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Study of *Artemisia annua* Proteome for Presence of Metal-Dependent Proteins in Intrusion Zone to Agrobiocenosis

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***Abstract* — The article presents the results of study metal-dependent proteins comprised in *Artemisia annua* proteome in the intrusion territories adjacent to the artificial agrobiocenosis in Volgograd region. Using mass spectrometry we detected more than 2200 proteins, including 134 metal-dependent ones. One hundred twenty one of them had a dependence on zinc, 9 proteins depended on copper, and 4 proteins were manganese-dependent. We found no any nickel- or cobalt-dependent proteins. Zinc-dependent proteins mainly worked in gene expression and energy metabolism regulation. Copper-dependent proteins involved in enzymatic reactions. Manganese-dependent proteins perform functions of DNA repair and enzymatic hydrolysis. According to these results, zinc-dependent proteins are the most prevalent from metal-dependent proteins of *Artemisia annua* in the intrusion territories adjacent to the artificial agrobiocenosis of Volgograd region. Further study can stimulate the development of point control methods to reduce *Artemisia* population and its effect from the technogenic intrusion zone to adjacent agrobiocenosis.**

Keywords: Artemisia annua, proteom, metal-binding proteins, manganese, copper, zinc

I. Introduction

Currently, ecological system approach to agrocenoses management is the most relevant in the development of the agro-industrial complex [1]. The system approach can improve new promising biotechnological solutions. In particular, integrated management by changing the relationship between biotic factors such as fungi and bacteria, mobile soil metals such as copper, zinc, manganese, nickel or cobalt, and other possible physical and chemical changes can significantly change the structure and properties of plant communities [2 – 8]. Interactions with adjacent natural and anthropogenic systems, which affect artificial biocenoses and become technogenic intrusions, take a special place in the dynamics of agrobiocenoses. These plant communities have a reduced species content, which usually presented with one-two explicit dominants [5, 9]. Genus *Artemisia* is a typical representative of dominants for an arid zone. Point management is optimal for sites of technogenic intrusions into agrobiocenoses, to minimize negative changes in agrocenosis [2, 10].

Agrobiocenoses of this area are characterized by a complex of stress factors with the serious effect on metabolism of microorganisms and plant communities on these areas [11, 12]. This fact confirms the necessity to study molecular effects of the soil metals on plant metabolism in agrobiocenosis.

The study of proteins depended on copper, zinc, manganese, nickel, and cobalt in the proteome of the dominant genus *Artemisia*, growing in the area of technogenic intrusion, will reveal the proteins involved in plant metabolism regulation. The obtained data can be used to control the growth of plants represented by the genus *Artemisia* by metabolism regulation [13].

The aim of the work is to find proteins depended on copper, zinc, manganese, nickel and cobalt in *Artemisia* proteome from technogenic intrusion to arid agrobiocenosis, and compare these findings to current collection of *Artemisia* proteins in UniProt database.

II. Material and Methods

A. Material

Plant specimens of the genus *Artemisia* were selected from the arid territory of the Volgograd region to study of the metal-dependent proteins comprised in the proteome.

B. Protein isolation

To prepare the protein-containing samples for mass spectrometry we sought the of protein isolation by centrifugation at 10,000 rpm for 20 minutes (Eppendorf 5417-C, Germany) and collected supernatant into new tubes. The adding acetone (PanReac AppliChem, Germany, -20 °C) in a 4:1 ratio (1.2 ml of acetone and 0.3 ml of sample) provided the protein precipitation. After mixing the solution on the vortex for 30 seconds, we incubated the samples for one hour at -20°C. Further, the samples were centrifuged at 10,000 rpm for 30 minutes, and the supernatant was collected in Eppendorfs. Following adding 50 µl of acetone resulted in protein precipitation. The solution was resuspended and repeatedly centrifuged at 10,000 rpm for 10 minutes. For acetone removing one dissolved the

precipitate in 9M urea and incubated at 60 °C for 20 hours. Solution of 50 mM ammonium bicarbonate (pH = 7.8) was added to the samples. The final urea concentration was 4 M.

B-mercaptoethanol in final concentration 20 mM was added to the samples to form disulfide bonds with the following incubation for one hour. The addition of iodacetamide solution (15 mM) provided the alkylation of cysteine residues. The reaction was carried out in a dark place for one hour. Three proteases: trypsin (1), proteinase K (2), and endoproteinase Glu-C (3) in the protein:enzyme ratio 30:1 used to perform the independent hydrolysis, the procedure took 20 hours at 37°C.

