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STSM TITLE: Determination of siderophores and organic acids production by selected fungal isolates

COST ACTION: FA1103 Endophytes in Biotechnology and Agriculture

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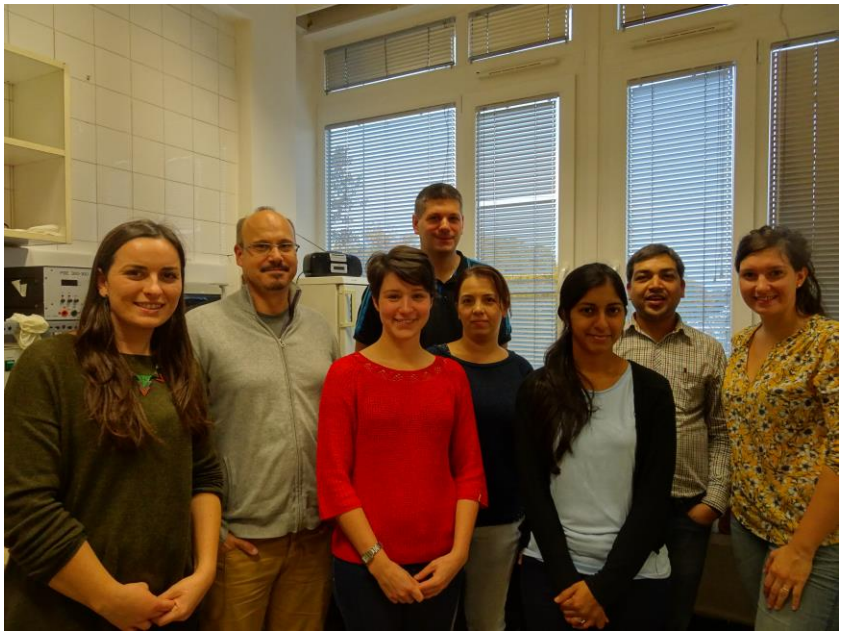
PERIOD AND PLACE: from 05/10/2015 to 30/10/2015 in Szeged (HU)



SCIENTIFIC BACKGROUND OF THE STSM:

The uptake and accumulation of heavy metals and nanoparticles (NPs) by fungal biomass is receiving increasing attention in biotechnological context since microbe based technologies may provide an alternative techniques of metal or NPs removal from polluted soils and waste waters or/and in production of different materials. Although copper (Cu) is an essential element for all living beings, in higher concentrations it can be one of the most toxic metals. The mechanism of Cu ion toxicity include oxidation of functional groups that blocks or inactivates enzymes, competition with other ions for binding sites and adverse effects on the functionality of the cell membranes. Moreover, redox cycling between Cu^{2+} and Cu^{+} can catalyse the production of highly toxic hydroxyl radicals, with subsequent damage to DNA, lipids, proteins and other biomolecules [1]. However, some microorganisms synthesize enzymes or produce biogenic oxidants/reductants which result in altered local redox environment and consequently detoxification of toxic compounds [2, 3]. One type of compounds with these capabilities are siderophores, which can affect the mobility of metals such as iron, copper, zinc, cadmium and lead [4]. Moreover, siderophores can also affect the speciation, bioavailability and long-term fate of metals in the environment. However, one of fungal tolerance mechanisms to copper is synthesis of organic acids, which can further form complexes with toxic metals (e.g. oxalic acid -> Cu-oxalate crystals) [5].

PURPOSE OF THE STSM:

In our study two selected fungal strains (copper tolerant *Botrytis cinerea* and copper sensitive *Alternaria alternata*), were exposed to CuO microparticles (MPs), CuO NPs, Cu NPs and Cu-salt, in liquid media as well as on solid agar. The experiments resulted in *B. cinerea* strain producing higher biomass, forming new blue-coloured compound, having lower level of lipid peroxidation in mycelia (determined by TBA spectrophotometric method). It was able to tolerate the toxic mechanisms of Cu nanoparticles as well as CuO nanoparticles. It actually degraded them and form unknown new blue copper compound. Moreover, lipid peroxidation, which indicates the oxidative stress in organisms, was lower in *B. cinerea* mycelia in comparison to *A. alternata*. We would like

to reveal the detoxification mechanisms of *B. cinerea* and our next aim in this study is the analyses of siderophores and organic acids production.

