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Biochemical characterization of neurolectins, neuron, glial cells, synaptosomes and cell subfractions of the brain

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A B S T R A C T

In the present work the results of experimental research in the field of quantitative and qualitative distribution of neurolectins in the neurons, glial cells, synaptosomes and subfraction of the rats, chickens and bovine brain are presented. Neurolectins were isolated from the enriched fraction of neural and glial cells The specific activity of neurolectins was 780 (neuron) and 713 (glia) units and showed diverse affinity for carbohydrates: neurolectin isolated from enriched fraction of neurons showed specific sensitivity to D-galactose (NL-Gal, 16.2 mM), neurolectin isolated from the enriched fractions of glial cells showed specific sensitivity to inositol (GL-I, 4.6 mm), and other carbohydrate haptens depending on the concentration. Two neurolectins were isolated from synaptic vesicles of chicken and bovine brain and were identified as specific to inositol (SVL-I) and N-acetyl-D-galactosamine (SVL-NAGA), which, according to our hypothesis, were actively involved in the capture and secretion of neurotransmitters and biological active substances. Since the quantitative distribution of neurolectins in the animal brain was established, it was of great interest to study the distribution of neurolectins at the level of subcellular fractions of nerve cells. It has been found that agglutination with mitochondrial neurolectin is specifically inhibited by glucose, galactose, and N-acetylglucosamine, N-acetyl-galactosamine and is called BML-GluG. Neurolectins of the nucleus nerve cells were separated on a Protein PAK-300-SW column. Nuclear neurolectins are divided into two protein fractions. Using the hapten-inhibitory method, it was shown that both neurolectins of the nucleus of the rat haptens of the brain are N-acetyl-D-glucosamine, D-galactose and L-fructose (NucL-G).

Keywords: Neurolectins, Brain, Neurons, Glial cells, Synaptic vesicles, Mitochondria.

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Introduction

Lectinology as a new area recently has been intensively developed. Lectins selectively and reversibly bind to carbohydrates and agglutinate cells and tissues. They actively participate in such vital processes as communication and signaling systems, adhesion, structural organization of brain, on transport and migration of nerve cells at the postnatal development and in brain termoregulation process etc.

Lectins are proteins with a low molecular weight and have glycoprotein nature. Based on the functions of lectins, great prospects arise for their use in medicine and biology. The anticancer, antiallergic and immunomodulating properties of lectins have already found practical application in clinical medicine for the diagnosis and treatment of cancer and infectious diseases.

Material and methods

Various compositions of extracting solutions for the extraction of neurolectins from the brain of embryonic and adult chickens, rats and bovine, were tested, among which the most promising were 0.5 mM Triton X-100 and 5 mM EDTA in potassium phosphate buffer (PPB). For further purification of lectins, a saturated solution of ammonium sulfate from 0 to 80% was used. Ratio of nerve cells to extracting solution was 1: 5 (mg/ml). The mixture was centrifuged at 16000 g for 15 minutes. For the gradual fractionation of ammonium sulphate at every step of sedimentation solution was added to the extract of soluble proteins. Excess of inorganic ions was removed by the dialysis on the G-10 Sephadex gel-filtration column (50x2.7 cm). The sediment

was dissolved in the minimum volume of PPB and its hemagglutination activity was determined.

Lectin activity was visually determined using microtitration method by Takachi [1] on immunologic slides using the hemagglutination test on trypsin-treated erythrocytes of rabbit. Lectin activity was evaluated by the minimum concentration of protein (mg/ml) which caused an agglutination of trypsin-treated rabbit erythrocytes. Lectin activity was determined in the following way: $SA=T^{-1}\cdot C^{-1}$, where T^{-1} (titre) is the degree of protein dilution in the last well of the titration plate, where hemagglutination still occurs, C- protein concentration in mg/ml.

Specificity of lectins to carbohydrates was studied using hapten-inhibitory method [2]. 0.6 M solutions of oligosaccharides on the basis of PPB were used for analysis. In the experiments the carbohydrates were used as haptens: D-galactose, methyl-D-galactose, N-acetyl-D-galactosamine, D-glucose, L-fucose, D-fructose, L-inositol, D-lactose, D-maltose. Hapten-specificity was judged by the minimum concentration of a carbohydrate (mM), which caused an inhibition of hemaggutinating activity of a lectin. For the agglutination reaction 2% suspension of trypsin-treated rabbit erythrocytes prepared on the PPB solution was used. Protein concentration was determined by Lowry method [3].

