ORIGINAL PAPER

Eur. J. Histochem.
46: 341-350, 2002

Zinc ions alter morphology and chitin deposition in an ericoid fungus

L. Lanfranco[1], R. Balsamo[1], E. Martino[1], S. Perotto[1] and P. Bonfante[1,2]

[1]Dipartimento di Biologia Vegetale - Università di Torino and [2]Istituto per la Protezione delle Piante - Sezione di Torino - CNR, V. le Mattioli 25, 10125 Torino, Italy

Accepted: 5/06/02

Key words: hyphal morphology, chitin, zinc, mycorrhizal fungus

SUMMARY

A sterile mycelium PS IV, an ascomycete capable of establishing ericoid mycorrhizas, was used to investigate how zinc ions affect the cellular mechanisms of fungal growth. A significant reduction of the fungal biomass was observed in the presence of millimolar zinc concentrations; this mirrored conspicuous changes in hyphal morphology which led to apical swellings and increased branching in the subapical parts. Specific probes for fluorescence and electron microscopy localised chitin, the main cell wall polysaccharide, on the inner part of the fungal wall and on septa in control specimens. In Zn-treated mycelium, hyphal walls were thicker and a more intense chitin labelling was detected on the transverse walls. A quantitative assay showed a significant increase in the amount of chitin in metal-treated hyphae.

INTRODUCTION

Filamentous fungi are characterised by a polarised growth pattern, where hyphae elongate by apical deposition of wall skeletal polysaccharides. Thanks to this growth mechanism and to the formation of lateral branches, most fungi have a potentially infinite capacity to explore and colonise substrates (Gow *et al*., 1999). Some environmental factors deeply influence fungal growth, such as availability of water, oxygen, nutrients, temperature, pH, salts and toxic metals. High concentrations of heavy metals (Cu, Cd, Pb, As and Zn) reduce fungal biomass and respiration rate (Nordgren *et al*., 1983; Babich & Stotzky, 1985). Although quite large information is available on the influence of heavy metal ions on fungal growth, studies about the effects of metals on the growth of mycelia at the microscopic level are rather sparse (Ramsay *et al*., 1999). Alterations in colony morphology and density have been reported by Gadd (1986). A disruption of normal polarized growth with twisting and looping of individual hyphae was observed in the basidiomycete *Schizophyllum commune* treated with cadmium (Lilly *et al*., 1992).

Mycorrhizal fungi are extensively studied because they can protect their host plants from the toxic effects of heavy metals (Leyval *et al*., 1997; Galli *et al*., 1994; Frey *et al*., 2000; Perotto and Martino, 2001). Significant differences in growth on metal containing media have allowed the identification of tolerant and sensitive strains both in ecto- and ericoid mycorrhizal fungi (Colpaert *et al*., 2000; Martino *et al*., 2000). Since growth is used as the main criterion to define tolerance or

Correspondence to: P. Bonfante
E-mail: p.bonfante@csmt.to.cnr.it

sensitivity, it is somewhat surprising that little attention has been paid to the effects of heavy metals on the molecular mechanisms of fungal growth and morphogenesis.

To better understand the interactions occurring between heavy metals and ericoid fungi, we investigated the influence of zinc ions on the hyphal growth of a sterile ascomycete isolate, mycelium PSIV. This isolate, previously described for its sensitivity to heavy metals in comparison to other tolerant strains, shows a strong growth reduction in the presence of zinc and cadmium (Martino *et al*., 2000).

Chitin is the most characteristic shape-determinant of fungal wall and its synthesis plays a key role in fungal growth and differentiation (Gooday, 1995). Due to its importance the deposition of this polysaccharide was located on the wall by means of cytochemical probes and quantitatively evaluated following metal exposure.

Our results demonstrate how the cytological modifications shown by hyphal tips and the change in chitin deposition may be relevant factors to explain growth inhibition after Zn treatment.

MATERIALS AND METHODS

Biological material and growth condition

Sterile mycelium PS IV (CLM1371.98), deposited in the Mycological Collection of the Department of Plant Biology, University of Turin, Italy, was isolated from a non polluted environment in Northern Italy (Perotto *et al*., 1990). The fungus was grown in liquid medium (2% malt extract) at 24°C with shaking. The effect of the heavy metals on hyphal morphology was studied in hyphae growing on solid agar medium containing 2% malt extract and 3, 5 or 7 mM $ZnSO_4$ $7H_20$ and on one month old liquid cultures were treated with $ZnSO_4$ $7H_20$ (0 mM, 7 mM or 14 mM) for 15 days.

Light and fluorescence microscopy

The samples were mounted on a glass slide and observed under a Zeiss light microscope equipped with Nomarski optics. For fluorescence microscopy, unfixed hyphae were treated with 10 μg/ml fluorescein-labelled wheat germ agglutinin (WGA-FITC, Vector Laboratories, Burlingame, CA). Propidium iodide was added (50 μg/ml) to reveal nuclear distribution. Samples were observed under an Olympus FluoView inverted confocal microscope, at 40x magnification with a digital zoom of 4x. Excitation light/emission filters wer at 488nm/510-550nm band-pass for WGA-FITC and at 512nm/610nm low-pass for propidium iodide. Image acquisition was performed with FluoView software.