WORK CARRIED OUT DURING STSM:

Siderophore production

The siderophores were detected by Chrome azurol sulphonate (CAS) method. First approach was solid CAS agar with aim to reveal the type of siderophore produced. Different protocols for CAS agar preparation were tried: original of Schwyn and Neilands (1987) [6], modified of Hu et al. (2001) [7] and modified of Cordero et al. (2001) [8]. Moreover, also overlay-CAS method by Perez-Miranda et al. (2007) [9] was tried. An agar plug of 7 mm diameter of 1-week-old mycelia was placed in the center of each plate containing CAS agar. The plates were kept in the dark at room temperature, and photographed daily. The change in colour of the blue dye to orange or purple indicated the presence of siderophores. The colouration zones (mm^2) around the mycelia were estimated using ImageJ software (http://www.macbiophotonics.ca/imagej/). Each of the siderophore tests was carried out as 4 replicates per fungal isolate, with fungus-free plate serving as controls.

Second approach was siderophores detection and quantification in liquid growing media. One tested unit represented 50 ml of MNM media without addition of agar and supplemented with 80 mg of Cu in different forms (CuO MPs, CuO NPs, Cu NPs) due to maintaining the same Cu concentration on media volume. Cu salt was added in 1 and 10% of Cu amount, in comparison to other treatments. Every flask was inoculated with one agar plug (6 mm in diameter) obtained from edge of 1-week old colony. Fungi were grown on orbital shaker on 24 °C, shaking on 175 rpm. At day 4, 7, 10 and 14, media sample was taken from each flask and centrifuged on 13000×g for 10 minutes.

To 100 µl of cell free culture supernatant, 100 µl of CAS reagent was added and absorbance was measured at 630 nm [10]. Uninoculated media with addition of CAS reagent served as reference. Siderophores content was expressed as percentage siderophore units according to Kumar et al. (2012) [11]:

$$\text{Siderophore units } (\%) = \left(\frac{\text{Ar}-\text{As}}{\text{Ar}}\right) \times 100$$

where Ar=absorbance of reference at 630 nm and As=absorbance of sample at 630 nm.

Organic acid analyses

The analyses were performed using high performance liquid chromatography (HPLC). Growing media from liquid experiment, supplemented with different copper forms were filtrated through 0.22 µm filter (Millipore, GTBP). During method development we tried 2 types of detectors (RID; UV-VIS), 5 concentrations of mobile phase (1.7; 2; 2.5; 5; 8 mM) and 5 different temperatures of column oven (55; 60; 65; 70; 75 ºC). However, our final measurements were performed by injection of 10 µl of filtrates to HPLC equipped with a UV-VIS detector (SPD-10Avp, Shimadzu). The organic acid separation was carried out on Agilent Hi-Plex Ligand Exchange column (Agilent Technologies) with 2 mM H_2SO_4 as mobile phase and use of column oven (CTO-10ASvp, Shimadzu) to heat the column on 70 °C. Retention time of each signal was recorded at wavelength of 210 nm and 240 nm. Chromatograms were compared to the 11 organic acid references of acetic acid, ascorbic acid, citric acid, fumaric acid, itaconic acid, levulinic acid, maleic acid, malic acid, oxalic acid, succinic acid and gluconic acid.

MAIN RESULTS:

Siderophore production

On solid CAS agar, both fungi showed capability to produce siderophores since the colour change of otherwise blue media. Seven days after inoculation *A. alternata* formed yellow halos surrounding the mycelia with an average area 224 ±13 cm^2 (7 days after inoculation), while *B. cinerea* mycelia were surrounded with purple halos with an average area of 1353 ±45 cm^2 (7 days after inoculation) (Fig. 1). The size of *B. cinerea* halos zone was 6-times bigger than halos of *A. alternata*, which means higher production and concentration of secreted siderophores by *B. cinerea*. The colour difference pointed out the production of two different types of siderophores, where yellow stands for hydroxymate type of siderophores and purple for catechol type [9].

