Iron Solubilization by Mycorrhizal Fungi Producing Siderophores*

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Abstract

In iron deficient conditions most of the microorganisms are able to produce iron chelating compounds called siderophores. Usually bacteria are considered to produce catecholates siderophores, and fungi hydroxamates siderophores.

In the reported experiments a mycorrhizal fungus, *Suillus granulatus*, was grown in iron deficient conditions in presence or in absence of ferric oxyhydroxide. The solubilization of this well crystallized oxyhydroxide (goethite) was studied to be related to the production of several organic compounds in the medium (siderophores, organic acids), and to the fungal growth. *Ustilago sphaerogena*, a smut fungus well known for its siderophores production, was used as reference. Results showed that bioweathering of the mineral (goethite) is mainly due to the siderophores production. Such microbial dissolution processes are certainly important in regards of iron acquisition by microorganisms but possibly also by higher plants under low iron availability in the soil.

Introduction

A large number of microorganisms are known to produce acidifying and/or chelating agents, which could be involved in the dissolution of minerals (Robert and Berthelin, 1986, Berthelin, 1988). By this way, such microorganisms may obtain mineral nutrients even in excess to their requirements.

Iron solubility is generally very low under aerobic conditions and microorganisms have to produce some efficient chelating agents to mobilize iron from primary or secondary iron sources in soils. So, under conditions of iron deficiency, Neilands (1974), Emery (1978) have observed that bacteria, fungi and algae can produce siderophores, which are low molecular weight compounds, having a very strong affinity for Fe^{III} (constants of the complexes as high as 10^{32}). Mycorrhizal fungi are also able to produce such compounds, that have generaly hydroxamic functional

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group. It appears that host plants could take advantage from this phenomenon (Powell et al., 1982).

Such processes of iron mobilization may be involved in soils, where an important insoluble iron source is represented by ferric oxyhydroxides. Therefore the purpose of this study is to determine the mechanisms of iron solubilization by an ectomycorrhizal fung, *Suillus granulatus*, and the involvement of its organic acids and siderophores, which are mainly produced under iron deficiency conditions, in the dissolution of ferric oxyhydroxides. *Ustilago sphaerogena*, a smut fungus, well known to produce two siderophores, ferrichrome and ferrichrome A (Emery, 1971) was used as reference.

Materials and Methods

Experimental design

Incubations were performed in batch culture devices in liquid media inoculated by the fungi, in absence or in presence of an oxyhydroxide (goethite) enclosed in a dialysis bag to avoid contamination of the mycelium by mineral particles.

Culture conditions

The two fungi *Suillus granulatus* and *Ustilago sphaerogena*, were cultivated respectively in two different media, designated as Pachlewski medium and Ustilago medium. The composition of the Pachlewski medium is as follow: $MgSO_4$, $7H_2O$ (0.5 g/l), KH_2PO_4 (1 g/l), ammonium tartrate (0.5 g/l), thiamine (5 μg/l), glucose (20 g/l), maltose (5 g/l). The pH of this medium after autoclaving is around 6–6.5. Ustilago medium contains 10 ml of the metallic solution (0.197 mg of $CuSO_4$; 1.08 mg of $MnSO_4,H_2O$; 88.3 mg of $ZnSO_4,7H_2O$, in 100 ml of distilled water); K_2SO_4 (0.1 g/l); K_2HPO_4 (4 g/l); ammonium acetate (0.3 g/l); citric acid (0.115 g/l); thiamine (0.2 μg/l); $MgSO_4,7H_2O$ (800 mg/l); sucrose (20 g/l).

The pH is adjusted to 6.8 with NH_4OH. Culture flasks of 250 ml, containing 120 ml of nutrient medium, closed with a coton plug were autoclaved at 120°C during 30 mn.

The goethite, particle size inferior at 0.2 μm, was synthetised as described by Atkinson et al., (1968). 100 mg of the mineral was enclosed in a dialysis bag (Visking, pores size 2.4 nm), then autoclaved in distilled water twice at 120°C.

