

M.O. Fomina

Institute of Microbiology and Virology, NASU, 154, Academ. Zabolotny str., Kyiv GSP,
D03143, Ukraine tel.: +38 (044) 526 92 16, e-mail: M.Fomina@ukr.net

NITROGEN SOURCE EFFECT ON COPPER SPECIATION WITHIN ECTOMYCORRHIZAL FUNGUS *RHIZOPOGON RUBESCENS*

Aim. The aim of this work was to study the effects of nitrogen source on the speciation of copper accumulated by fungi and ectomycorrhizas grown in the presence of copper phosphate. **Materials and Methods.** Ectomycorrhizal fungus *Rhizopogon rubescens* and its symbiotic ectomycorrhizal association with Scots Pine were grown in the presence of copper phosphate at different nitrogen sources: either ammonium or nitrate. The coordination of copper released from copper phosphate and bioaccumulated by fungus and ectomycorrhiza was determined by using synchrotron-based X-ray absorption spectroscopy (XAS). **Results.** XAS data showed that the change of nitrogen source from ammonium to nitrate was shifting copper coordination by mixed oxygen ligands towards oxalate coordination of copper. **Conclusions.** It was found that the conditions of growth of fungi and mycorrhizas may change the nature of ligands available for sequestering toxic metals and coordination of copper within ectomycorrhizal fungus *R. rubescens* and its symbiotic association with pine roots depended on nitrogen source. **Key words:** fungi, ectomycorrhiza, copper phosphate, nitrogen, metal speciation, bioaccumulation, X-ray absorption spectroscopy.

The ability of fungi to create mutualistic symbiotic associations with plant roots (mycorrhizas) has major consequences for the biogeochemical cycling of elements [7, 11, 13]. Mycorrhizal fungi solubilize phosphate and essential metals for the host plant in the course of so called “heterotrophic leaching” as a result of protonation, chelation and metal accumulation by the biomass [1, 5, 13]. It has been previously shown that mycorrhizal fungi were able to solubilize various toxic metal phosphate minerals resulted in releasing mobile toxic metals into environment and their bioaccumulation by fungi and mycorrhizas [2, 4].

Copper is one of the most common pollutants of the terrestrial environments. It has been reported that soluble fraction of copper in sludge-amended soils found almost exclusively in low molecular weight complexes with amino acids, small peptides or polycarboxylic acids is a major contributor to copper mobility and bioavailability threatening lower trophic levels of the food chain. Fungi can play a significant role in copper transformations and



many soil fungi were able to withstand copper toxicity in heavily contaminated soils (500–11.500 mg Cu/kg soil) [3–5]. Nearly 60–70% of tested mycorrhizal fungal cultures tolerated copper phosphate and cuprite with more than half of them being able to solubilize copper phosphate and over 40% of them solubilizing cuprite [4].

Because of the amorphous state or poor crystallinity of metal complexes within biomass and relatively low metal concentrations, the determination of metal speciation in biological systems remains a challenging problem with only a few studies on fungal biomass that mainly clarify the nature of adsorption sites on cell walls [6]. However, synchrotron-based X-ray absorption spectroscopy (XAS) provides the means for studying element complexation in environmental samples varying from biological to mineralogical in nature and is increasingly used to study biological systems giving information about the oxidation state of the target element and its coordination environment, including the number and identity of neighbouring atoms [9, 12].

The aim of this study was to elucidate the effect of nitrogen source on copper speciation by fungal and ectomycorrhizal biomass using X-ray absorption spectroscopy.

Materials and methods

Biomass used in present work was ectomycorrhizal fungus *Rhizopogon rubescens* B32ö in axenic culture and its ectomycorrhizal associations with Scots pine (*Pinus sylvestris*). Fungus and ectomycorrhiza were exposed to copper phosphate $[\text{Cu}_3(\text{PO}_4)_2 \times 2\text{H}_2\text{O}]$ at a final metal concentration equivalent to 15mM. Axenic culture of fungus was grown at 23–25 °C in sterile Petri-dish microcosms on top of cellophane membranes on modified Melin-Norkrans medium for mycorrhizal fungi (MMN) [4] and harvested after 2 months.