The detection and quantification of siderophores in liquid growing media did not work for our experiment, since one of the compounds in CAS reagent interacted with Cu in our treatments, resulting in very strong light blue colour (Fig. 2a). Due to that, all Cu supplemented media were not valid for quantification of siderophores production. We quantified siderophores only in control media without Cu and the siderophores detection

was positive only in *B. cinerea* media, with 60% siderophore units. In control liquid media of *A. alternata* there was no siderophore production (Fig. 2b)

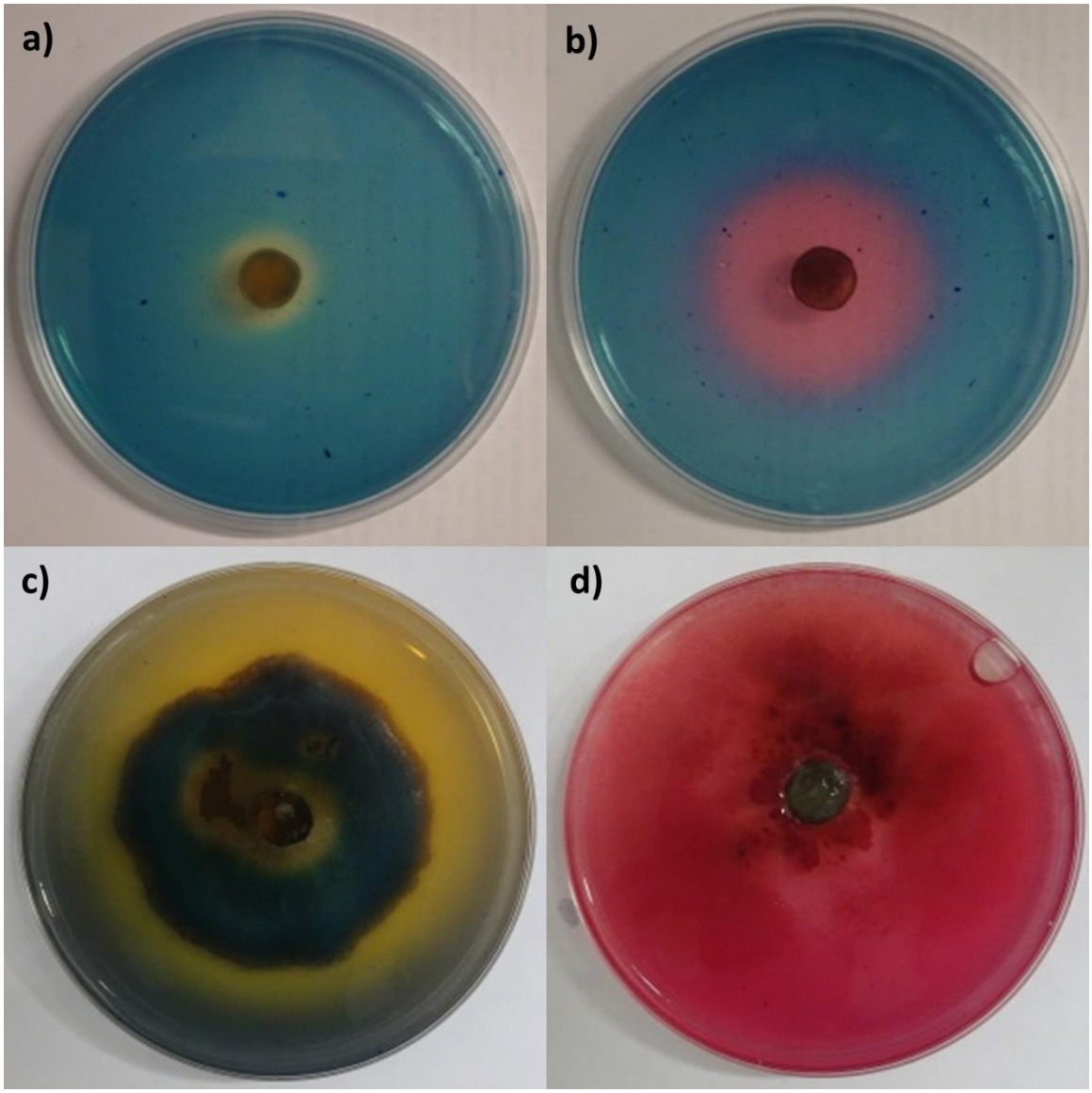


Fig. 1. Siderophores detection by CAS agar plates a) *A. alternata* mycelia with yellow halos, and b) *B. cinerea* mycelia with purple halos; and by overlay-CAS method c) *A. alternata* overlay coloured yellow, and d) *B. cinerea* overlay coloured purple.