Culture flasks inoculated by the two fungi, *Ustilago sphaerogena* (ATCC 12421) and *Suillus granulatus* (Garbaye, INRA, Nancy Champenoux, France), were incubated on a shaking incubator (Bioblock) at 120 rpm at 24°C in the dark. Three repetitions were done per treatment. Cultures were performed during 22 days for *Ustilago sphaerogena*, and 32 days for *Suillus granulatus*, and harvested respectively after 6/12/18/22 days and 7/14/21/32 days.

To eliminate iron impurities all the glassware were rinced with acid (HCl 6N) then with bidistilled deionized water.

Analysis

Fungal biomass was separated from the supernatant by centrifugation (1200 rpm during 15 mn), then washed on a filter paper with distilled water.

The weight of the dried biomass was measured and then mineralized according to Clément's method (Clément, 1977), before analysis.

After measurement of the pH, solubilized iron and iron accumulated by the biomass (bioaccumulated iron) were determined by Emission Spectrometry (Inductively Coupled Plasma, Jobin Yvon). The organic acids were characterized by HPLC (Gold Beckman). Colorimetric methods were used to estimate the amounts of sugars (Dubois et al., 1956), of diphenols (Folin, 1912), and of siderophores (hydroxamates and phenolates) by the methods of Csaky and Hathway (Reeves et al., 1983).

Results

Suillus granulatus growth (Fig. 1) was better than *Ustilago sphaerogena* one (Fig. 2). Comparatively to the inoculum (or to the control without goethite) *Suillus granulatus* and *Ustilago sphaerogena* growths were respectively significant or no significant (Student test). *Suillus granulatus* grew continuously during the incubation time (Fig. 1), but decrease in biomass, certainly as a result of autolysis, occured after 12 days incubation for *Ustilago sphaerogena*. Although both fungi were growing in their specific medium, *Suillus granulatus* did not use sugars so quickly as *Ustilago sphaerogena* that had utilized more than 90% of them after twelve days of incubation.

The time course of medium pH varied during growth. A noticeable acidification occured in the first days with both fungi, but it was slightly higher with *Suillus granulatus* (Fig. 1). pH medium stabilized around pH 3.0 with this latter, while it increased after 12 days incubation for *Ustilago sphaerogena*. The presence of goethite did not affect both fungi growth and medium pH.

In the controls without goethite, iron concentration in the solution and in the biomass did not change and remained very low (around 100 ppb) during the growth of both fungi (Figs 1a and 2a). In contrast, increased solubilization in the medium and accumulation in the microbial biomass of iron were observed in the presence of goethite, as a result of its dissolution. However the time course of iron dissolution was quite different for both fungi.

With *Suillus granulatus* the solubilization and bioaccumulation of iron were very slow until 20 days of incubation. Then the dissolution of goethite increased remarkably; the amount of soluble iron increased more quickly than that of bioaccumulated iron. The concentration of iron in the medium reached 2.32 ppm (Fig. 1).