Transmission (TEM) electron microscopy

Treated and control hyphae were fixed in 2% glutaraldehyde in cacodylate buffer (0.1 M pH 7.2) for 2 h at room temperature. Specimens were post-fixed with buffered 1% OsO_4 in the same buffer and dehydrated with ethanol. Other samples were embedded in Araldite and processed for TEM. Ultrathin sections were handled with plastic rings an stained with the PATAg method (Roland and Vian, 1991) to visualize polysaccharides or with uranyl acetate-and lead citrate. The sections were observed with a CM 10 Electron Microscope (Philips) at the Laboratorio di Microscopie Avanzate (www.bioveg.unito.it).

For WGA ultrastructural labelling, thin sections were treated with the WGA-colloidal gold complex (Polysciences, Warrington, PA). Controls were performed as detailed in Arlorio *et al*. (1992).

Chitin content assay

Chitin content was measured as described by Bulawa (1992) on liquid coltures. The mycelium was washed with sterile water and treated with 1 ml of 6% KOH at 80°C for 90 min. After the addition of 0.1 ml of acetic acid the sample was centrifuged, washed twice with 1.5 ml of 50 mM $NaPO_4$ pH 6.3 and then resuspended in 540 μl of 50 mM $NaPO_4$ pH 6.3. Then 40 μl of chitinase (42.5 mg/ml SIGMA) and of glusulase (Roche) were added and the sample was incubated at 37°C for 3 hrs. The samples were boiled for 1 min and centrifuged at 10,000g for 5 min. The N-acetylglucosamine content of the supernatant was measured by using the Reissig solution at 585 nm.

RESULTS

Effects of Zn ions on mycelial growth and hyphal morphology

Growth of isolate PSIV on 2% malt medium was inhibited in the presence of $ZnSO_4$ at all concen-