Results and discussion

Protein with agglutination ability was discovered in 1888 by Peter Hermann Stilmark in the seeds and oils of the Ricinus communis plant and was named Ricin (hemagglutinin). Since the detection of proteins with agglutinating ability is assigned to lectins [4], we called lectins excreted from nerve tissues neurolectins [5- 8].

The first neurolectin was isolated from the Electric Organ [9]. Lectin agglutinates rabbit erythrocytes treated with trypsin and is called electrolectin. Electrolectin was partially bound to the membrane, but it was also found in the soluble fractions of homogenates of electrical organs. Neurolectin was purified by means of affinity chromatography and isolated as a separate fraction with a molecular weight of 33,000 kDa. Large amounts of electrolectin were found in the microsomal fraction of the electric organ obtained after the centrifugation (70 min 100000 g) and it was increased to 11% of the total amount of proteins.

In 1987, Janeta and his colleagues isolated two endogenous soluble neurolectins CSL1 (Mr = 33,000) and CSL2 (Mr = 31,500) from a cerebellum of the brain of young rats with a 0.5% solution of Triton X-100. Using the immunoaffinity method, another minor component with a molecular mass of Mr = 45,000 was isolated from the same tissue, the minor components of which were CSL1 (mainly lysosomal) and CSL2- (mainly cytoplasmic) subunits. The aforementioned neurolectins turned out to be mannose-specific and were effectively extracted with a 0.5 M mannose concentration [10-13].

The discovery of neurolectins at different stages of the embryonic development of chickens has become a subject of our interest. First, in order to reveal the maximum activity of neurolectins in the brain of embryonic and adult chickens, various compositions of extracting solutions were tested, among which the most promising were 0.5 mM EDTA and potassium phosphate buffer (PPB). For further purification of lectins, a saturated solution of ammonium sulfate from 0 to 80% was used. Neurolectin activity under the conditions of embryonic development of chickens in the brain is found to be deprived on the 5th day and it reaches its maximum before hatching [14].

Noteworthy results were obtained by studying the distribution of neurolectins in the rat brain fractions P1 and P2 within 2-16 days before hatching [14]. Fractions P1 and P2 were obtained by the method of Teichberg and others [9]. P fractions were dissolved in 0.5 mM EDTA and then homogenized. After centrifugation, the precipitate was dissolved in PPB with sodium chloride and the lectin activity of the fractions P1 and P2 was studied [6, 7]. Significantly higher activity was manifested on the 7th day of embryonic development in the P2 protein fraction. If the specific activity of neurolectins before hatching of the P1 and P2 fractions was 79.37 and 132.50 units, respectively, then, after hatching, this indicator decreased to the maximum and increased to no more than 16.04 and 80.62 units [14]. It is assumed that a decrease in the activity of neurolectins is explained by an increase in brain mass and brain volume. A similar opinion was expressed by other authors [15].

The content of neurolectins was also studied in the brain of chickens under conditions of embryonic and postembryonic development [14, 16-18]. Neurolectins were extracted from the chicken brain with a solution of 5 mM EDTA PPB. Subsequently, the proteins were purified with ammonium sulfate at a saturation of 80-90%. The isolated neurolectin was specific for D-galactose (30 mM), D-lactose

(15 mM), N-acetyl-D-glucosamine and, therefore, it was designated as LC-GLN. Neurolectin was purified by lactose-sepharose column 4B affinity chromatography. As a result, lectin activity increased by more than 25 times. Using the method of electrophoresis in polyacrylamide gel, the molecular weight of neurolectin was 15 kDa. Lectin is a glycoprotein with a monosaccharide content of approximately 20%. Neurolectin showing an optimal activity at pH of 7.0-8.0, was particularly sensitive to Ca^{+2} ions, in the presence of a Ca^{+2} EGTA chelator, at a concentration of 0.2 mM, an agglutination of trypsinized red blood cells was completely inhibited [14, 18-20].

In 1982, mannose-specific neurolectin was isolated from the brain of embryonic and adult chickens, which was purified 1700 times using APL Sepharosa 4B. Such a polyacrylamide gel neurolectin preparation in the presence of sodium dodecyl sulfate was divided into two protein fractions with a molecular weight of 15 kDa and 13 kDa of lectin activity. Neurolectins extracted from different tissues of embryonic and adult chickens were identical. It should be noted that neurolectins from chicken muscles, the brain, and liver had similar chemical and immunological properties [15, 16].

Neurolectins from neurons, glial cells and synaptic vesicles

After the discovery of neurolectins in the brain of chickens and rats, we examined the quantitative distribution of neurolectins in enriched fractions of neurons, glial cells and in synaptic vesicles. Enriched fractions of neurons and glial cells were obtained by the modified Rose method [21, 22].

