requirements which are considered important factors in plant bioremediation (Escobar and Dussan 2016; Houda *et al.*, 2016; Kalubi *et al.* 2016; Yang *et al.* 2015; Mleczek *et al.,* 2017). This work also contributed to a knowledge on *Q. cerris* accumulation and distribution of trace elements, macronutrients and heavy metals which seem to reflect a specific growth pattern in plant species (Subramanian *et al.,* 2022). Further detailed biochemical and molecular studies are needed to decipher the physiological and molecular mechanisms underlying variation in nutrient uptake and translocation in response to Pb contamination and *T. borchii* mycorrhization.

Experimental procedures

Mycelial strains

Five mycelial strains designated 1BO (ATCC 96540), 10RA, 17BO, 43BO and Tbo5118 isolated from *T. borchii* fruiting bodies harvested in central Italy were used. These soils contain less than 30 mg/kg of Pb (Amorosi *et al.,* 2012) (Table S1). Identity of the strains was molecularly verified by sequencing their ITS region (Bonuso *et al.*, 2006, 2010; in this work for Tbo5118). They were maintained by subculturing on half strength Potato dextrose agar (PDA, Difco).

In vitro assessment of Pb on T. borchii *mycelial growth*

313 The response of *T. borchii* strains to various concentration of Pb (0.04, 0.08, 0.16, 0.32, 0.64, 1.28,
314 2.56, 5.12, 10.24, 20.48 mM) was assessed. Before adding the lead to the growth medium it was
315 melted in the form of $Pb(NO_3)_2$, in a solution with a chelating agent (EDTA sodium salt) at the
316 same molar concentration of $Pb(NO_3)_2$ as described by Vassil *et al.* (1998).
317 The isolates were grown in the dark at 24°C, with no agitation, in 100 L-flasks containing 70 ml
318 of a modified Murashige-Skoog liquid medium (MS/2) (Sisti *et al.,* 1998). Each flask was
319 inoculated with one plug (0.7 cm in diameter) from 30-d old cultures grown on PDA. Colony
320 diameter of each strain was measured every five days for a month in cultures grown in Petri dishes
321 (9 cm in diameter) on agarized MS/2 at the same conditions applied for liquid cultures. The pH of
322 each medium was adjusted to 5.6-5.8 after the addition of chelated lead and before autoclaving
323 them < 120 °C for 20 min.
324
325 *Gene expression assay*
326 RNA isolation
327 Total RNA was isolated from 1-month-old liquid cultures of Tbo5118 (the strain then used for
328 plant inoculation) added with 5.12 mM $Pb(NO_3)_2$ or without lead addition (control), using RNeasy
329 Plant-mini kit from QIAGEN, following the manufacturer's instructions. The final concentration
330 and quality of RNA samples were estimated either spectrophotometrically by a NanoDrop®ND-
331 1000 (Celbio) or by agarose gel electrophoresis, staining with ethidium bromide. Total RNA was
332 treated with DnaseI, DNA-*free*™ kit (Ambion), according to the manufacturer's instructions. Three
333 biological replicates were processed for each treatment.
334
335 Real Time PCR analysis

The reverse transcription reactions were performed in 20 µl volume reactions. Firstly, 1 µg of DNase-treated total RNAs from *T. borchii* strain grown with or without Pb addition and 1 µl of random hexamers (12 µM, Promega) were combined and incubated at 65° C for 2 min; 1 µl RT-buffer, 0.5 mM dNTPs, 1 U RNase inhibitor and 4 U of MMLV Reverse Transcriptase (QIAGEN) (for a total of 6 µl) were then added. The reactions were incubated at 37° C for 1 h and then at 72° C for 5 min. Finally, the cDNAs were diluted 1:2 for the subsequent PCR reaction.

Suitable primer pairs for each selected gene with a high melting temperature (> 60 °C) were designed. The primers used to amplify Tb*Thio* (BM56For 5'-CTTCCATCACACATCCATCAA-3', BM56Rev 5'-AATCAGTTTGCAGGGACCAC-3'), Tb*Glut* (BM55For 5'-ACCCCGTTGCTTATCTTTTCC-3', BM55Rev 5'-CTCCTTGAGAGCAGCCTGG-3'), Tb*NL* (AF23For 5'–TGCCTGGTGACATTATCGAA-3', AF23Rev 5'-CCCCAGTCAAAAGTGCTTCA-3') genes were designed on Tb*Thio* (BM266256), Tb*Glut* (BM266155, Lacourt *et al.,* 2002) sequences and AF23 Na(+)/Li(+)-exporting P-type ATPase EST sequences (AF487323 Zeppa *et al.,* 2002), using the software Primer 3.0 (http://www.bioinformatics.nl/cgi-bin/primer3plus.cgi/). Moreover TbRhoGdi and TbCdc42 primer pairs were used to amplify Tb*RhoGdi* and Tb*Cdc42* genes, as reported in Menotta *et al.* (2007). The 18S rRNA gene from *T. borchii* was selected as a reference (18S RT F 5'-TGGTCCGGTCGGATCTT-3', 18S RT R 5'-CATTACGGCGGTCCTAGAAA-3') (Menotta *et al.,* 2008). To avoid amplification of genomic DNA, all the primers used, were designed including a splice junction.