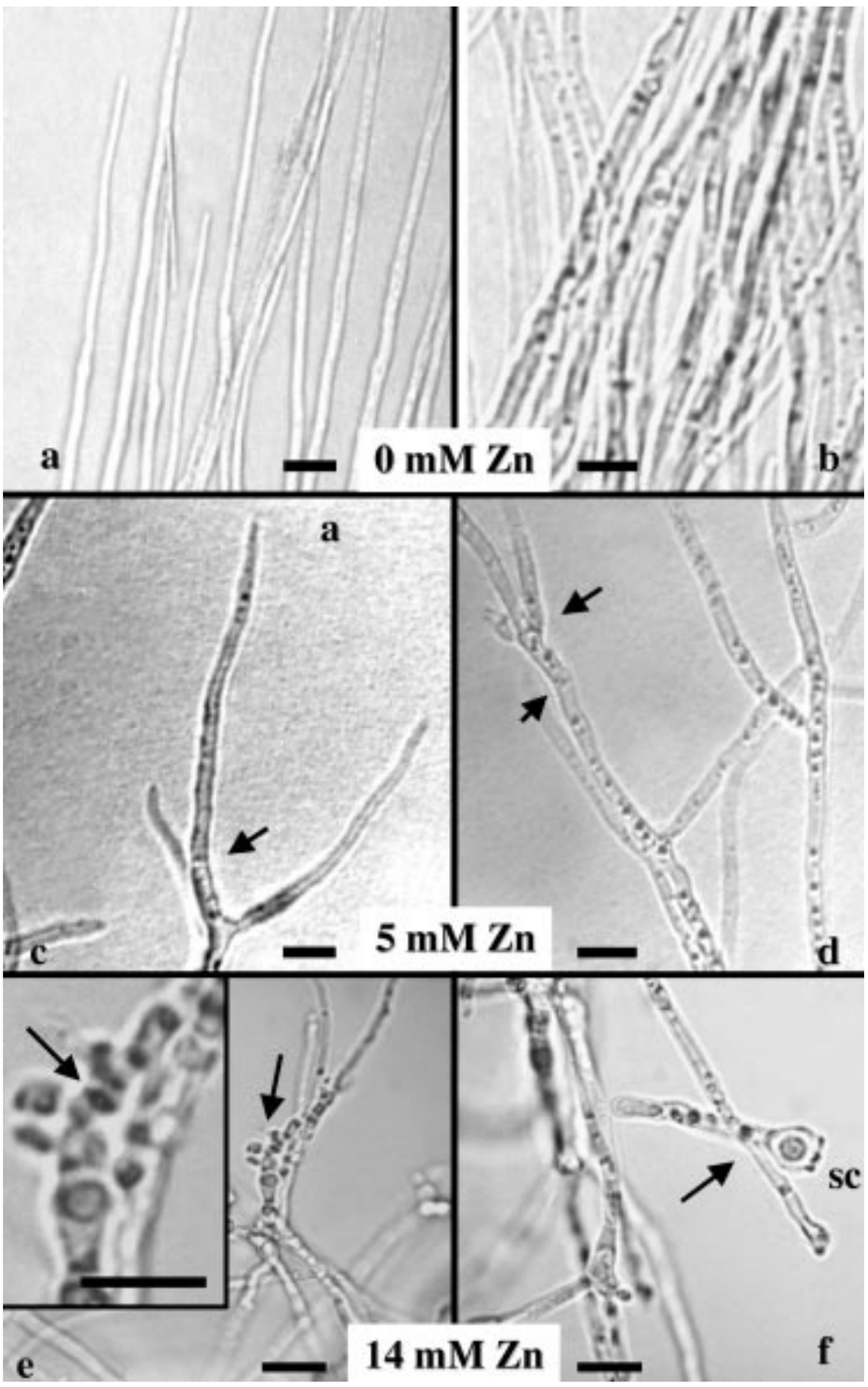
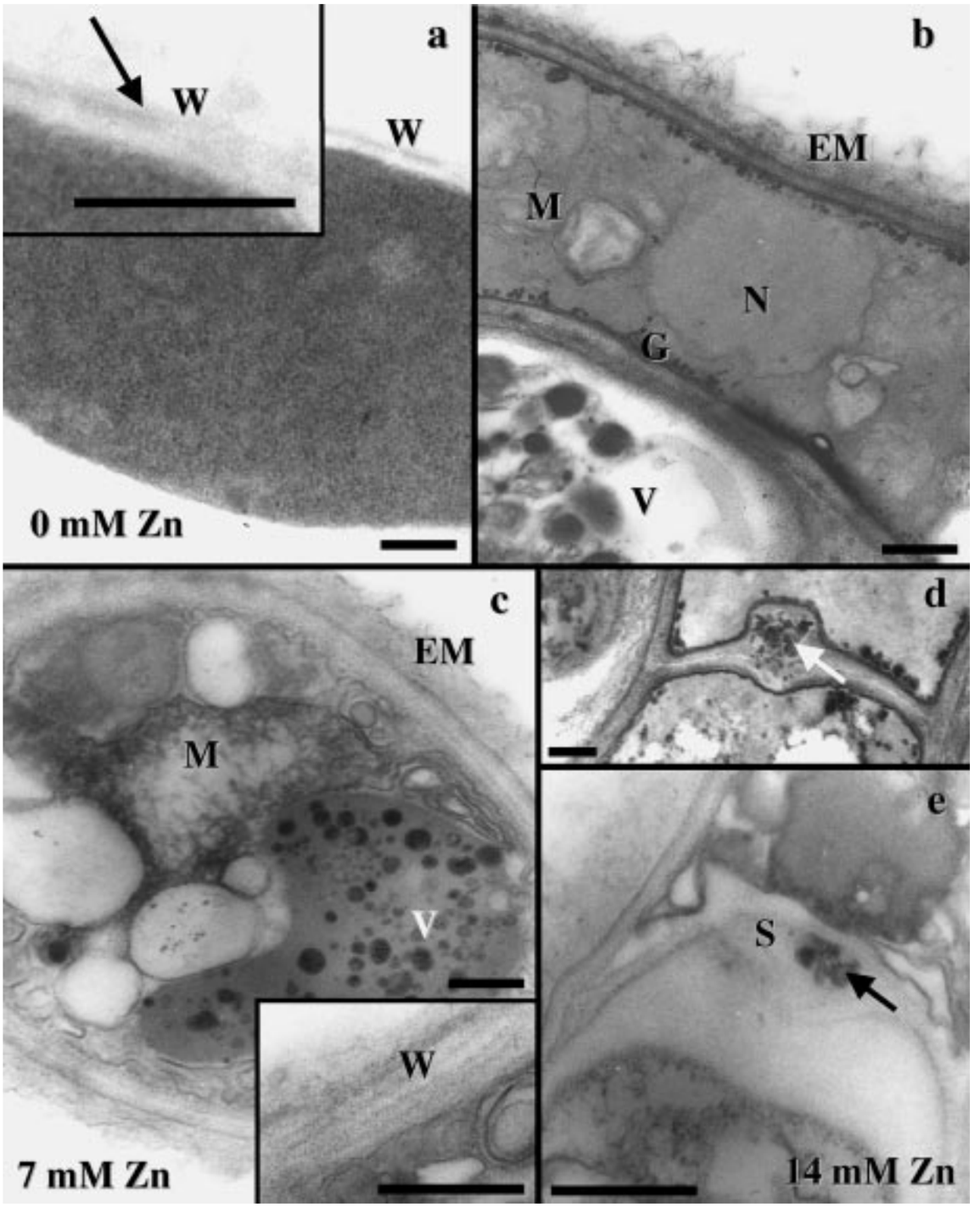


Fig. 1 - Morphology of PSIV hyphae growing in the absence (a-b) and in the presence of Zn (c-f) as seen under light microscope. a. Slender growing hyphae run in parallel, unbranched bundles. b. In the subapical region of the colony, hyphae are grouped in parallel, tighter bundles; c. Zn treatment causes branching and septation (arrows) already in the apical region; d. In the subapical part, the colony appears loose and the bundle organization is lost. Refractile vacuoles are abundant; e. the strongest Zn treatment leads to highly irregular hyphae where extension is affected, thickened septa are very close (arrows and details in the inset); f. detail showing a swollent tip with scars (sc) and a large vacuole inside. Bars = 10 μm, except e inset = 5μm.


a
W
W
0 mM Zn
b
EM
M
N
G
V
c
EM
M
V
W
7 mM Zn
d
e
S
14 mM Zn

trations tested (data not shown), as already reported for a different growth medium (Czapek-pectin) by Martino *et al.* (2000).