Samples of hydrolysates were purified from urea and other agents on ZipTip© type micro-columns with a C18 carrier before mass spectrometry. A sample contained 50 µg proteins was applied to the micro-column. Then we washed the column with a solution of 4% acetonitrile and 0.1% trifluoroacetic acid in deionized water. After purification from salts and denaturants, peptides were washed off the column with a solution of 80% acetonitrile and 0.1% trifluoroacetic acid, and then dried on a vacuum concentrator.

C. Proteome analysis

To get proteome of *Artemisia* we underwent protein hydrolysates by mass chromatography using nanoflow chromatograph Easy-nLC 1000 (Thermo Scientific, USA) with high-field mass spectrometer Orbitrap Elite ETD (Thermo Scientific, Germany) as a detector. Protein separation was carried out on a capillary column 150 mm long, 75 µm in diameter, filled with Aeris 1.7 µm PEPTIDE XB-C18 phase (Phenomenex, USA). The column was prepared for analysis in the laboratory.

The hydrolysate of the analyzed sample was applied to a column with reversed phase. Changing the concentration of two buffer systems ‘A’ and ‘B’ to separate peptides in the acetonitrile gradient. Buffer "A" contained a solution of 0.05% formylic acid, 0.05% trifluoroacetic acid, and 3% acetonitrile (Merck, Germany) in water for MS analysis (Merck, Germany). Buffer " B " contained a solution of 90% acetonitrile (Merck, Germany), 0.05% formylic acid and 0.05% trifluoroacetic acid (PanReac AppliChem, Germany) in water for MS analysis. The work regimen included separation in 95% buffer ‘A’ and 5% buffer ‘B’, the uniform increase of buffer ‘B’ concentration to 30% from 5 to 180 min, and the increase of buffer ‘B’ concentration to 90% for next 20 minutes. Peptides eluted from the column fell into the ionization chamber. Then ions were analyzed by tandem mass spectrometry.

D. Biochemical analysis

The computer software PeaksStudio 7.5. (Bionformatics Solutions Inc., Canada) used to obtain mass spectrometric data including masses of peptide and ion fragments. All peptides were identified using two databases. The first one contained all protein sequences of the organism. The second database contained the amino acid sequences of proteins.

We considered the protein determination as reliable if the value of identification authenticity degree (- 10l gP) was greater than or equal to 20.

E. Bioinformatics analysis

Identified proteins from *Artemisia annua* growing on the technogenic intrusion, we compared with the UniProt protein database (www.uniprot. org). There were five analysed metals: manganese, nickel, cobalt, copper, and zinc. We executed virtual screening according to the following criteria: the presence of the required metals in the protein (1) and the occurrence of interaction with the metal (2). To capture necessary information we used the synonymous constructs containing metal and species name, for example ‘[zinc AND organism: “*Artemisia* (Sweet wormwood) OR 35608]’. The comparison involved separate analysis of metals as cofactors i metalloprotein composition, and as atoms in metal-binding sites. Consequently, the screening of all metal-dependent proteins and the degree of protein annotation was carried out. The annotated protein was considered to have 3 or more points.

III. Results and Discussion

A. Results of mass spectrometry

As a result of obtaining proteome from *Artemisia annua* we selected 2224 proteins. They included 461 proteins, which could not be identified, so they named as ‘Uncharacterized proteins’. One hundred and thirty two identified proteins were metal-depended.

Table 1 presents the results of mass spectrometry in part of zinc-dependent proteins, separated from *Artemisia annua* species, growing in technogenic intrusion to arid agrobiocenosis. Among the metal-dependent proteins involved in metabolism, Acyl-CoA N-acyltransferase and E3 ubiquitin-protein ligase were the most significant, they were annotated. DNA repair protein Rad50, Histone deacetylase HDT1, RNA-directed DNA polymerase, eukaryota, Reverse transcriptase zinc-binding domain protein and others can be distinguished as metal-dependent proteins involved in the regulation of gene expression. Also, there were found proteins, which are structural elements known as ‘Zinc finger’.

Table 2 contains information about nine copper-dependent proteins are predominantly involved in metabolism. As enzymes as Laccase (Benzenediol: oxygen oxidoreductase, EC 1.10.3.2, or Diphenol oxidase, or Urishiol oxidase) and Superoxide dismutase [Cu-Zn] (EC 1.15.1.1) predominated among the identified copper-dependent proteins. Chloroplast-targeted copper chaperone protein was connected to energy metabolism.