Quantitative real time PCR (qPCR) was performed in a Bio-Rad iCycler iQ Multi-Color Real Time PCR Detection System (Bio-Rad) using, for all genes, the following thermal parameters: 95° C for 10 min, followed by 50 cycles of 95° C for 30 s and 60° C for 30 s.

Each sample was analysed in triplicate in 25 µl reaction consisting of 1 µl diluted cDNA, 2X QuantiTect SYBR Green PCR kit, 300 nM of primers and 0.6 U of Hot Rescue Real Time DNA polymerase (Diatheva). The specificity of the amplification products was confirmed by examining thermal denaturation plots and by sample separation in a 3% DNA agarose gel. The amount of each target transcript was related to that of the reference gene using the method described by Pfaffl (2001). The Pfaffl method is a comparative method calibrated on the single gene efficiency. Three independent replicates of amplification products were used to calculate the means and standard errors.

Greenhouse experiments

Quercus cerris acorns were germinated in sterile sand in a greenhouse. Six-week-old seedlings were transplanted individually in plastic pots (Bamaplast, Italy), 7 cm diameter and 18 cm deep, filled with 500 ml of a calcareous loam soil (pH in water, 7.79; total $CaC0_3$, 9.9%; organic matter, 2.18%; electrical conductivity, 0.33 dS m^{-1}; texture: sand 34.8%, silt 47.0%, clay 18.2%) mixed with vermiculite and sand (8:1:1). The substrate was autoclaved at 120 °C for 2 h. Fifteen seedlings were inoculated with *T. borchii*, using the mycelium of the strain Tbo5118 as inoculum, before transplanting. This strain was chosen because it was isolated more recently than the other strains and thus it is more infective (Iotti *et al*., 2012). Mycelial inoculum was prepared following the instruction reported in the Italian patent application nr. 102021000023342 filed on September 9, 2021. Fifteen uninoculated seedlings were also prepared and run in parallel as controls.

Six months after inoculation the high of the stem and the collar diameter of each seedling were measured and all seedlings were removed from their pots to assess *T. borchii* mycorrhiza formation and the presence of contaminants. The identity of the obtained *T. borchii* mycorrhizas was

morphologically (Zambonelli *et al.,* 1993) and molecularly confirmed under a dissecting microscope (× 20) and by direct PCR strategy (Iotti and Zambonelli, 2006) using *T. borchii* species-specific primers (Amicucci *et al.,* 1998), respectively. The degree of mycorrhization was measured by counting the number of colonized and non-colonized root tips of 3-4 root fragments randomly selected roots from the top, middle and bottom sector of the root system. The results were expressed as a percentage of colonized tips out of the total number of tips.

Ten seedlings mycorrhized with *T. borchii* (1mic) and ten control seedlings completely free of ectomycorrhizal contaminants (0mic) were selected for the following experiments. An half of these seedlings (five 1mic and five 0mic) were treated during 6 months with an increasing dose of $Pb(NO_3)_2$ (1Pb) (Table S2) whereas the other seedlings (five1mic and five 0mic) were not treated with Pb (0Pb). EDTA sodium salt was added to the seedlings of the first two treatments at the same molar concentration of $Pb(NO_3)_2$. In total each treated seedling received around 3.5 g of $Pb(NO_3)_2$. Each pot was watered taking care to minimize water leakage and, in treated pots, Pb leaching.

Two months after the last treatment the high of the stem and the collar diameter of each seedling were measured; all the seedlings were removed from their pots and, after accurately washing the roots system under tap water, the degree of ectomycorrhizal infection was assessed as previously described. Then, each seedling was separated into roots, stems and leaves which were dried in an oven at 60°C until constant weight for Pb content analyses.

Morphological analyses of mycelium and ectomycorrhizas

Before morphological observation, the mycelium from liquid cultures was gently washed in distilled water. Morphological features of ectomycorrhizas were firstly observed under a dissecting

405 microscope (20x) and then hand-made cross sections of fresh unramified ends were carried out by
406 using a razor blade. Sodium rhodizonate was applied to mycelia and ectomycorrhizal sections to
407 visualize the lead particles forming a pink-colored lead–rhodizonate complex (Glater and
408 Hernandez 1972; Tung and Temple, 1996). All the microscopic observations were carried out
409 under an Eclipse TE 2000-E microscope (1000 X) (Nikon) and images captured with a DXM1200F
410 digital camera (Nikon).
411
412 *Pb content measurements in soil and plant tissues*
413 The different dried organs (root, stem and leaves) were ground in a titanium ball mill. 250 mg of
414 dried plant tissues were mineralized with 6 ml of HNO_3 (Suprapur, Merck, Kenilworth) and 2 ml
415 of H_2O_2 (Carlo Erba, MI, Italy) using a microwave oven (Milestone 2100, Sorisone). After
416 digestion, the sample solution was made up to 20 ml with Milli-Q ultrapure distilled water and
417 filtered with Whatman 42 filter paper. Pb concentration was determined in mineralized samples
418 by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Spectro, Ametek, MI,
419 Italy), after appropriate calibration. The quality of the data analysis was assessed using an
420 International Standard (CRM 482, Community Bureaux of Reference, BCR).
421 Soils of the four treatments (0Pb-0mic, 1Pb-0mic, 0Pb-1mic and 1Pb-0mic) were air-died and
422 ground (less than 2 mm in size) with a titanium ball mill (Vittori Antisari *et al.*, 2012, 2013, 2014).
423 Briefly, 250 mg of soil samples was mineralized with aqua regia solution (2 ml 65% HNO_3 plus 6
424 ml 37% HCl, suprapur grade, Carlo Erba) using a microwave oven (Milestone 2100, Sorisone).
425 After digestion, the soil sample solution was made up to 20 ml with Milli-Q ultrapure distilled
426 water and filtered with Whatman 42 filter paper. Pb concentration was determined by ICP-OES;