In the absence of zinc in the medium, fungal hyphae grew by apical growth with a typical tapered apex and absence of branching in the first 100 μm from the tip. The pattern resulted in parallel-oriented hyphae that were loose at the edge of the colony (Fig. 1a) and organized in bundles that sometimes coiled in the inner part (Fig. 1b). There, hyphae became increasingly vacuolated with rounded refractive vacuoles.

Exposure to Zn ions modified the fungal morphology in relation to the range of Zn concentrations. At the lowest concentrations (from 2 to 5 mM), hyphae still showed the typical slender tip, although branching became evident, septa could be found close to the tip and the ordered parallel organization was lost (Figs. 1c,d). At 5 mM $ZnSO_4$ swollen tips separated from the subapical hypha by septa were commonly observed. The most significant alterations in hyphal morphology were found at 7 and 14 mM $ZnSO_4$: some hyphae appeared swollen with frequent septa, rich in dense refractive bodies and highly branched. Irregular arthrospore-like structures were often evident (Figs. 1e,f).

Ultrastructural analysis confirmed some previous descriptions of this ericoid fungus (Perotto *et al*., 1990) with a hyphal morphology that depended on the distance from the apex: close to the tip, PSIV hyphae showed a thin wall and a cytoplasm rich in organelles (Fig. 2a), whereas they were more vacuolated and rich in glycogen as distance from the tip increased. The wall became thicker, lined by a thin electron-dense layer and by an extracellular fibrillar material. This was reactive to the silver staining after PATAg reaction, thus indicating the presence of polysaccharidic molecules (Fig. 2b). Zinc ions affected this phenotype mostly at the higher concentration range (from 7 to 14 mM) both on solid and liquid media. Severe modifications were observed in the cytoplasm: swollen mitochondria, electron dense vacuoles, thickened walls with a regular layering were present (Fig. 2c). Septa doubled their size thanks to the deposition of amorphous masses that first became evident in the middle of the transverse wall (Fig. 2d) leading to highly irregular structures (Fig. 2e).

Different patterns were observed under a fluorescence confocal microscope after treatment with FITC-conjugated WGA, a lectin used to localise chitin. In the control mycelium, hyphae were weakly labelled along the longitudinal walls (Fig. 3a), whereas wall labelling became more intense at 7 mM $ZnSO_4$, particularly on the septa (Fig. 3b). At 14 mM, highly fluorescent swollen tips were frequently observed, as well as strongly labelled scars resulting from the detachment of arthrospore-like structures (Fig. 3c). As a consequence of the frequent septation in the fungal hyphae, nuclear density was higher in the metal-treated samples compared to the untreated controls (Fig. 3a,b,c).

Chitin distribution on the fungal wall was confirmed by ultrastructural analysis of thin sections treated with gold-conjugated WGA. The labelling of septum well illustrates the changes induced by the metal treatment. Scattered gold granules were found over the thin transverse hyphal walls in the control samples (Fig. 4a). By contrast, longitudinal and trasverse walls of fungal mycelia exposed to $ZnSO_4$ were strongly labelled: septa featured a double wall, which allowed separation of the arthrospore-like structures (Fig. 4b,c).

Chitin quantification

To understand whether the modifications in wall architecture and in chitin location corresponded to a change in the amount of chitin produced by the fungus after exposure to zinc ions, the chitin content of mycelia grown in liquid medium was quantified. A higher amount of chitin was found in the mycelium exposed to $ZnSO_4$, when compared to untreated samples (Table I).

Fig. 2 - Ultrastructural features of hyphae growing in the absence (a-b) and in the presence of Zn (c-e). a. Longitudinal section of a hypha, close to the apex. The cytoplasm is dense and rich in ribosomes; wall (W) is thin with a more electron-dense layer at the surface (inset, arrow). b. Longitudinal section of a hypha in a subapical portion, showing a nucleus (N), mitochondria (M), glycogen particles (G) and an abundant extracellular fibrillar material (EM). A hypha cut in a more basal part shows a high number of vacuoles (V). c. Trasverse section of a hypha after a 7 mM Zn treatment: mitochondria (M) are swollen, vacuoles (V) have an electron dense content, walls (W) are thickened with a regular layering (inset). d. Amorphous masses are laid down in the middle of the transversal wall (arrow). e. The transversal wall (S) appears very thick and irregular in structure. The electron dense material is still evident after the 14 mM Zn treatment. Bars = 0.25 μm.