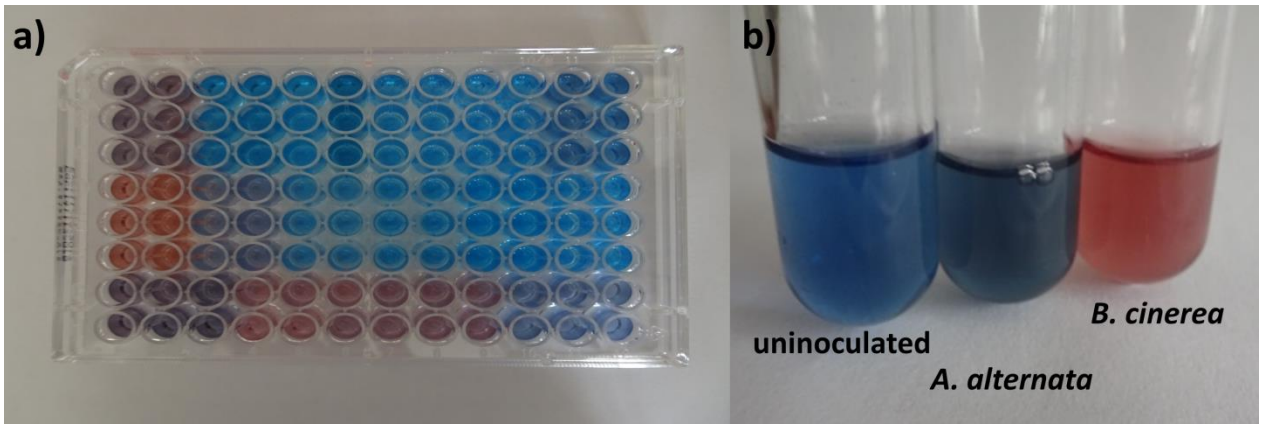


Fig. 2. Detection of siderophores in liquid media. a) Microtiter plate with growing media, supplemented with different copper forms and addition of CAS reagent. b) Colouration of growing media after addition of CAS reagent (uninoculated media, control media of *A. alternata* and control media of *B. cinerea*).

Organic acid analyses

References (oxalic, maleic, citric, gluconic, malic, ascorbic, succinic, itaconic, fumaric, acetic and levulinic acid) were mixed in MNM media, same as media used for experiment with fungi (Fig. 3).