427 each soil sample was analysed three time and the data calibrated using International Reference
428 Materials (BCR) and internal laboratory standards (Ferronato *et al.*, 2021).
429
430 *Calculation of phytoextraction efficiency*
431 Capacity of mycorrhized and non-mycorrhized oak seedlings for Pb phytoextraction was estimated
432 based on the lead bioconcentration factor in roots (BFC) [BFC = Pb concentration in the roots (mg
433 kg^{-1}) / Pb concentration in the soil (mg kg^{-1})]. The translocation capacity of Pb and other analyzed
434 elements from roots to stems and to leaves was calculated as translocation factor (TF) [TFs = Pb
435 concentration stem (mg kg^{-1}) / Pb concentration in the root (mg kg^{-1}) and TFl = Pb concentration
436 leaves (mg kg^{-1}) / Pb concentration in the roots (mg kg^{-1})] (Placek *et al.,* 2016). These parameters
437 were expressed as percentage values.
438
439 *Statistical analyses*
440 The dry weight of the mycelia as a function of the different strains (considered as a random factor),
441 and as a function of the Pb concentration (considered as covariate) was compared with a univariate
442 GLM model.
443 The data of plant growth (height and stem diameter), soil Pb content and BFC were compared
444 using a two-way ANOVA with Tukey's post hoc test. To establish the effects of Pb on *T. borchii*
445 mycorrhizal colonization a Before-After-Control-Impact (BACI) design (Smith, 2002) was
446 applied. BACI provides a way of comparing data obtained before treatment with data obtained
447 after treatment, as repeated measurement analysis of variance ANOVA with Tukey's post-hoc test.
448 The effects of Pb treatment and mycorrhizal symbiosis on the element uptake and distribution in
449 roots, shoots and leaves was analyzed by MANOVA analysis; mycorrhization and Pb treatment

were both binary predictive factors, while dependent variables were all the elements measured (Table 1); significance of mycorrhization, Pb treatment and their interaction have been calculated. RT-PCR expression signals were analyzed by Wilcoxon test for one sample, using Bonferroni correction for multiple comparison. All elaborations were performed using SPSS statistical package version 22.0 or XLSTAT.

ACKNOWLEDGEMENTS

We thank E. Vecchi for its help in laboratory analyses.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY

Some of the data that support the findings of this study are available in the supplementary material of this article. Any additional information will be made available upon request.

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Legends of the figures

Fig. 1 - Dry weights (mean and standard deviation) of the 5 *Tuber borchii* strains, cultivated at different Pb concentration. The complete dataset is reported in Table S3.

Fig. 2 - *Tuber borchii* hyphae treated with Pb. The pink-colored elements correspond to the lead–rhodizonate complex inside the vacuoles (a), or to oxalate crystals outside the hyphae (b).

718 Fig. 3 – Relative expression of Tb*Cdc42*, Tb*RhoGdi*, Tb*Glu,* Tb*Thio*, Tb*NL*genes in *T. borchii*
719 strain Tbo5118 grown on MS/2 liquid medium treated with lead compared to the controls (dotted
720 line). Data are mean ± standard error of at least three independent experiments each performed in
721 triplicate. The expression data were expressed as fold expression versus C. Asterisks indicate
722 significant differences with the control (** $p<0.01$, *** $p<0.001$).
723 Fig. 4 - Difference between the percentages of mycorrhizal colonization of the control plants (0Pb,
724 green column) and those treated with Pb (Pb1) before the first Pb treatment and two months after
725 the last Pb treatment (a). *Tuber borchii* mycorrhizas untreated with Pb (0Pb) (b) and treated with
726 Pb (1Pb) (c,d, e, f and g). The pink-colored elements correspond to the lead–rhodizonate complex
727 inside the vacuoles of extra-radical hyphae (c), to the oxalate crystals on the cystidia surface (d
728 and f), lead–rhodizonate complex in the hyphae forming the Hartig net (e and g) and in the
729 innermost part of the mantle (g).
730 Fig. 5 - Lead concentration in the pot soil (a) and BFC (b) of the plants treated (1Pb) or untreated
731 with Pb (0Pb), mycorrhized (1mic) or not mycorrhized (0mic) with *T. borchii*. Bars indicate
732 standard error. *p* values determined by two-way ANOVA were the following: $p(Mic) = 0.000$,
733 $p(Pb) = 0.0001$, $p(Mic\ x\ Pb) = 0.002$ for the soil lead concentration (a); $p(Mic) = 0.038$,
734 $p(Pb=) 0.003$, $p(Mic\ x\ Pb) = 0.05$ for the BFC (b).
735 Fig. 6 - TFs (black bar) and TFl (grey bar) values (log10 scale) of the principal element measured;
736 in ordinate symbols of element and status of plants analyzed (0mic-0Pb: non mycorrrhizal plant,
737 without Pb; 1mic-0Pb: mycorrrhizal plant, without Pb; 0mic-1Pb: non mycorrrhizal plant, with Pb;
738 1mic-1Pb: mycorrrhizal plant, with Pb). For figure clarity, some elements are omitted (Ni, V, Zn,
739 and Sr).
740

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 - Metadata of the *Tuber borchii* strains selected in this work. Herbarium numbers are of dried specimens in the Mycology Center, University of Bologna, Italy (CMI-Unibo). The accession number is referred to the ITS sequences deposited in GenBank.

