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Ph.D. in Biology and Biotechnology of Fungi

Serpentine soils, derived from the weathering of ultramafic rocks (i.e. rock high in iron and magnesium silicates) offer environmental cases for the study of the interaction mineral/fungi or metal/fungi. Such soils have strong limitations of fertility because of their mineral composition, rich in magnesium and containing heavy metals that reach hazard levels, such as nickel and chromium. In Italy, serpentine sites are found in the North-Western Alps and in the Northern Appenin, and they are considered to be a source of biological variability and biodiversity. Serpentine rocks can bear asbestos minerals, which represent a hazard for health if exposed, airborne and inhaled.
The mechanisms of asbestos toxicity are not fully established. The fate of the fibres inhaled into the lung depends mainly on their size, shape, biopersistence and surface reactivity. One of the most harmful reactions occurring at the fibres surface is the iron-catalyzed free radical generation, that can lead to the oxidation of biomolecules (lipids, proteins and DNA), to the alteration of cellular homeostasis, and can contribute to the oxidative stress related to the chronic inflammation, all together leading to fibrosis.
Asbestos is thus harmful for health, and the presence of fibrous asbestos minerals, mainly as a consequence of human activity (e.g. asbestos mines) represents an important environmental issue. In those cases asbestos cannot be removed, and at the moment there are no strategies of inactivation *in situ*. Any remediation method should aim to modify the characteristics of asbestos minerals that are related to its toxicity, such as fibrous shape, crystalline structure, biopersistence, iron content and surface reactivity.
Iron ions extraction could be important because of its role in catalyzing damaging reactions at the fibres surface. In fact, asbestos treatment with chelators in vitro can subtract iron ions from fibres surface and subsurface and blunt the fibres surface chemical reactivity. Asbestos in soil could be modified by fungal chelators, directly released by closely growing fungi.
The aims of this thesis were: 1) investigate the fungal diversity associated to serpentine rocks collected in the Western Alps; 2) functionally test some serpentinic strains for their ability to extract iron and magnesium from the fibres; 3) study the chemical composition and reactivity, both in acellular and in cellular systems, of fungi-modified asbestos fibres; 4) investigate the metabolic response of fungi to fibres.

1) Isolation and identification of fungi from serpentine soils.
A possible strategy to find suitable agents for bioremediation of a contaminated site is the isolation of indigenous microrganisms from the contaminated soil itself and the in vitro selection of strains of interest for their degradative/detoxifying capabilities and their competitive growth. For these reasons, many samples of serpentine rocks collected in the Wastern Alps area were used to characterize the associated fungal population. The genera *Mortierella, Myrothecium* and
Penicillium
and the species
Verticillium leptobactrum
,
Aspergillus fumigatus
and

Paecilomyces lilacinus
dominated in the observed population for both abundance and frequency.
The striking dominancy of *V. leptobactrum*, previously only rarely isolated, raised the interest for this fungus, not only as possible bioremediation tool to modify asbestos fibres (see below), but also for its phylogenetic characterization. The analysis of ITS ribosomal sequences was used to confirm the morphological identification of the specie and to investigate its taxonomic relationship with related species. The taxonomy of the section Prostrata of the genus *Verticillium*, that includes *V. leptobactrum*, was revised by Gams and collegues (2001). They divided the section in five new genera: *Simplicillium, Lecanicillium, Pochonia, Haptocillium* and *Rotiferophtora*. The "serpentine" fungi form a cluster that is very close to, but distinct from, the other genera. In this group, most of the isolates' ribosomal DNA sequences cluster with the sequence deposited in gene banks for *V. leptobactrum*, thus suggesting their belonging to this specie.