Formation of ectomycorrhiza was carried out under sterile conditions using a test tube technique in autoclaved (120 °C, 60 min) glass tubes filled with 30 ml vermiculite and peat mixture 5:1 and 10 ml of Ingestad medium [13].

After successful colonization, uniform pine seedlings were carefully taken out from the tubes, washed with sterile water and transferred into sterile square Petri dishes mesocosms. Square Petri dishes were filled with Ingestad agar amended with copper phosphate to a final metal concentration equivalent to 15mM. The roots of mycorrhizal seedlings were placed over sterile cellophane membrane on top of the agar medium. The upper cellophane membrane and an autoclaved piece of foam, soaked in sterile water, were placed on top of the roots to prevent them from desiccation and to fix the position of the root system. The shoot was kept outside by



means of a hole in the side of the plastic dish and sealed around the stem with sterile lanolin.

Mesocosms were incubated for at least 3 months in the growth chamber with $200 \mu\text{mol} \times \text{m}^{-2} \times \text{s}^{-1}$ PAR, at least 60% relative air humidity and at day/night regime of 18/6 h and a temperature of 23/15 °C. In the experiments with axenic fungal cultures and the *R. rubescens*/*P. sylvestris* associations we also used a nitrate-only version of MMN and Ingestad media substituting KNO_3 ($0.8 \text{ g} \cdot \text{l}^{-1}$ and $17.34 \text{ mg} \cdot \text{l}^{-1}$) for $(\text{NH}_4)_2\text{HPO}_4$ and NH_4NO_3 , respectively.

Freshly harvested biomass, enclosed in cello tape and quenched in liquid nitrogen, was used for X-ray absorption spectrometry.

XAS investigates atomic local structure and electronic states by measuring absorption of the electromagnetic radiation by electrons as a function of energy, and is usually performed at synchrotron radiation sources (SRS), provided intense and tunable X-ray beams.

X-ray absorption spectra at Cu K-edges were collected on Station 7.1 at the CCLRC Daresbury SRS operating at 2 GeV with average current of 140 mA, using a vertically collimating plane mirror and a sagittally bent focussing Si(111) double crystal monochromator detuned to 80% transmission to minimize harmonic contamination. Sample data were collected with the station operating in fluorescence mode using a 9-element solid state Ge diode detector with high count-rate XPRESS processing electronics. The monochromator was calibrated using a 5 mm Cu foil. The experiments were performed using a liquid nitrogen cooled cryostat. Single scans were collected for the model compounds, and 3–4 scans were collected and summed for each sample. Background-subtracted EXAFS spectra (Extended X-ray Absorption Fine Structure) were analysed in EXCURV98 using full curved wave theory. Fourier transformation of EXAFS spectra were used to obtain an approximate radial distribution function around the central metal (Cu – the absorber atom): the peaks of Fourier transformations can be related to the “shells” of surrounding back scattering atoms characterised by atom type, number of atoms in the shell, the absorber-scatterer distance, and the Debye-Waller factor ($2\sigma^2$). The data were fitted for each sample by defining the theoretical model and comparing the calculated EXAFS spectrum with the experimental data. The shells of backscatterers were added around the absorber and by refining an energy correction E_f (the Fermi energy), the absorber-scatterer distance, and Debye-Waller factor for each shell, a least squares residual (the *R*-factor was minimised). Where appropriate, multiple scattering effects were included in the fits.

Results and discussion

In the present study, all tested samples of fungal and ectomycorrhizal biomass grown in the presence of copper phosphate showed Cu coordination with oxygen ligands (tab. 1). The effect of nitrogen source on the coordination of accumulated copper was examined for both ectomycorrhizal



Table 1. Cu K-edge EXAFS parameters*.

Sample	Scatterers	r / E	2 σ^2 / E ²	Residual
Biomass of axenic <i>R. rubescens</i> exposed to copper phosphate:				
Grown on nitrate MMN agar medium	4 \times O	1.96	0.012	29.8
	2 \times O	2.31	0.022	
	4 \times C	2.72	0.019	
	2 \times Cu	3.82	0.039	
Grown on ammonium MMN agar medium	4 \times O	1.94	0.013	27.1
	2 \times O	2.37	0.037	
Biomass of <i>R. rubescens</i>/<i>P. sylvestris</i> ectomycorrhizas exposed to copper phosphate:				
Grown on nitrate Ingestad agar medium	4 \times O	1.99	0.021	27.2
	2 \times O	2.39	0.028	
	4 \times C	2.78	0.006	
	2 \times Cu	3.79	0.028	
Grown on ammonium Ingestad agar medium	4 \times O	1.94	0.012	33.7
	2 \times O	2.42	0.054	
	2 \times Cu	3.79	0.034	