Table S2 - Timetable of the Pb treatments

Table S3 – Dry weight (mean ± standard deviation) of the mycelia of different *T. borchii* strains grown on liquid MS/2 untreated (Control) or treated with different Pb concentrations.

Table S4 – Colony diameter (mean ± standard deviation) of the different *T. borchii* strains grow on agarized MS/2 untreated (Control) or treated with different Pb concentrations.

Table S5 – Height and collar diameter of the plants at the end of the experiment (one year after inoculation and eight months after the first Pb treatment).

Fig. S1 – Grow curve of the different *T. borchii* strains grown on agarized MS/2 untreated or treated with 2.53, 10.24 and 20.48 mM of Pb. The data obtained at all Pb concentrations are reported in Table S4.

TABLE 1 Element concentration in the organs of the plants treated (1Pb) or untreated with Pb (0Pb), mycorrhized (1mic) and non-mycorrhized (0mic) with *Tuber borchii*. In bold the significant values of *p*

	0mic-0Pb (ppm)	0mic-1Pb (ppm)	1mic-0Pb (ppm)	1mic-1Pb (ppm)	*p*(mic)	*p*(Pb)	*p*(mic × Pb)
Leaf							
Al	0.07 ± 0.02	0.07 ± 0.09	0.11 ± 0.04	0.08 ± 0.02	0.429	0.602	0.505
Ca	10.4 ± 0.85	15.8 ± 4.07	11.8 ± 5.87	16.7 ± 3.04	0.556	**0.018**	0.898
Fe	0.15 ± 0.03	0.14 ± 0.10	0.19 ± 0.07	0.13 ± 0.03	0.638	0.335	0.409
K	4.86 ± 0.88	6.41 ± 1.29	5.98 ± 0.99	9.02 ± 0.84	**0.003**	**0.001**	0.167
Mg	3.26 ± 0.50	2.93 ± 0.53	2.60 ± 0.26	3.17 ± 0.42	0.345	0.594	0.062
Mn	0.31 ± 0.06	0.26 ± 0.02	0.17 ± 0.04	0.19 ± 0.02	**<0.001**	0.544	0.088
Na	2.36 ± 0.82	1.97 ± 1.31	3.83 ± 1.55	4.09 ± 1.54	**0.017**	0.923	0.632
P	0.51 ± 0.10	0.43 ± 0.04	0.93 ± 0.16	0.62 ± 0.07	**<0.001**	**0.001**	**0.026**
S	0.94 ± 0.14	1.21 ± 0.08	0.99 ± 0.10	1.23 ± 0.03	0.508	**<0.001**	0.696
B	54.63 ± 14.6	59.8 ± 23.4	65.9 ± 23.5	77.8 ± 14.7	0.154	0.392	0.734
Ba	37.6 ± 4.16	43.1 ± 14.0	40.5 ± 23.7	37.3 ± 9.25	0.840	0.873	0.553
Cr	2.31 ± 1.35	2.72 ± 3.00	4.12 ± 3.32	1.95 ± 1.26	0.673	0.478	0.304
Cu	4.92 ± 0.48	6.40 ± 0.52	8.81 ± 1.24	8.84 ± 0.93	**<0.001**	0.086	0.099
Li	3.27 ± 0.60	5.96 ± 1.07	4.24 ± 1.81	6.42 ± 1.44	0.277	**0.002**	0.696
Mo	0.43 ± 0.16	0.34 ± 0.10	0.42 ± 0.14	0.35 ± 0.01	0.952	0.165	0.911
Ni	0.28 ± 0.30	1.36 ± 0.78	0.54 ± 0.41	1.02 ± 0.42	0.895	**0.010**	0.271
Pb	0.71 ± 0.26	295 ± 215	0.28 ± 0.24	167 ± 30.0	0.290	**0.002**	0.293
Sb	0.36 ± 0.03	0.35 ± 0.07	0.69 ± 0.61	0.39 ± 0.09	0.226	0.313	0.316
Se	0.36 ± 0.10	0.26 ± 0.06	0.18 ± 0.17	0.19 ± 0.06	**0.030**	0.423	0.330
Sr	45.7 ± 3.76	84.0 ± 26.3	55.7 ± 27.1	89.1 ± 21.6	0.498	**0.006**	0.824
V	0.03 ± 0.02	0.08 ± 0.16	0.06 ± 0.10	0.01 ± 0.01	0.740	0.929	0.356
Zn	15.5 ± 1.63	18.9 ± 5.10	22.2 ± 1.86	49.5 ± 26.7	**0.013**	**0.033**	0.085
Stem							
Al	0.15 ± 0.05	0.23 ± 0.11	0.18 ± 0.07	0.13 ± 0.01	0.304	0.618	0.069
Ca	14.1 ± 1.34	14.5 ± 1.47	10.4 ± 0.77	12.1 ± 1.40	**<0.001**	0.116	0.301
Fe	0.21 ± 0.05	0.29 ± 0.12	0.21 ± 0.04	0.14 ± 0.01	0.058	0.892	0.066
K	2.35 ± 0.14	3.00 ± 0.44	2.65 ± 0.54	3.94 ± 0.52	**0.013**	**0.001**	0.157
Mg	1.19 ± 0.24	0.93 ± 0.14	0.98 ± 0.16	0.94 ± 0.14	0.236	0.098	0.224
Mn	0.25 ± 0.13	0.15 ± 0.02	0.17 ± 0.06	0.14 ± 0.06	0.259	0.109	0.346
Na	0.74 ± 0.09	1.09 ± 0.61	1.00 ± 0.47	1.32 ± 0.43	0.298	0.160	0.923
P	0.23 ± 0.05	0.24 ± 0.02	0.49 ± 0.08	0.60 ± 0.06	**<0.001**	**0.035**	0.094
S	0.37 ± 0.03	0.46 ± 0.04	0.44 ± 0.17	0.55 ± 0.11	0.137	0.064	0.931
B	18.3 ± 1.80	12.7 ± 2.67	17.4 ± 2.55	12.1 ± 2.31	0.533	**<0.001**	0.911
Ba	58.7 ± 9.90	40.9 ± 6.24	32.8 ± 2.34	28.0 ± 5.67	**<0.001**	**0.004**	0.064
Cr	2.00 ± 1.06	3.55 ± 1.58	2.88 ± 1.56	1.50 ± 0.44	0.366	0.890	**0.035**
Cu	4.14 ± 0.40	5.70 ± 1.33	6.14 ± 0.74	9.01 ± 1.07	**<0.001**	**<0.001**	0.197
Li	1.22 ± 0.07	1.39 ± 0.14	1.24 ± 0.18	1.11 ± 0.14	0.086	0.778	**0.044**
Mo	0.29 ± 0.05	0.45 ± 0.08	0.26 ± 0.08	0.37 ± 0.02	0.125	**0.001**	0.416
Ni	1.14 ± 0.60	0.80 ± 0.37	0.84 ± 0.21	0.44 ± 0.17	0.096	0.067	0.869
Pb	0.58 ± 0.62	1334 ± 324	0.46 ± 0.52	2185 ± 212	**0.001**	**<0.001**	**0.001**
Sb	0.49 ± 0.13	0.51 ± 0.08	0.44 ± 0.04	0.51 ± 0.09	0.602	0.372	0.609
Se	0.15 ± 0.08	0.20 ± 0.13	0.13 ± 0.07	0.12 ± 0.11	0.323	0.739	0.606
Sr	96.2 ± 8.47	98.2 ± 13.8	91.3 ± 8.51	95.9 ± 10.9	0.511	0.544	0.808
V	0.48 ± 0.19	0.63 ± 0.30	0.63 ± 0.26	0.34 ± 0.11	0.548	0.540	0.076
Zn	9.05 ± 1.62	9.59 ± 1.37	12.0 ± 0.94	14.4 ± 0.92	**<0.001**	**0.032**	0.153