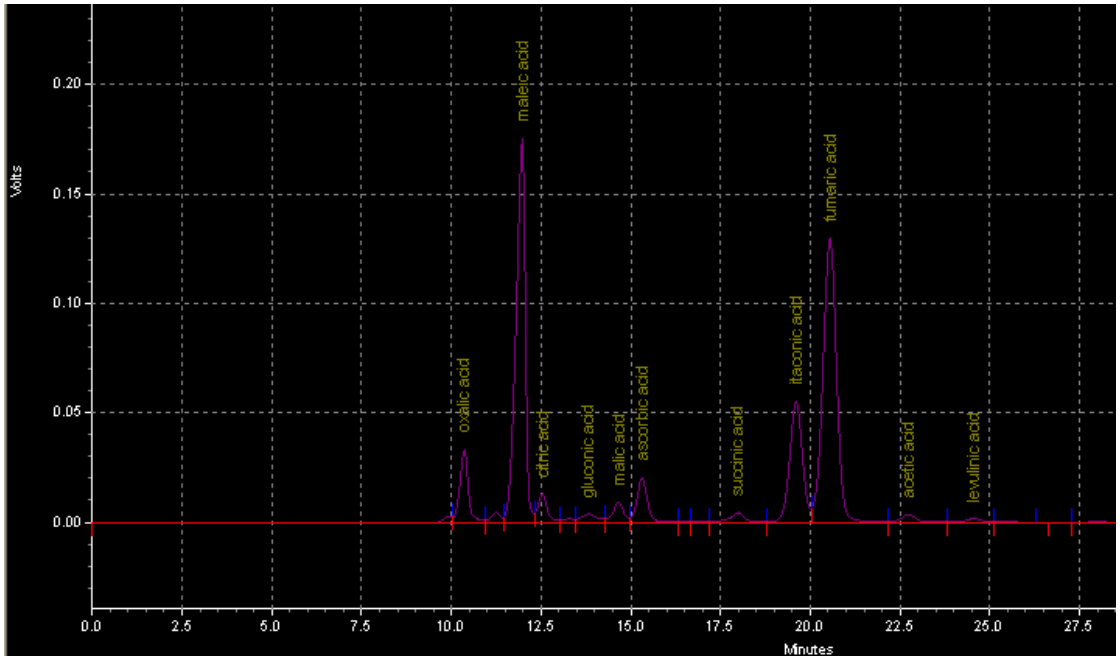


Fig. 3. Chromatogram of references (oxalic, maleic, citric, gluconic, malic, ascorbic, succinic, itaconic, fumaric, acetic and levulinic acid).

Chromatograms of each media with 5 biological repetitions were recorded and compared (Fig. 4).

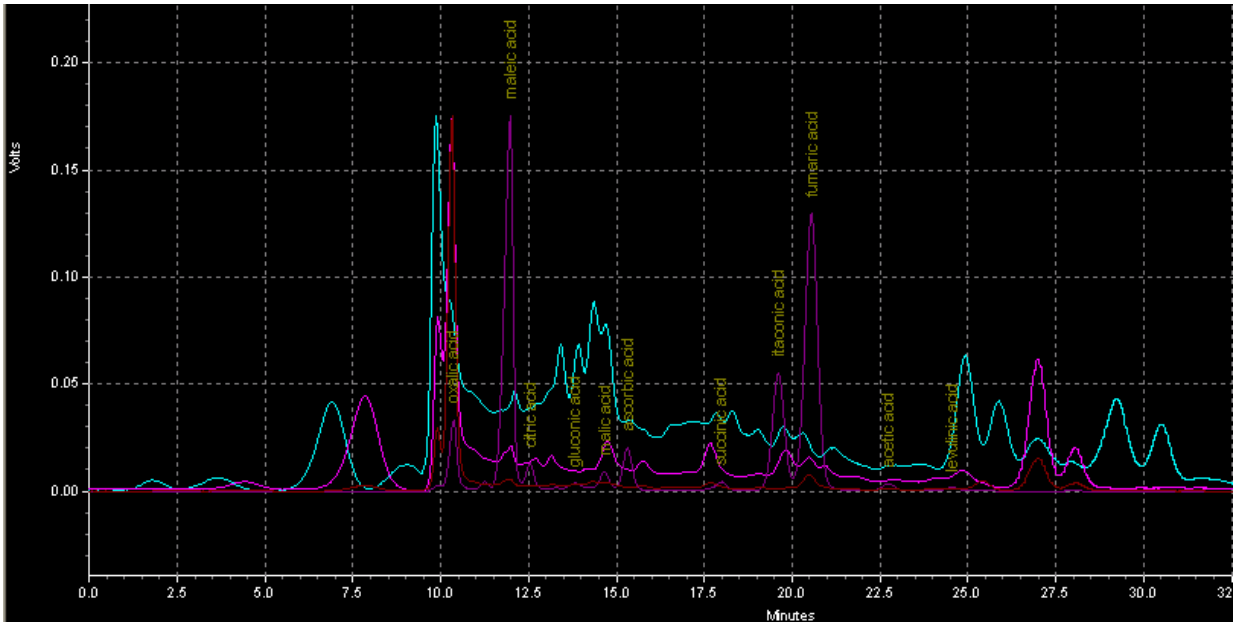


Fig. 4. Comparison of chromatograms of organic acids references (dark red), *A. alternata* control (blue) and *B. cinerea* control (pink).