With *Ustilago sphaerogena* iron was solubilized in the medium at the beginning of the incubation, mainly during the first twelve days and reached a concentration in the medium of 2.1 ppm. During the stationary and death phase respectively a slight then
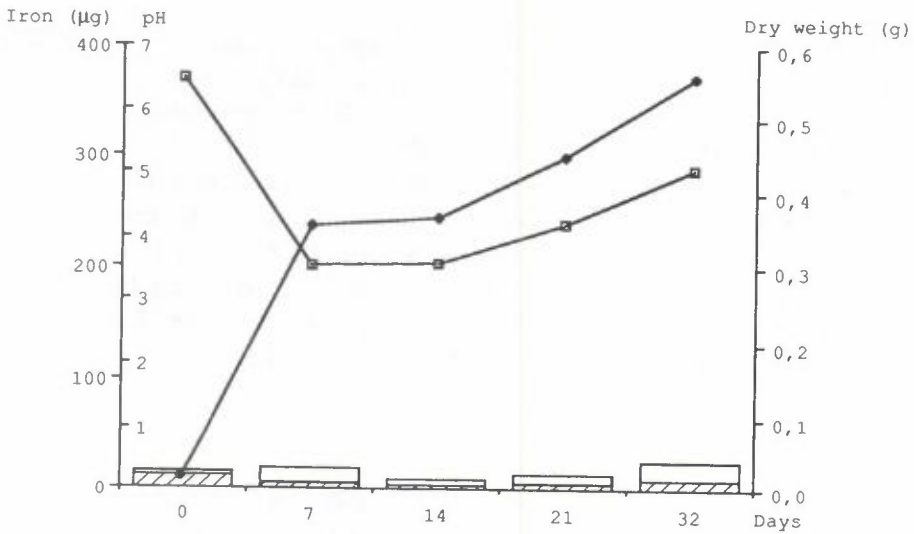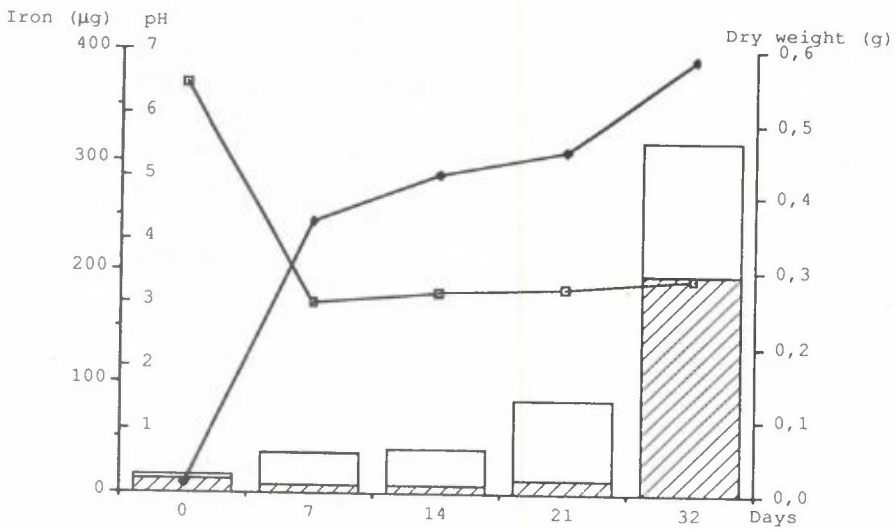
Fig. 1a

Fig. 1b

Figure 1. Time course of pH of the medium, fungal growth and iron solubilization per culture flask obtained for *Suillus granulatus*, without (1a) or with (1b) goethite.
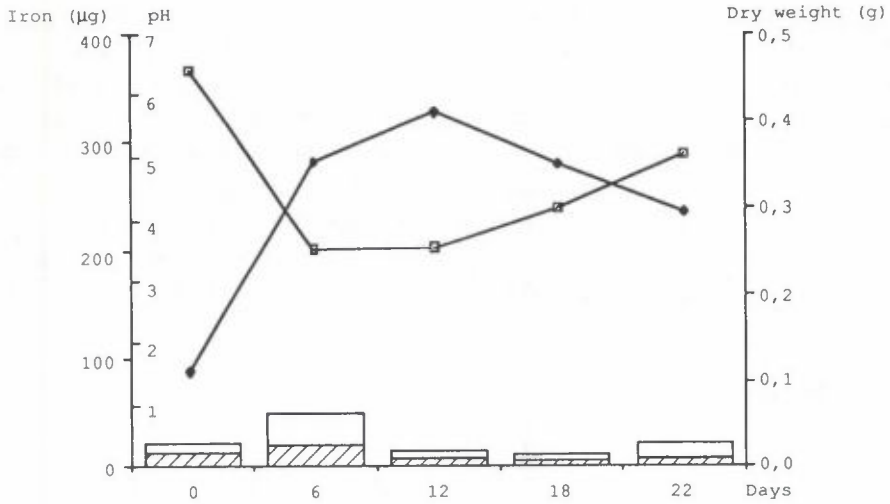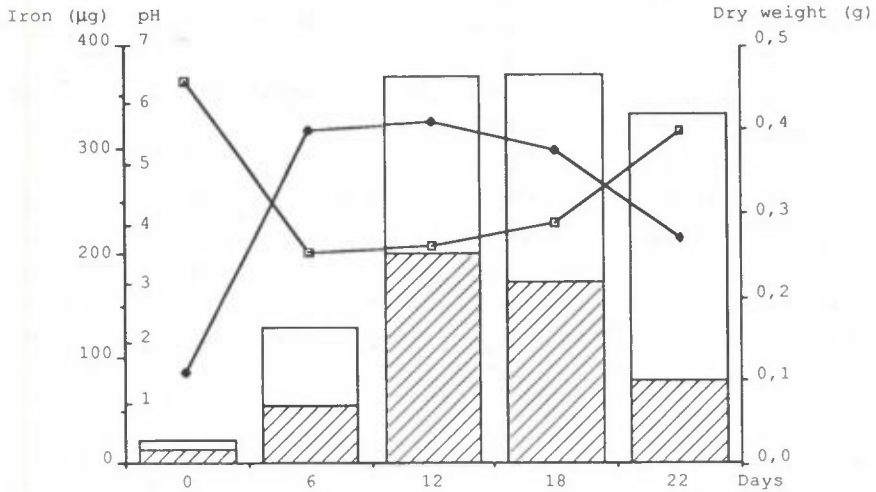
Fig. 2a