* r is the copper–scatterer distance in Angstroms (± 0.02 E inner shells, ± 0.05 E outer shells). $2\sigma^2$ is the Debye–Waller type factor ($\pm 15\%$ inner shells, $\pm 30\%$ outer shells). Residual is a least squares residual from fitting the spectrum of the model to the experimental data

fungus *R. rubescens* in axenic culture and in its mycorrhizal association with Scots pine (tab. 1, fig. 1). The nitrogen source is a factor that can affect organic acid production by fungi with nitrate considerably increasing oxalate excretion [10, 14]. Biomass of both axenic *R. rubescens* and its ectomycorrhizal association grown on nitrate and copper phosphate gave the best fit to the inner shell with a coordination shell of 4 oxygens bound to the copper at a distance of 1.96 E and two oxygens bound at 2.31 E. This is a typical distortion from perfect octahedral symmetry for a six-coordinate copper (II) centre, and is known as a Jahn-Teller distortion [8]. The EXAFS contributing to two further peaks in the Fourier transform were best fitted with 4 carbons at 2.72 E and 2 coppers at 3.82 E, showing carboxylate coordination close to oxalate (tab. 1, fig. 1 d, h).

If ammonium was used as a nitrogen source, the best fit to the inner shell was with a Jahn-Teller distorted coordination shell of 4 oxygens at 1.94 E and two oxygens at 2.42 E. There was a small outer shell best fitted with two coppers at 3.79 E. However, attempts to fit phosphorus atoms at 3.1 E (phosphate) or carbon atoms at 2.7 E (oxalate/carboxylic acid) did not improve the fit (tab. 1, fig. 1). For other samples of biomass grown on ammonium-containing medium, the fit with carbons was slightly better and more consistent with oxalate coordination than with phosphate, but was not conclusive probably indicating mixed carboxylate/phosphate coordination of copper.



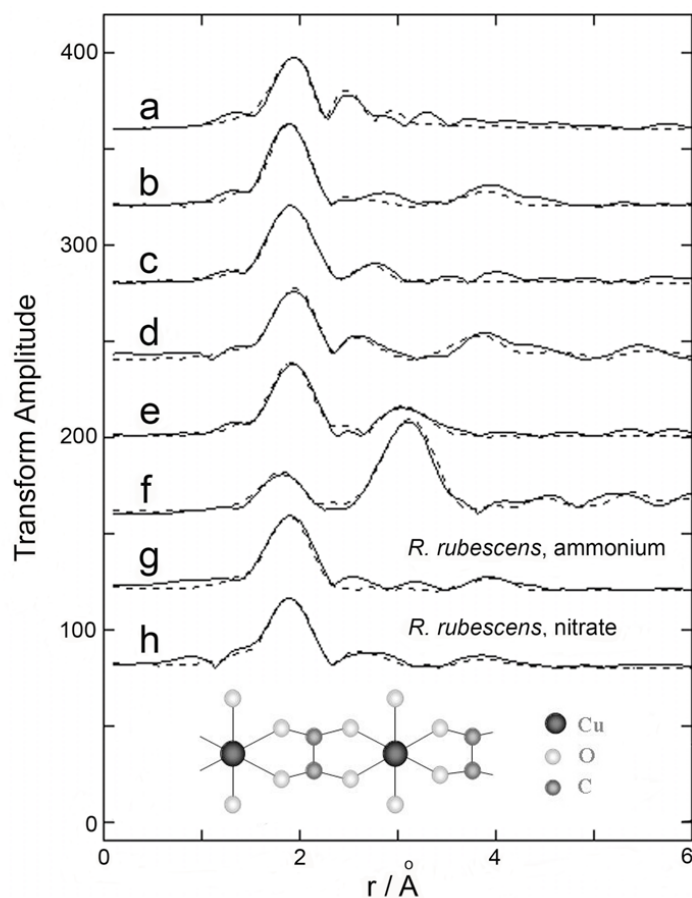


Fig. 1. Copper coordination within fungal biomass:

Fourier transforms of Cu K-edge XANES, EXAFS (solid lines) and fits (broken lines) for standard compounds (a) Cu(acetate), (b) Cu(gluconate), (c) Cu(malate), (d) Cu(oxalate), (e) Cu(phosphate), and (f) Cu(I)oxide. Samples are *R. rubescens* exposed to copper phosphate grown on MMN agar medium with (g) ammonium or (h) nitrate. Insert: model of Cu oxalate coordination within biomass of *R. rubescens* grown on media containing nitrate.