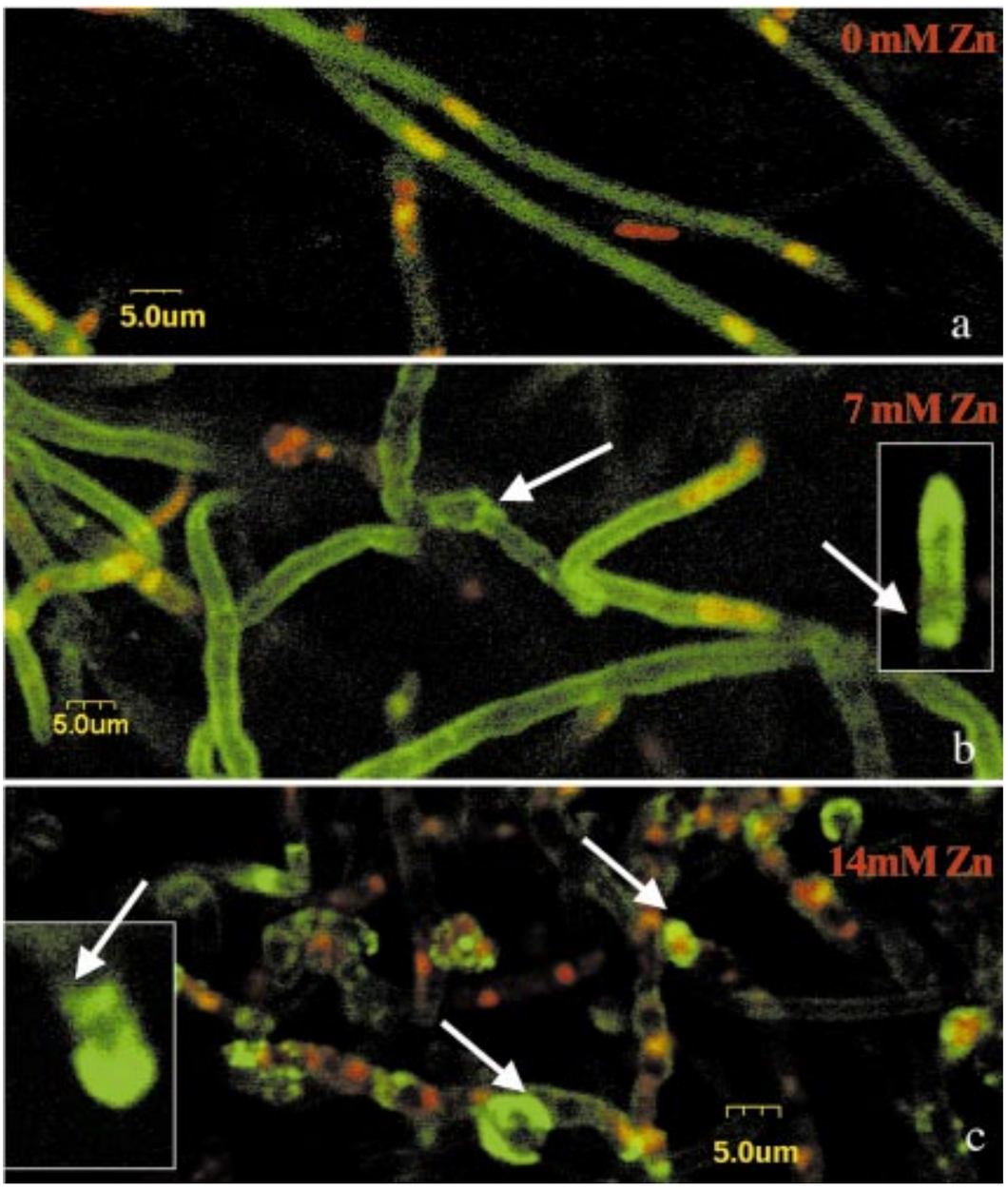


Fig. 3 - Morphology of PSIV hyphae growing in the absence (a) and in the presence of Zn (b-c) as seen under confocal microscope after WGA-FITC treatment for chitin location and propide iodure staining for nuclear distribution. a. A weak uncospicuous labelling is seen along the walls ofthe control colonies. Nuclei are elongate in shape. Septa are not easily visible. b. At 7 mM wall labelling is intense, particularly on the septa (arrows). Hyphal tips are swollen and fluorescent with closer septa. c. At 14 mM strongly labelled scars are very common together with strongly labelled septa. Nuclei are round in shape and very closed one to the other. Swollen tips are frequent.

Table I

$ZnSO_4$ concentration	*N*-acetylglucosamine content*
0 mM	1.97 ± 0.08
7 mM	12.17 ± 0.73
14 mM	8.94 ± 0.48

* μg / mg of dry weight

DISCUSSION

Although it has been often reported that supra-optimal concentrations of heavy metals interfere with fungal growth by causing a general decrease in biomass, investigations on the cellular processes affected by these metals are scanty (see Ramsay *et al*., 1999). In this study, we have shown that toxic concentrations of zinc deeply affect the hyphal morphology in the sterile ericoid mycorrhizal ascomycete PSIV, a strain sensitive to heavy metals (Martino *et al*., 2000). At the molecular level, they cause an unexpected increase in the amount of chitin deposited in the cell wall.