(Continues)

TABLE 1 (Continued)

	0mic-0Pb (ppm)	0mic-1Pb (ppm)	1mic-0Pb (ppm)	1mic-1Pb (ppm)	*p*(mic)	*p*(Pb)	*p*(mic × Pb)
Root							
Al	0.56 ± 0.41	0.66 ± 0.11	0.74 ± 0.32	0.94 ± 0.56	0.225	0.406	0.792
Ca	7.51 ± 1.26	9.09 ± 0.69	8.58 ± 0.70	10.7 ± 1.74	**0.035**	**0.006**	0.666
Fe	0.69 ± 0.43	0.77 ± 0.12	0.84 ± 0.37	0.97 ± 0.66	0.400	0.612	0.915
K	2.81 ± 0.31	3.31 ± 0.40	3.33 ± 0.47	3.96 ± 0.56	**0.017**	**0.021**	0.756
Mg	1.22 ± 0.50	1.31 ± 0.19	1.20 ± 0.15	1.49 ± 0.40	0.647	0.261	0.544
Mn	0.05 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.723	0.501	0.501
Na	0.79 ± 0.29	1.22 ± 0.41	0.86 ± 0.29	1.37 ± 0.31	0.533	**0.013**	0.825
P	0.23 ± 0.03	0.26 ± 0.03	0.54 ± 0.10	0.71 ± 0.14	**<0.001**	**0.024**	0.119
S	0.46 ± 0.04	0.55 ± 0.07	0.47 ± 0.08	0.62 ± 0.10	0.323	**0.007**	0.426
B	15.1 ± 1.26	15.3 ± 1.77	16.25 ± 2.04	12.9 ± 3.24	0.571	0.157	0.114
Ba	18.7 ± 6.40	23.1 ± 2.84	20.2 ± 1.38	29.1 ± 8.62	0.178	**0.026**	0.411
Cr	9.28 ± 8.66	9.00 ± 4.18	8.61 ± 4.21	10.7 ± 7.15	0.868	0.770	0.703
Cu	8.08 ± 2.07	24.2 ± 7.09	12.4 ± 1.68	24.9 ± 3.72	0.271	**<0.001**	0.428
Li	1.48 ± 0.40	1.61 ± 0.16	1.73 ± 0.37	2.03 ± 0.81	0.180	0.372	0.718
Mo	0.62 ± 0.13	0.62 ± 0.07	0.62 ± 0.08	0.67 ± 0.20	0.670	0.652	0.758
Ni	3.25 ± 1.81	2.87 ± 0.78	3.47 ± 1.14	3.47 ± 1.91	0.571	0.792	0.800
Pb	1.14 ± 0.66	1383 ± 1025	1.20 ± 0.38	2461 ± 1467	0.244	**0.001**	0.244
Sb	0.60 ± 0.15	0.58 ± 0.07	0.61 ± 0.09	0.60 ± 0.15	0.787	0.787	0.919
Se	0.10 ± 0.12	0.29 ± 0.08	0.22 ± 0.03	0.35 ± 0.17	0.126	**0.014**	0.643
Sr	51.2 ± 8.48	65.0 ± 6.82	61.8 ± 6.40	78.4 ± 12.5	**0.015**	**0.003**	0.754
V	2.31 ± 0.85	2.18 ± 0.06	2.63 ± 0.49	2.47 ± 1.34	0.457	0.719	0.971
Zn	18.5 ± 14.2	15.2 ± 2.04	14.0 ± 2.26	22.5 ± 9.34	0.742	0.524	0.168