We found four manganese-dependent proteins: double-strand break repair protein, S-locus glycoprotein domain-containing protein, metallopeptidase M24 family protein and protein phosphatase. Double-strand break repair protein, S-locus glycoprotein domain-containing protein, metallopeptidase M24 family protein, and protein phosphatase are involved in DNA repair and protein hydrolysis.

We didn’t identify any cobalt- and nickel-dependent proteins. The absence of these proteins may be related to the lack or absence of these metal ions in sufficient quantities in the soil at the area of technogenic intrusion.

Ministry of Education and Science of the Russian Federation (2017-2019)

TABLE I. *ARTEMISIA ANNUA* ZINC-DEPENDENT PROTEINS DETECTED BY MASS SPECTROMETRY ANALYSIS.

Function	***UniProt ID***
Metabolism	A0A2U1KA36, A0A2U1KA46, A0A2U1KA58, A0A2U1KAF8, A0A2U1KBY1 A0A2U1KG96 A0A2U1KIQ6 A0A2U1KK48 A0A2U1KLG6 A0A2U1KT89, A0A2U1KTL4 A0A2U1L8B6 A0A2U1L9B4, A0A2U1LB26, A0A2U1LB92, A0A2U1LBW8, A0A2U1LCI6, A0A2U1LGG2, A0A2U1LGJ4, A0A2U1LHJ4, A0A2U1LHK8, A0A2U1LII0, A0A2U1LIK0, A0A2U1LKP6, A0A2U1LKS6, A0A2U1LPN6, A0A2U1LQT2, A0A2U1LQY6, A0A2U1LT65, A0A2U1LTL2, A0A2U1LTL3, A0A2U1MMP5, A0A2U1MPL7, A0A2U1MQK1, A0A2U1MRN0, A0A2U1MVR9, A0A2U1MY11, A0A2U1MYY8, A0A2U1N212, A0A2U1QM71
Regulation of gene expression	A0A2U1KAL8, A0A2U1KAN0, A0A2U1KBM8 A0A2U1KI41, A0A2U1KPE6, A0A2U1KQ55, A0A2U1KS31, A0A2U1KXN3, A0A2U1L1L7, A0A2U1KV89, A0A2U1L3W5, A0A2U1L6A4, A0A2U1L6S6, A0A2U1L8R0, A0A2U1LIU2, A0A2U1LK64, A0A2U1M5E4, A0A2U1M5U9, A0A2U1M6Y2, A0A2U1M904, A0A2U1MBH7, A0A2U1MK39, A0A2U1MK94, A0A2U1ML46 A0A2U1MNK0, A0A2U1MRQ8, A0A2U1MYS9, A0A2U1N249, A0A2U1N667
Structural element	A0A2U1KFA2 A0A2U1KFZ6 A0A2U1KI52 A0A2U1KLI2, A0A2U1KMY6, A0A2U1KP12, A0A2U1KZB5, A0A2U1L056, A0A2U1L089 A0A2U1L7M3, A0A2U1M7F8, A0A2U1M8V1, A0A2U1N6N9, A0A2U1N801, A0A2U1N8N1, A0A2U1N9V7, A0A2U1NDW0, A0A2U1NDW5, A0A2U1NEF5, A0A2U1NEN8, A0A2U1NF23, A0A2U1NGB3, A0A2U1NI99, A0A2U1NIS5, A0A2U1NPZ6, A0A2U1NR80, A0A2U1NSN9, A0A2U1NXF3, A0A2U1NYN5, A0A2U1P3K4, A0A2U1P5T9, A0A2U1PAT2, A0A2U1PB29, A0A2U1PCF9, A0A2U1PNU9, A0A2U1PPH4, A0A2U1PQ69, A0A2U1PWC9, A0A2U1PWR6, A0A2U1Q2I4, A0A2U1Q2W3, A0A2U1QLW3 A0A2U1QAD2, A0A2U1QAM7, A0A2U1QBW7, A0A2U1QCS9, A0A2U1QFF4, A0A2U1QG30, A0A2U1QIR7, A0A2U1QK07, A0A2U1QK22

B. Virtual screening of the UniProt database for metal-dependent proteins in the genus Artemisia

Database UniProt analysis regarding *Artemisia annua* revealed more than 5000 metal-dependent proteins, containing one of the 5 studied metals. It showed about 98% of these proteins to have no annotations (Table 3).