Fig. 2b

Figure 2. Time course of pH of the medium, fungal growth and iron solubilization per culture flask obtained for *Ustilago sphaerogena*, without (2a) or with (2b) goethite.

a significant (p = 0,01) decrease of solubilization were observed, corresponding probably not only to a decrease of growth and consequently of goethite dissolution but also connected to bioaccumulation processes. In fact iron immobilization in the biomass began just as the solubilization started and continued during all the experiment (Fig. 2).

Bioaccumulation at the end of the fungal culture was more important for *Ustilago sphaerogena* (253 μg of iron per flask) than for *Suillus granulatus* (119 μg per flask). In regard of the growth, the sum of soluble and immobilized iron reached 1259 and 539 μg/g of dry matter with *Ustilago sphaerogena* and *Suillus granulatus* respectively (table 1). Such amounts correspond respectively to 0.25% and 0.20% of the total iron of the initial ferric oxyhydroxide.

Table 1. Amounts of solubilized iron in μg/g dry biomass. The first/second/third/fourth samplings corresponded respectively for *Ustilago sphaerogena* and *Suillus granulatus* to 6/12/18/22 days and 7/14/21/32 days.

Samplings	First	Second	Third	Fourth
Suillus − goethite	53	27	33	24
Suillus + goethite	95	91	184	539
Ustilago − goethite	128	33	31	78
Ustilago + goethite	321	891	988	1260

Similar amounts of organic compounds (aliphatic acids, hydroxamic acids and diphenol compounds) were produced by *Ustilago sphaerogena* and *Suillus granulatus* in the culture in presence or in absence of goethite. But the behaviour of the fungi in their production was quite different.

Ustilago sphaerogena produced small amounts of diphenols at the beginning of the incubation without any change during all the incubation. With *Suillus granulatus*, these compounds appeared in larger amount only at the end of the incubation (table 2). The Csaky method revealed the presence of hydroxamates for both fungi. They were present for *Ustilago sphaerogena* at the first sampling (t = 6 days), then they were no more detected. For *Suillus granulatus*, the amounts of hydroxamates has increased strongly at 32 days, and phenolates compounds that could correspond to the other type of siderophore were found in larger amount at the end of the incubation (table 2).

In addition some results obtained by HPLC analysis showed that organic acids,

Table 2. Contents in mM/1 of diphenols and phenolates produced by *Suillus granulatus*

	Samplings (days)	7	14	21	32
Diphenols	Suillus − goethite	0.055	0.054	0.060	1.17
	Suillus + goethite	0.025	0.032	0.055	1.24
Phenolates	Suillus − goethite	0.018	0.017	0.008	0.12
	Suillus + goethite	0.016	0.015	0.023	0.20