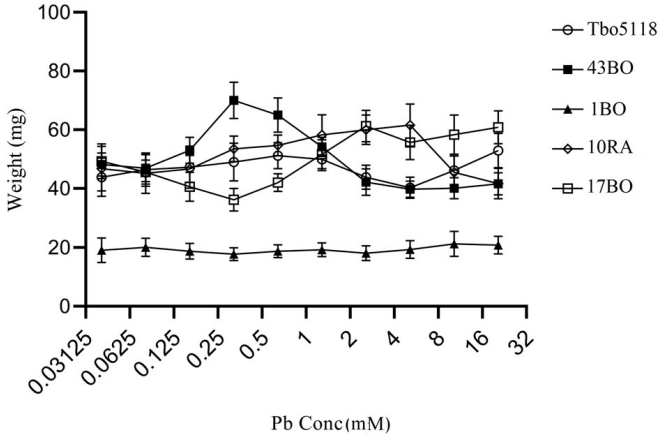

FIGURE 1 Dry weights (mean and SD) of the 5 *Tuber borchii* strains, cultivated at different Pb concentration. The complete dataset is reported in Table S3.

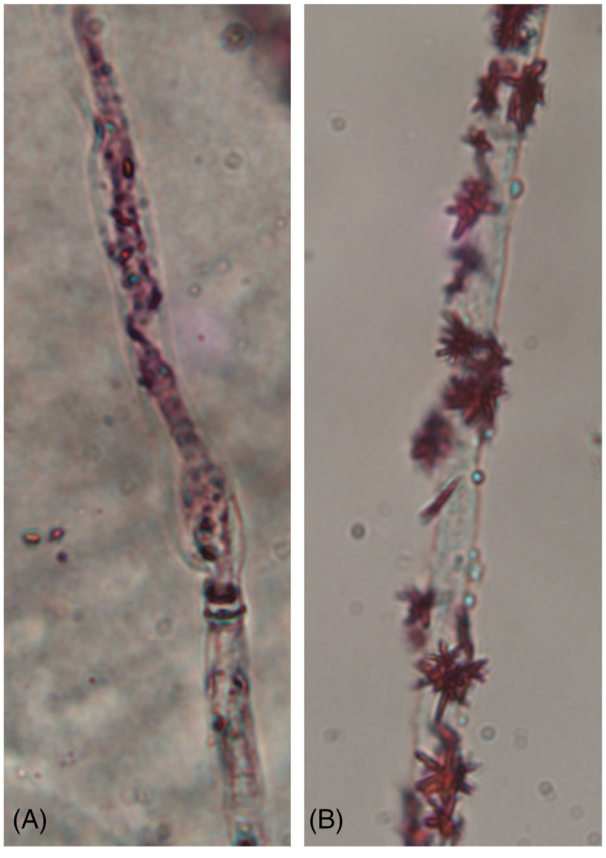

FIGURE 2 *Tuber borchii* hyphae treated with Pb. The pink-coloured elements correspond to the lead–rhodizonate complex inside the vacuoles (A), or to oxalate crystals outside the hyphae (B).

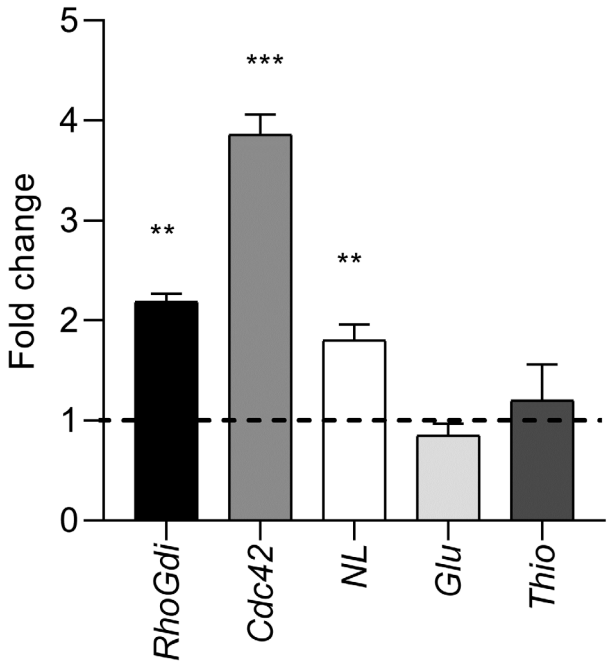

FIGURE 3 Relative expression of Tb*Cdc42*, Tb*RhoGdi*, Tb*Glu*, Tb*Thio*, Tb*NL*genes in *Tuber borchii* strain Tbo5118 grown on MS/2 liquid medium treated with lead compared to the controls (dotted line). Data are mean ± SE of at least three independent experiments each performed in triplicate. The expression data were expressed as fold expression versus C. Asterisks indicate significant differences with the control (**$p < 0.01$, ***$p < 0.001$).