2) Fungi-mediated fibres modification
Fungi are able to release organic compounds that modify metals mobility and bioavailability. *F. oxysporum*, already tested in similar assays with other kind of fibres, confirmed its ability to extract iron from crocidolite and chrysotile, and also showed a good ability to extract magnesium. Among the fungal strains isolated from a serpentine soil, *V. leptobactrum* and *P. lilacinus* were able to extract iron from crocidolite and iron and magnesium from chrysotile. The most active was V. leptobactrum. The amount of iron and magnesium extracted from the fibres depends not only on the activity of the fungus, but also on the physico-chemical characteristics of the different fibres, namely the amount of ion in the fibres and the surface area. If these features are considered in the data analysis, the results show that fungi induce deeper modification in chrysotile than in crocidolite, data confirmed by SEM-EDS and SEM-XPS analysis, showing that the effect of the fungal treatment involves mainly the fibres surface.

3) Fungi can modify asbestos fibres chemical reactivity.
The reactivity of fibres depleted of iron by fungi was investigated by focusing on the ability of the fibres to generate free radicals. The results suggest that the chelating activity of fungi involves reactive sites at the fibre surface, because there is a general decrease of reactivity for both chrysotile and crocidolite. The only exception observed was chrysotile incubated with V. leptobactrum which exhibited enhanced reactivity. This could be caused by the strong chelating ability of the fungus that, by modifying the fibre surface, may lead to the exposure of poorly coordinated iron ions that may represent new reactive sites. A similar effect was observed after treatment of crocidolite with ascorbic acid.

The ability of fibres depleted of iron by fungi to induce an oxidative damage to naked DNA in the presence of hydrogen peroxide was investigated by measuring 8-oxo-7,8-dihydro-2'-deoxyguanosine (8oxodGuo) formation, a marker of genotoxicity. Fibres treated with fungi induced significantly lower amount of 8oxodGuo than control fibres.
The oxidation of an isolated molecule of DNA by fibres depends on the chemical properties of the fibres surface and on the medium, while the reactions occurring in a cellular context are much more complex. The oxidation of DNA in HL60 (macrophage-like cells) and A549 (lung epithelial cells) was investigated, with the aim to reveal possible differences between fungus-treated and untreated fibres effects. Even though modified fibres have a lower oxidative activity on naked DNA, it was not possible to draw a direct correlation between chemical modification of fibres and cellular effects.

4) Metabolic responses of fungi to asbestos fibres.
Iron extraction from asbestos by fungi lead to an increased concentration of these ions in the culture medium. Some of the markers of oxidative stress were considered in order to understand the posible oxidative stress response in fungi.
Malondialdehyde (MDA), a product of lipid peroxidation, is highly toxic and used as a marker of oxidative stress. Mycelia incubated with crocidolite showed a higher amount of MDA than control mycelia, whereas chrysotile did not significantly induced MDA formation. Since lipoperoxidation is initiated by iron-generated radicals, the lower amount of iron contained in chrysotile likely explains the difference from crocidolite.
The expression and activity of antioxidant enzymes, such as superoxide dismutase, catalase and glutatyhione peroxidase, were investigated, obtaining different results for different fibres and different fungal strains. Altogether the data concerning the fungal metabolic response to asbestos fibres, even if not complete, suggest the involvement of the investigated antioxidant systems, which are also involved in the mammalian cells response.

One of the issues raised by the presence of asbestos in soils is the remediation of the sites involved, because the contaminant is neither degraded nor removable.
The experiments reported in this thesis show the ability of fungi to interact with asbestos fibres and to modify them after just 20 days. As a consequence of chemical modification of the fibres, the damage caused to DNA in vitro was strongly reduced. This result did not find a direct correspondence with the results obtained in cell cultures. However, other experiments suggest that iron extraction from the fibres could be prolonged until no more bioavailable iron remains, thus further inactivating the fibres. The in vitro modification and the bioweathering activity of fungi in the soils, allows some speculation on their possible effectiveness on asbestos minerals in the soil, even though no in field assays have been performed so far. The isolation and identification of the fungal population inhabiting ophiolite sites contribute to the characterization of these ecologically important sites, but also to the identification of metal tolerant strains, interesting for the development of bioremediation strategies.