Thus, the conditions of growth in both *in vitro* and *ex vitro* may change the nature of ligands provided by fungal and mycorrhizal biomass. The change of nitrogen source from ammonium to nitrate may increase the proportion of oxalate excretion and subsequently shift towards oxalate coordination of toxic metal (copper) within fungal and mycorrhizal biomass.

The author would like to acknowledge the funding of CCLRC Daresbury SRS user grants 40107, 43079, and personally Prof Geoffrey M. Gadd (University of Dundee, UK) for the opportunity to do this work, Prof Hekan Wallander (Lund University, Sweden) for providing the fungal strain and Dr John Charnock (University of Manchester, UK) for his valuable help with EXAFS spectra analysis.

УДК [602:4:631.466:661.63-034.3]:631.416.1

М.О. Фоміна

Інститут мікробіології і вірусології НАН України,
вул. Академіка Заболотного, 154, Київ ДСП, Д03680, Україна,
тел.: +38 (044) 526 92 16, e-mail: M.Fomina@ukr.net

ВПЛИВ ДЖЕРЕЛА НІТРОГЕНУ НА ХІМІЧНЕ ЗВ'ЯЗУВАННЯ КУПРУМУ ЕКТОМІКОРИЗНИМ ГРИБОМ *RHIZOPOGON* *RUBESCENS*

Реферат

Мета. Метою цієї роботи було вивчення дії джерела нітрогену на зв'язування купруму, біоакумульованого грибами та ектомікоризою, що були вирощені у присутності фосфату купруму. **Матеріали і методи.** Ектомікоризний гриб *Rhizopogon rubescens* та його симбіотична ектомікоризна асоціація з сосною звичайною були вирощені у присутності фосфату купруму на різних джерелах нітрогену: амонію або нітраті. Координація купруму, звільненого з фосфату купруму та біоакумульованого грибом та ектомікоризою, визначалась з використанням синхротронної рентгено-абсорбційної спектроскопії (ХАС). **Результати.** Дані ХАС показали, що переміна джерела нітрогену з амонію на нітрат змінювала координацію купруму неоднорідними оксиген-вмісними лігандами на оксалатну. **Висновок.** Встановлено, що умови зростання гриба та мікоризи можуть змінювати природу лігандів, наявних для зв'язування токсичних металів. Координація купруму в ектомікоризному грибі *R. rubescens* та в його симбіотичній асоціації з коренями сосни залежала від джерела нітрогену.

Ключові слова: гриби, ектомікориза, фосфат купруму, нітроген, хімічне зв'язування металу, біоаккумуляція, рентгено-абсорбційна спектроскопія.

УДК [602:4:631.466:661.63-034.3]:631.416.1

М.А. Фомина

Институт микробиологии и вирусологии НАН Украины, ул. Академика Заболотного, 154,
Київ ДСП, Д03680, Україна, тел.: +38 (044) 526 92 16, e-mail: M.Fomina@ukr.net

ВЛИЯНИЕ ИСТОЧНИКА АЗОТА НА ХИМИЧЕСКОЕ СВЯЗЫВАНИЕ МЕДИ ЭКТОМИКОРИЗНЫМ ГРИБОМ *RHIZOPOGON* *RUBESCENS*

Реферат

Цель. Целью данной работы было изучение действия источника азота на связывание меди, биоаккумулированной грибами и эктомикоризой, которые были выращены в присутствии фосфата меди. **Материалы и методы.** Эктомикоризный гриб *Rhizopogon rubescens* и его симбиотичес-