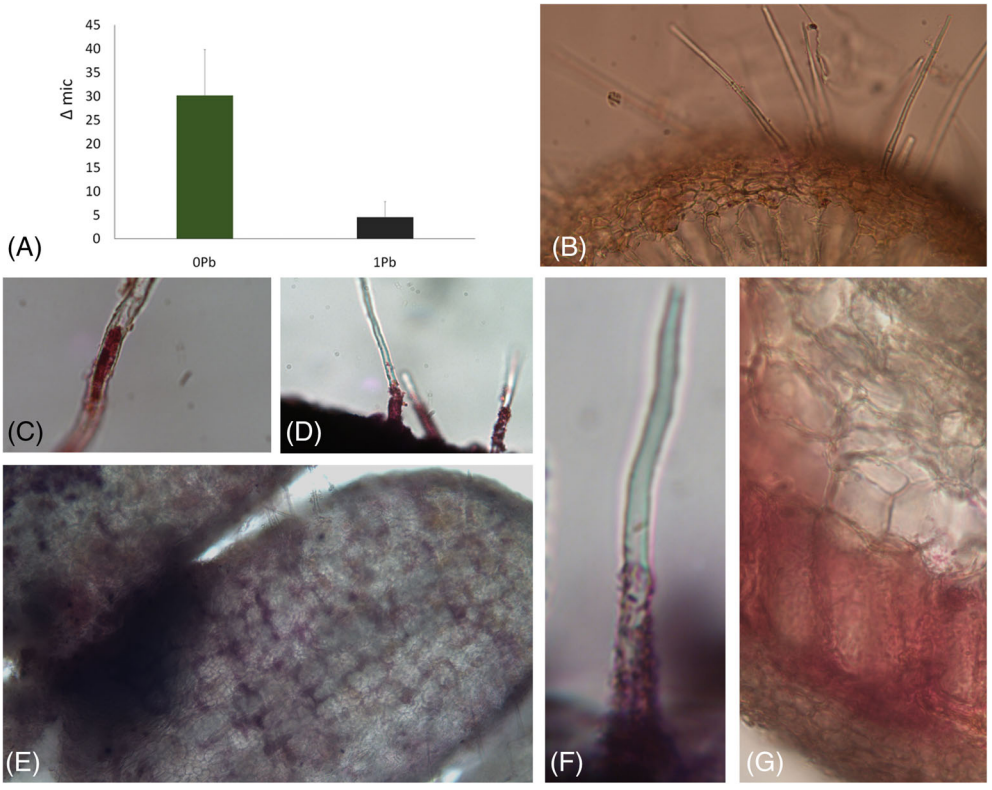

FIGURE 4 Difference between the percentages of mycorrhizal colonization of the control plants (0Pb, green column) and those treated with Pb (Pb1) before the first Pb treatment and 2 months after the last Pb treatment (A). *Tuber borchii* mycorrhizas untreated with Pb (0Pb) (B) and treated with Pb (1Pb) (C–G). The pink-coloured elements correspond to the lead–rhodizonate complex inside the vacuoles of extra-radical hyphae (C), to the oxalate crystals on the cystidia surface (D and F), lead–rhodizonate complex in the hyphae forming the Hartig net (E and G) and in the innermost part of the mantle (G).