Apical growth is the hallmark of fungi. This apparently simple process is actually the result of complex interactions between internal and external signals. Changes in the balance of these components may therefore alter the shape of the hyphal tip or the direction of growth. The presence of high concentrations of zinc did modify the hyphal morphology, and the most characteristic features of zinc-treated hyphae, when compared to control samples, were the overall increase in hyphal branching, swelling and septation. Many septa showed a double wall structure and were responsible of a schizogenous separation of hyphal compartments, reminiscent of the formation of asexual propagules. This phenomenon may be of ecological relevance, because it may represent a strategy to escape from adverse conditions by increasing fungal dispersion.

Tip swelling is a common response of filamentous fungi to environmental stress, but can be also associated with specific morphogenetic processes (see Moore 1998). An example is the formation of conidia during asexual reproduction, or the development of infection structures such as appressoria in plant pathogens (Perfect *et al*., 1999) and mycorrhizal fungi (Bonfante and Perotto, 1995). Swelling in response to chemical or physical stress follows the "stop-swell-branch" sequence observed originally by Robertson (1965), which may also explain the increase in branching observed in the zinc-treated mycelia. Ramsay *et al*. (1999) actually reported for *Trichoderma viride* and *Rhizopus arrhizus* a decrease in branching when grown on low-nutrient media in the presence of Cd and Cu ions. However, the differences in the taxonomic position of the fungal species investigated, as well as the different metal ions and culture conditions used may explain these different behaviour.

Swelling may be explained by different mechanisms. According to the model proposed by Bartnicki-Garcia (1973; see Moore 1998), hyphal growth involves a continuous balance between the activity of synthetic and lytic enzymes. Chitin synthases and chitinases are key players in this process because they are involved in chitin synthesis and chitin degradation, respectively. Loosening of the cell wall, due to excessive degradation or reduced local synthesis of wall components, may thus result in swelling. Treatments of growing mycelia with wall loosening enzymes such as chitinase have been reported to induce pronounced swelling – or even bursting - in several fungal species (Arlorio *et al*., 1992). Swelling has also been observed in genetic mutants defective in the expression of specific chitin synthase (*chs*) genes (Yarden and Yanofsky, 1991; Borgia *et al*., 1996; Aufauvre-Brown *et al*., 1997). Experimental evidences indicate that PSIV, like other filamentous fungi, features a multigene family of chitin synthases (*chs*), with at least two genes. The expression of one of these genes, corresponding to a class II Chs, seemed to be down regulated in the mycelium grown in the presence of high concentraton of zinc ions in the liquid medium (Lanfranco *et al*., unpublished results). It would be tempting to speculate that the swelling observed in metal-treated hyphae may derive from an unbalance