TABLE II. COPPER-DEPENDENT PROTEINS OF *ARTEMISIA ANNUA* IDENTIFIED BY MASS SPECTROMETRY

Function	***UniProt ID***
Metabolism	A0A2U1PHG2, A0A2U1KHZ4, A0A2U1NLG8, A0A2U1NZP9, A0A2U1LF58, A0A2U1Q0M8, A0A2U1PPH4,A0A2U1PFS0, A0A2U1M6V1

TABLE III. *ARTEMISIA* PROTEOME METALL-DEPENDENT PROTEINS IN DATEBASE UNIPROT

Metal	**Number of proteins**		
	Reviewed	***Unreviewed***	***Total***
Zn	28	5805	5833
Mn	14	221	235
Cu	9	208	217
Co	3	22	25
Ni	1	16	17

Comparative analysis of the results of mass spectrometry of proteins and the database showed that only a small part of the metal-dependent proteins in *Artemisia annua* was identified. We found 1% of zinc-dependent proteins, 1.5% of manganese-dependent proteins, and 4% of copper-dependent ones from possible. We also assume that some of these metal-dependent proteins could not be identified, being in the group 'Uncharacterized protein'. Some of them could not express because *Artemisia annua* grew in the territory of technogenic intrusion, where deficiency or excess of soil metals is commonplace.

C. Discussion

Starting the discussion, we emphasize that we have identified the dominant plant of intrusive phytocenoses in the arid zone as *Artemisia annua*. The fact that we found only a small part of the possible metal-dependent proteins indicates significant changes in metal-dependent metabolism in the territory of technogenic intrusions. It also clearly demonstrates to us that a detailed study of the protein composition of *Artemisia annua* has not been carried out to date. As a result, we do not know the full range of functions that metal-dependent proteins in this plant can perform.

We found 4 proteins which depended on manganese. This dependence is fuzzy because it could not be decisive in the ability of a plant to grow in the natural environment of its habitat [6, 12]. Manganese and zinc are essential metals, but the peculiarities of their metabolism in different plants lead to differences in their needs and to changes in their concentration in the soil in different reactions [14]. According to the studies, a lot of metal-dependent proteins, especially containing manganese, involved in plants photosynthesis and energy exchange. Zinc-dependent proteins are involved in specific functions such as lignification, plant aromatics synthesis, and salt stability

maintenance, regulation of fatty-acid metabolism and gene expression regulation. Other important functions provided by the presence of metal-dependent proteins are metabolism and transport. Only evolutionary processes can explain all generic and specific mechanisms of adaptation to environmental conditions, based on the processes of manganese and zinc biochemical transformation. The main mechanism of this process is a regulated flow of microelements from the soil (technogenic intrusion), which is largely determined by the composition and activity of the soil microbiota [4]. Copper-dependent proteins are involved in the photosynthesis, metabolism of proteins and lipids. In our study, we found a small amount of copper-dependent proteins, perhaps this is related to the fact that the area of technogenic intrusion contains a small amount of copper, what directly affects the activity of proteins synthesis [15].

The revealed dependences can form the basis of such biotechnological strategies that would be able to limit the consort influence of a number of "undesirable" plant dominants on agricultural plant communities [1, 5]. We believe it is possible to develop on this basis, stimulating the number and activity of subdominants, which, according to our previous studies [9, 16], was accompanied by increased intraspecific competition within the phytocenosis and weakening its impact on the contact of plant communities.

IV. Conclusion

A. *Results of mass spectrometry analysis of metal-dependent proteins in the Artemisia annua proteome.*

Mass spectrometric analysis of *Artemisia annua* proteins identified 2,224 proteins. Of these proteins, 121 were zinc-dependent, 9 proteins were copper-dependent, and 4 ones were manganese-dependent. Proteins dependent on cobalt and Nickel were not detected. 21% of the proteins could not be identified. Zinc-dependent proteins mainly regulate gene expression and participate in energy metabolism. Proteins containing copper are involved in enzymatic reactions. Manganese-dependent proteins participate in DNA repair and enzymatic hydrolysis.

B. *Analysis Uni Prot database.*

An analysis of the UniProt database for the presence of metal-dependent proteins included in the *Artemisia annua* proteome revealed 6,327 proteins, metal-dependent proteins including copper, zinc, manganese, cobalt, and Nickel made up 2% of all annotated proteins. The comparative analysis showed a slight coincidence of isolated and Uniprot proteins, which amounted to 1% for zinc-dependent proteins, 1.5% for manganese-dependent proteins, and 4% for copper-dependent ones.

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