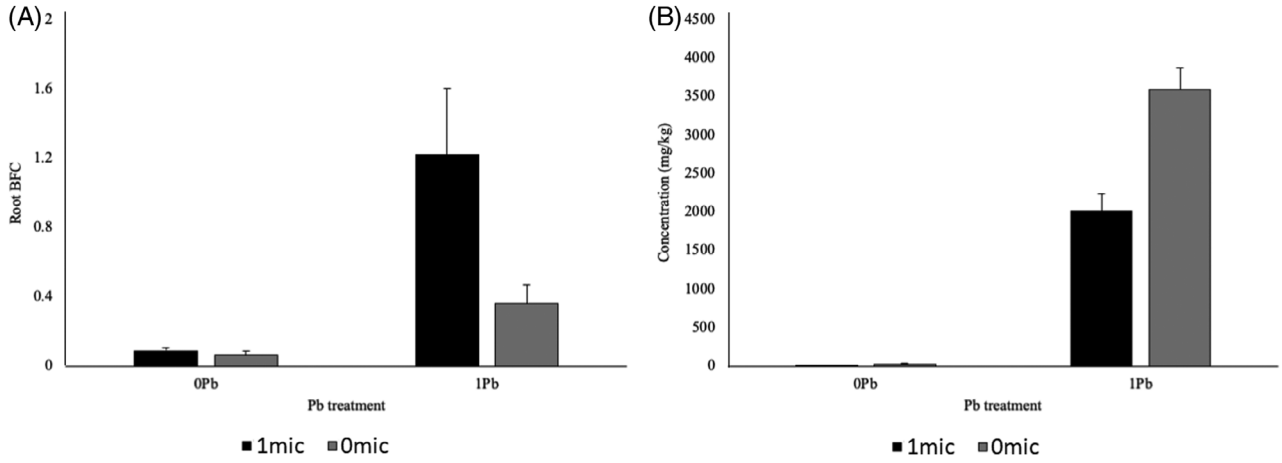

FIGURE 5 Lead concentration in the pot soil (A) and BFC (B) of the plants treated (1Pb) or untreated with Pb (0Pb), mycorrhized (1mic) or not mycorrhized (0mic) with *Tuber borchii*. Bars indicate standard error. *p*-Values determined by two-way ANOVA were the following: $p(\text{mic}) = 0.000$, $p(\text{Pb}) = 0.0001$, $p(\text{mic} \times \text{Pb}) = 0.002$ for the soil lead concentration (A); $p(\text{mic}) = 0.038$, $p(\text{Pb}) = 0.003$, $p(\text{mic} \times \text{Pb}) = 0.05$ for the BFC (B).

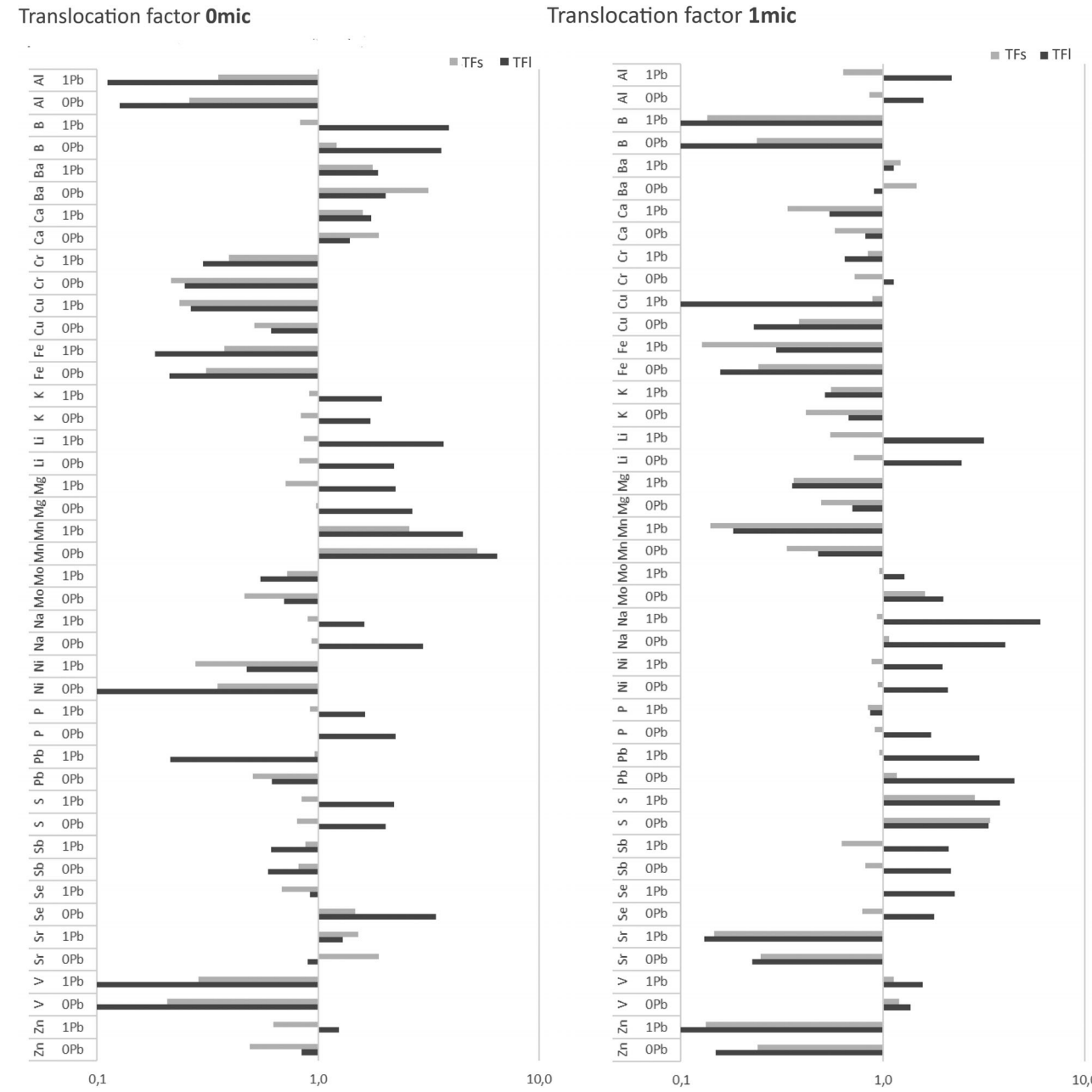

FIGURE 6 Translocation factor (TFs) (grey bar) and TFl (black bar) values (log10 scale) of the principal elements measured in not mycorrhized (0mic) and in mycorrhized plants (1mic); in ordinate symbols of element and status of plants analysed (0Pb: untreated plants, 1Pb: treated Pb plants).