identified as oxalic acid, citric acid and malic acid were present in the culture media of *Ustilago sphaerogena* in the amounts of, respectively, 6, 130 and 230 ppm, at 12 days. Their production occured at the beginning of the culture, it increased strongly during the first twelve days and then decreased gradually, and so, seemed to be related to the iron solubilization. For *Suillus granulatus* the chromatograms showed especially qualitative differences because more acids were detected and their number increased during the incubation. Some acids disappeared and seemed to be used as nutrients. Such results were confirmed by the potentiometric titration of the different acidities, showing the disappearence of at least a part of the organic acids at the end of the incubation. To verify the possible role of organic aliphatic acids in the weathering of the goethite, incubations were performed in axenic conditions (without microorganism). Several organic acids, known as acid and complexing agents, were tested at the concentrations they were found in the growth media after the fungal culture: oxalic, citric and malic acid respectively at 10, 150, 300 ppm. After one month of incubation, no iron solubilization was observed. The pH of the medium were respectively 3.8, 3.5 and 3.2 and were similar to the pH of the culture media, showing that the acidity alone is not involved directly in the iron dissolution. There is, in the experiments with fungi, a disproportion between the quantity of acids and the iron release in the nutrient media, that verify the low efficiency of these aliphatic acids, in goethite dissolution.

Discussion and Conclusion

Both fungi *Suillus granulatus* and *Ustilago sphaerogena* are able to solubilize and to accumulate iron from goethite at a significant extent. But their respective behaviour during these processes are under a kinetic and chronological aspect quite different.

With *Ustilago sphaerogena*, despite a slower biomass production, the dissolution and immobilization (or bioaccumulation) of iron occured as soon as the beginning of the incubation, and are accompagnied by an important consumption of sugars (sucrose), a production of siderophores detected as hydroxamic acids (certainly ferrichome and ferrichrome A), and a production of aliphatic acids (citric, oxalic, malic and unidentified acids). So it seems that soluble ferric iron, certainly associated with ferrichrome, that is recognized as an efficient iron transport agent, was absorbed by *Ustilago sphaerogena*. At the second sampling period, hydroxamates were not significantly detected. Then in further incubation steps, the content of soluble iron decreased in the medium, as hydroxamates disappeared and as organic aliphatic acids decreased, but iron immobilization (or bioaccumulation) was continuing. At this time *Ustilago sphaerogena* utilized at least in part aliphatic acids as nutrients, and the connection between the curves of iron dissolution and of organic acids production make us assume that iron could get also chelated by these compounds. They seem able to relay at least in part the hydroxamates, which get depleted by fungal absorption. The

siderophores can also promote the possible weathering effect of the aliphatic acids. Such hypotheses need to be verified by further experiments and analysis.

Suillus granulatus has a different behaviour. Despite a good initial growth and a significant pH decrease, due to aliphatic acids production at the beginning of the incubation, iron was neither solubilized in the medium nor immobilized by the fungi during the first steps of the experiment. A slight solubilization and a relatively more important bioaccumulation appeared during the second period. Then as the growth become relatively low, only at the end of the incubation time, it was observed, during the last incubation step, an important iron solubilization. Simultaneously hydroxamic and also diphenolic compounds appeared at a significant extent in the medium. Such compounds can be related to the production of hydroxamates and phenolates siderophores, as it was observed for *Boletus edulis* (Szanislo et al., 1981). So what, with *Suillus granulatus* even if the kinetics of iron solubilization and immobilization and of siderophores production are quite different from those of *Ustilago sphaerogena*, ferric oxyhydroxide (goethite) weathering due to ferric iron dissolution, involved mainly siderophores production by the fungi. The possible involvement of the organic acids is not so clearly demonstrated as far they were not efficient in the performed axenic chemical tests and as their production at the beginning of the culture is not associated to iron solubilization.

In conclusion, from the results, it appears that mycorrhizal and saprophytic fungi are able to weather significantly ferric oxyhydroxides (e.g. goethite), a common mineral source of iron in different soils, by their production of siderophores that form hydroxamates or phenolates soluble ferric complexes. Such weathering is also increased by their iron bioaccumulation capacity that also involved siderophores as transport agents. Aliphatic acids produced simultaneously are not, or are scarcely involved in the dissolution of these well crystallized ferric minerals. They have in these processes certainly no more than a side effect, the main role being attributed to siderophores. One may also underline that the fungi do not have the same type of metabolism in regard of the production of siderophores and of the ferric iron solubilization and immobilization. In soils, such processes can play a significant role in the weathering of oxyhydroxides, but they can also more certainly play a major role in iron availability either for the microorganisms, or also for the plants, by their presence in the rhizosphere, or by their involvement (e.g. *Suillus granulatus*) in the mycorrhiza formation.

Acknowledgement

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