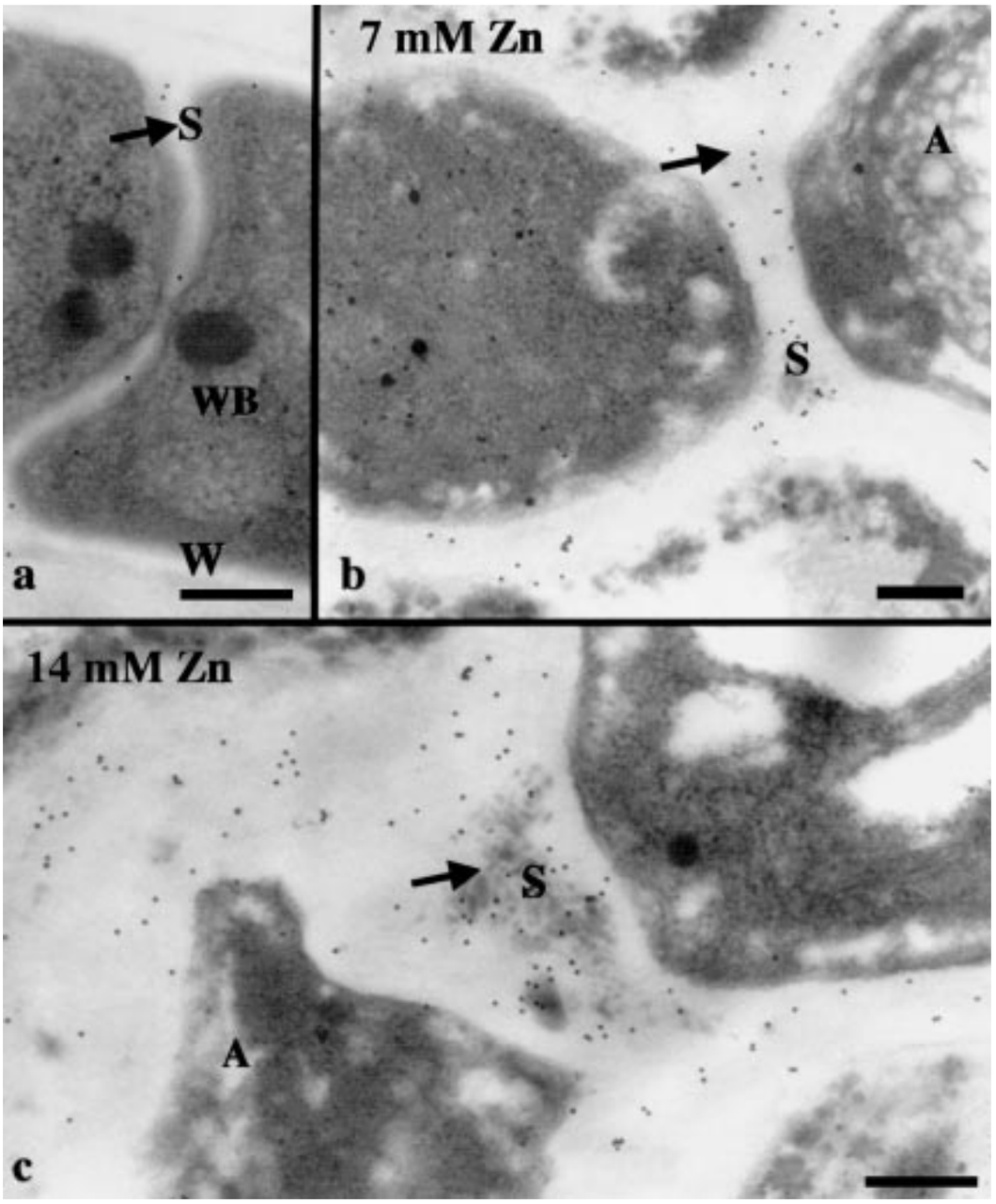


Fig. 4 - Ultrastructural features of hyphae growing in the absence (a) and in the presence of Zn (b-c) after gold-bound WGA treatment for chitin location a. Loose gold granules (arrow) are seen over the thin transversal (S) and longitudinal (W) walls of the control hyphae. WB: Woronin bodies. b. An intense gold granule deposition is seen over the thickened septum (S) which separates a swollen tip, which appears as an arthrospore-like hypha (A). The double septum may allow the detachment. c. Magnification of a septum (S) immediately below a strongly modified tip (A) after the 14 mM treatment. All the walls around this arthrospore-like structure are strongly labelled as well as the electron dense material (arrow) occurring over the septum. Bars = 0.25 μm.

between chitin synthase and chitinase activities. This unbalance may also lead to changes in overall wall architecture and in the interaction of chitin with other wall components.

A distinctive feature of zinc-treated hyphae in PSIV was the significant increase in the amount of chitin deposited in the cell walls, particularly abundant on the numerous septa found under these conditions. Similarly an increase in chitin (2.4 times higher) was also found in *Fusarium oxysporum* grown in the presence of copper ions (Hefnawy and Razak, 1998) although its precise localization was not determined. A hypothesis to explain the increase in chitin deposition which is mostly located on the transverse hyphal walls of the ericoid strain and does not exit into a hyphal extension, is the up-regulation of specific and still unidentified chitin synthases genes. Alternatively, the literature provides evidence that chitin synthase enzymes, that are distributed throughout the plasma membrane, can be activated by post-translational modifications that involve proteolytic cleavage upon different stimuli (see Gooday, 1995). Zn ions could act as a stimulus for a localised Chs activity.

In conclusion, a combination of morphological observations and cytochemical and quantitative assays for chitin demonstrate that Zn affects fungal growth with multiple attacks: at the tip it produces swelling, in the subapical parts it increases the number of branches and the thickness of transverse walls, leading to an inhibition of hyphal extension. Further experiments are needed to demonstrate whether the altered phenotype is caused by a differential expression of Chs isozymes.

ACKNOWLEDGEMENTS

The authors thank Dr C. Carotti and L. Popolo for the help in chitin quantification, Dr A. Genre, Mrs A. Faccio and S. Panero for assistance in confocal and electron microscopy. The research was funded by the Finalized Project Biotechnology (Subproject 2: Environmental Biotechnology) and National Council of Research (CNR) to Paola Bonfante.

REFERENCES

Arlorio, M., Ludwig, A., Boller, T., Bonfante, P.: Inhibition of fungal growth by plant chitinases and β-1,3-glucanase. A morphological study. Protoplasma *171*, 34-43, 1992.

Aufauvre-Brown, A., Mellado, E., Gow, N.A.R., and Holden D.W.: *Aspergillus fumigatus chs*E: a gene related to *CHS*3 of *Saccharomyces cerevisiae* and important for hyphal growth and conidiophore development but not pathogenicity. Fung. Genet. Biol. *21*, 141-152, 1997.

Babich, H., and Stotzky, G.: Heavy metal toxicity to microbe-mediated ecologic processes: a review and potential application to regulatory policies. Environ. Res. *29*, 111-137, 1985.