кая эктомикоризная ассоциация с сосной обыкновенной выращивались в присутствии фосфата меди на различных источниках азота: аммонии или нитрате. Координация меди, освобождённой из фосфата меди и биоаккумуляированной грибом и эктомикоризой, определялась с использованием синхротронной рентгено-абсорбционной спектроскопии (XAS). **Результаты.** Данные XAS показали, что перемена источника азота с аммония на нитрат изменяла координацию меди смешанными кислородосодержащими лигандами на оксалатную. **Вывод.** Установлено, что условия роста гриба и микоризы могут изменять природу лигандов, доступных для связывания токсичных металлов. Координация меди в эктомикоризном грибе *R. rubescens* и его симбиотической ассоциации с корнями сосны зависела от источника азота.

Ключевые слова: грибы, эктомикориза, фосфат меди, азот, химическое связывание металла, биоаккумуляция, рентгено-абсорбционная спектроскопия.

REFERENCES

1. *Burford E.P., Fomina M., Gadd G.M.* Fungal involvement in bio-weathering and biotransformation of rocks and minerals // *Mineral. Mag.* — 2003. — 67. — P. 1127–1155.
2. *Fomina M.A., Alexander I.J., Hillier S., Gadd G.M.* Zinc phosphate and pyromorphite solubilization by soil plant-symbiotic fungi // *Geomicrobiol. J.* - 2004. — 21. — P. 351–366.
3. *Fomina M., Burford E.P., Gadd G.M.* Toxic metals and fungal communities // *The Fungal Community. Its Organization and Role in the Ecosystem* / Dighton J., White J.F., Oudemans, P. (eds). — Boca Raton: CRC Taylor & Francis, 2005. — P. 733–758.
4. *Fomina M.A., Alexander I.J., Colpaert J.V., Gadd, G.M.* Solubilization of toxic metal minerals and metal tolerance of mycorrhizal fungi // *Soil. Biol. Biochem.* — 2005. — 37. — P. 851–866.
5. *Fomina M., Hillier S., Charnock J.M., Melville K., Alexander I.J., Gadd G.M.* Role of oxalic acid over-excretion in toxic metal mineral transformations by *Beauveria caledonica* // *Appl. Environ. Microbiol.* — 2005. — 71. — P. 371–381.
6. *Fourest E., Serre A., Roux J.C.* Contribution of carboxylic groups to heavy metal binding sites in fungal cell walls // *Toxicol. Environ. Chem.* — 1996. — 54. — P. 1–10.
7. *Gadd, G.M., Fomina, M., and Burford, E.P.* Fungal roles and functions in rock, mineral and soil transformations // *Micro-organisms and Earth Systems — Advances in Geomicrobiology* / Gadd G.M., Semple K.T., Lappin-Scott H.M. (eds.). - Cambridge: Cambridge University Press, 2005. — P. 201–232.
8. *Greenwood N.N., Earnshaw A.* Chemistry of the Elements. — Oxford: Pergamon Press, 1984. — 1087 p.



9. *Kemner K.M., O'Loughlin E.J., Kelly S.D., Boyanov M.I.* Synchrotron X-ray investigations of mineral-microbe-metal interactions // *Elements*. – 2005. – 1. – P. 217–221.

10. *Lapeyrie F., Ranger J., Vairelles D.* Phosphate-solubilizing activity of ectomycorrhizal fungi *in vitro* // *Can. J. Bot.* – 1991. – 69. – P. 342–346.

11. *Meharg A.A., Cairney J.W.G.* Co-evolution of mycorrhizal symbionts and their hosts to metal-contaminated environments // *Adv. Ecol. Res.* – 2000. – 30. – P. 69–112.

12. *Sarret G., Schroeder W.H., Marcus M.A., Geoffroy N., Manceau A.* Localization and speciation of Zn in mycorrhized roots by μ SXRF and μ EXAFS // *J. Phys. IV France*. – 2003. – 107. – P. 1193–1196.

13. *Smith S.E., Read D.J.* Mycorrhizal Symbiosis. - London: Academic Press, 1997. – 605 p.

14. *Whitelaw M.A., Harden T.J., Helyar K.R.* Phosphate solubilization in solution culture by the soil fungus *Penicillium radicum* // *Soil Biol. Biochem.* – 1999/ – 31. – P. 655–665.

Стаття надійшла до редакції 20.05.2013 р.