Table 1. Concentrations of oxalic, gluconic, fumaric and acetic acids in media.

Organic acid		**Oxalic acid**	**Gluconic acid**	**Fumaric acid**	**Acetic acid**
Detection limit (µg/ml)		5.68	28.4	1.42	14.2
Fungi	**Treatment**				
A. alternata	control		37.34±13.91	0.27±0.53	
A. alternata	MPs CuO	2.53±4.4			
A. alternata	NPs CuO	6.9±3.89	9.76±1.79	0.9±0.13	
A. alternata	NPs Cu	3.69±3.61	1.3±2.31	0.8±0.16	
A. alternata	salt (1 %)		6.27±11.92		51.25±58.3
A. alternata	salt (10 %)		12.72±14.98		
B. cinerea	control	86.89±21.54	27.78±8.23	0.96±0.36	2.87±6.59
B. cinerea	MPs CuO	66.49±21.42		1.75±2.13	41.18±51.58
B. cinerea	NPs CuO	59.92±23.84			8.51±8.34
B. cinerea	NPs Cu	38.92±34.47		0.41±.29	
B. cinerea	salt (1 %)	145.66±8.23		1.65±1.62	15.22±15.39
B. cinerea	salt (10 %)	35.66±17.26	1.83±3.17	0.31±0.4	

Data are means±SE (n=5).

Citric, itaconic and levulinic acids were not present in detectable concentrations in any media analyzed. Our detection limits for those acids were 14.2, 1.42 and 28.4 µg/ml, respectively. Maleic acid was present in all samples, except *A. alternata* control and

MPs CuO, but under quantification concentrations, which was 0.71 µg/ml. Malic acid was present only in control media of *A. alternata*, but under quantification concentrations, which was 28.4 µg/ml. Ascorbic and succinic acid were present only in *A. alternata* control media and media supplemented with 1% Cu salt, but under quantification limits, which was 227.2 µg/ml for both. Values measured for oxalic, gluconic, fumaric and acetic acids are presented in Table 1. Oxalic acid was produced in much higher concentrations by *B. cinerea* with concentrations of 36, 39, 60, 66, 87 and 146 µg/ml, in increasing order in media Cu salt (10%), NPs Cu, NPs CuO, MPs CuO, control and Cu salt (1%), respectively. In media of *A. alternata* oxalic acid was present only in media supplemented with MPs, CuO, NPs CuO and NPs Cu in very low concentrations (Table 1). Gluconic acid was present in almost all *A. alternaria* media and in *B. cinerea* media in control and treatment Cu salt (10%), but only in control media values of both fungi were close to quantification limit. Fumaric acid was detectable in both fungi in several treatments, but below quantification limit. Acetic acid was detected in *B. cinerea* control, NPs CuO and Cu salt (1%) media of *B. cinerea* and quantified only in *B. cinerea* MPs CuO media and *A. alternata* Cu salt (1%) media.

FUTURE COLLABORATION AND FORESEEN PUBLICATIONS RESULTING FROM THE STSM:

STSM helped us to complement the study of *B. cinerea* detoxification mechanisms. Interaction with the host institution will continue with preparation of manuscript, including the data obtained during STSM. We expect to publish one paper with the collaboration of the host institute.

CONFIRMATION BY THE HOST INSTITUTION OF THE SUCCESSFUL EXECUTION OF THE STSM:

Attached on end of this document.

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October 30, 2015

CERTIFICATE

I, Tamás Papp, herewith confirm that **Ms. Eva Kovačec** (University of Ljubljana, Biotechnical Faculty, Department of Biology, Ljubljana, Slovenia) carried out her research activity "**Determination of siderophores and organic acids production by selected fungal isolates**" at the Department of Microbiology, University of Szeged (Szeged, Hungary) between 05.10.2015 and 30.10.2015.

During this period, she fully completed her short term research planned in the frame of the COST ACTION: FA1103 Endophytes in Biotechnology and Agriculture.

Yours sincerely,

SZEGEDI TUDOMÁNYEGYETEM Természettudományi és Informatikai Kar Mikrobiológiai Tanszék SZEGED

Dr. Tamás Papp
Associate Professor