Bartnicki-Garcia, S.: Fundamental aspects of hyphal morphogenesis. In Microbial Differentiation (Eds. Ainsworth, J.M. and Smith, J.E.), Cambridge University Press. Cambridge, UK, pp. 245-267, 1973.

Bonfante, P., and Perotto S.: Strategies of arbuscular mycorrhizal fungi when infecting host plants. New Phytol, *82 (130)*, 3-21, 1995.

Borgia, P.T., Iartchouk, N., Riggle, P.J., Winter, K.R., Koltin, Y., and Bulawa, C.E.: The *chs*B gene of *Aspergillus nidulans* is necessary for normal hyphal growth and development. Fung. Genet. Biol. *20*, 193-203, 1996.

Bulawa, C.E.: CSD2, CSD3 and CSD4, genes required for chitin synthesis in *Saccharomyces cerevisiae*: the CSD2 gene product is related to chitin synthases and to developmentally regulated proteins in *Rhizobium* species and *Xenopus laevis*. Mol. Cell. Biol. *12*, 1764-1776, 1992.

Colpaert, J.V., Vandenkoornhuyse, R., Adriansen, K., and Vangronsveld, J.: Genetic variation and heavy metal tolerance in the ectomycorrhizal basidiomycete *Suillus luteus*. New Phytol. *147*, 367-379, 2000.

Frey, B., Zierold, K., and Brunner, I.: Extracellular complexation of Cd in the Hartig net and cytosolic zinc sequestration in the fungal mantle of *Picea abies – Hebeloma crustuliniforme* ectomycorrhizas. Plant Cell Environ. *23*, 1257-1265, 2000.

Gadd, G.M.: Toxicity screening using fungi and yeasts. In Toxicity Testing Using Microorganisms (Eds. Dutka, B.J. and Bitton, G.), CRC Press, Boca Raton, Florida, pp. 43-77, 1986.

Galli, U., Schuepp, H., and Brunold, C.: Heavy metal binding by mycorrhizal fungi. Physiol. Plant. *92*, 364-368, 1994.

Gooday, G.W.: The dynamics of hyphal growth. Mycol. Res, *99 (4)*, 385-394, 1995.

Gow, N.A.R., Robson, G.D., and Gadd, G.M.: The Fungal Colony. Cambridge University Press, Cambridge, UK, 1999.

Hefnawy, M.A., and Razak, A.A.: Alteration of cell-wall composition of *Fusarium oxysporum* by copper stress. Folia Microbiol. *43*, 453-458, 1998.

Leyval, C., Turnau, K., and Haselwandter, K.: Effects of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. Mycorrhiza *7*, 139-153, 1997.

Lilly, W.W., Wallweber, G.J., and Lukefahr, T.A.: Cadmium absorption and its effects on growth and mycelial morphology of the basidiomycete fungus, *Schizophyllum commune*. Microbios *72*, 227-237, 1992.

Martino, E., Turnau, K., Girlanda M., Bonfante, P. and Perotto, S.: Ericod mycorrhizal fungi from heavy metal polluted soils: their identification and growth in the presence of zinc ions. Mycol.Res. *104*, 338-344, 2000.

Moore D.: Fungal Morphogenesis. Cambridge University Press, Cambridge, UK, 1998.

Nordgren, A., Bååth, E., and Söderström, B.: Microfungi and microbial activity along a heavy metal gradient. Appl. Environ. Microbiol. *45*, 1829-1837, 1983.

Perfect, S.E., Hughes, H.B., O'Connell, R.J., and Green, J.R. *Colletotrichum*: A model genus for studies on pathology and fungal-plant interactions. Fung. Genet. Biol. *27*, 186-198, 1999.

Perotto, S., and Martino, E.: Molecular and cellular mechanisms of heavy metal tolerance in mycorrhizal fungi: what perspectives for bioremediation? Minerva Biotec. *13*, 55-63, 2001.

Perotto, S., Peretto, R., Moré, D., and Bonfante, P. Ericoid fungal strains from an alpine zone: their cytlogical and cell surface characteristics. Symbiosis *9*, 167-172, 1990.

Ramsay, L.M., Sayer J.A., Gadd G.M.: Stress responses of fungal colonies towards toxic metals. In The Fungal Colony (Eds. Gow, N.A.R. Robson, G.D. and Gadd, G.M.), Cambridge University Press, Cambridge, UK, pp. 178-200, 1999.

Robertson, N.F.: The mechanisms of cellular extension and branching. In The Fungi, Vol. 1 The Fungal Cell (Eds. Ainsworth, G.C. and Sussman A.S.), Academic Press, New York, pp. 613-623, 1965.

Roland, J.C., and Vian, B.: Affinodetection of the sites of formation and of the further distribution of polygalacturonans and native cellulose in growing plant cells. Biol. Cell. *71*, 43-55, 1991.

Yarden, O., and Yanofsky, C.: Chitin synthase 1 plays a major role in cell wall biogenesis in *Neurospora crassa.* Genes Dev. *5*, 2420-2430, 1991.