Neurolectins were isolated from the enriched fraction of neural and glial cells with a 0.5% solution of Triton X-100. After homogenization, the mixture was centrifuged (10000 g/30 min), the supernatant was fractionated with ammonium sulfate in different saturations. The residue was dissolved in an agglutinating PPB solution, and after dialysis, the activity of neurolectin was determined in each individual fraction. The specific activity of neurolectins was 780 (neuron) and 713 (glia) units, respectively. Neurolectins isolated from enriched fractions of neurons and glial cells showed a diverse affinity for carbohydrates. Namely, the neurolectin isolated from the enriched fraction of neurons, showed a specific sensitivity to D-galactose (16.2 mM) and N-acetyl-D-glucosamine (8.1 mm), neurolectin isolated from enriched fractions of glial cells showed specificity to D-fructose (16.2 mm), D-xylose (16.2 mm), N-acetyl-D-galactosamine (9.3 mm) and inositol (4.6 mm). To obtain proteins with the highest lectin activity of neurons and glial cells, the neurolectins were purified by affinity chromatography on a column of tris-acryl-inositol and tris-acryl-galactose. Inositol-specific neurolectin was isolated from the enriched fractions of glial cells (GL-I), and the neurolectin specific to galactose was isolated from the enriched fractions of neurons (NL-GAL). The molecular weights of the neurolectins were measured using an HPLC system (Millipor-Waters, USA). Their molecular weights, respectively, were GL-I = 11.5 kDa, and NL-Gal = 13.5 kDa. Both lectins were glycoproteins containing carbohydrates 28% and 26%, respectively.

It should be noted that NL-Gal neurolectin contains the following amino acids: leucine, isoleucine, valine, phenylalanine, tryptophan, glutamic acid, threonine, glycine, serine, glutamine, asparagine and arginine, whereas in GL-I, with the exception of these amino acids, in addition, proline and tyrosine were found. The presence of SH groups was noted in both neurolectins, which was established by the Ellman method [23]. The number of SH groups per mg / protein was 35×10^{-4} M for NL-Gal, while 15×10^{-4} M for GL-I. Both neurolectins showed particular sensitivity to Ca^{+2} ions and were most inhibited in the presence of 0,2 mM Ca^{+2} EGTA chelators.

It is noteworthy that the neurolectins of glial cells of the embryo are predominantly presented in soluble form, which is usually characteristic of the brain of embryonic animal cells. It is more likely that the soluble forms of neurolectins in the process of postnatal development turn into a bound form.. It is worth noting that endogenous neurolectin was also found in Schwan cells. It has been suggested that Schwan cells neurolectins are involved in the stabilization of compact myelin [11, 12].

Taking into account the strategic role of glial cells in providing trophic function of cells, an attempt was made to study the effect of amino acids, neurotransmitters and biologically active substances on the hemagglutination of trypsinized rabbit erythrocytes [14, 20, 22], suggesting that the results of these experiments should give an answer about the possible role of neurolectins in capture and their inactivation. Using the hapten-inhibitor method, it has been experimentally shown that the hemagglutination activity of trypsinized rabbit erythrocytes NL-Gal is inhibited by arginine (37.5 mm), L-ser-

ine (18.7 mm), L-glutamine (18.7 mm), glycine (0.31 mm), hemagglutination of neurolectins GL-I is inhibited by L-arginine (37.5 mm), L-serine (18.7 mm), L-glutamine (9.4 mm), tyrosine (37, 5 mM), acetylcholine (0.62 mm), beta-alanine (0.156 mm), serotonin (0.32 mm), epinephrine (1.25 mm), norepinephrine (0.62 mm), dopamine (0.156 mm), tyramine (0.63 mmol), hydroxytyramine (0.156 mmol), choline bromide (0.08 M) and hydroxytryptamine (1.25 mM). It is important to note that a number of other biologically active substances had also an inhibitory effect on the activity of neurolectin GL-I, but with relatively high concentration: gamma-aminobutyrate (> 100 mM) and methoxytyramine (> 100 mM). The quotation marks provide data on the concentration of hemagglutination inhibitors [24-26].

Based on the presented material, the determination of the orientation of the active center of GL-I and NL-Gal on cell membranes was of great interest. It has been shown that native glial cells cause agglutination of trypsinized rabbit erythrocytes. GL-I agglutination is completely inhibited by inositol (0.6 mM), which suggests that the GL-I active center on the membrane of glial cells is oriented to the outer side of the membrane in the direction of the intercellular space and the active center of NL-Gal is oriented towards the cell cytoplasm [7, 14, 16].

The question arises: what role can the neurolectins play in the trophic function of glial cells. After the hapten-inhibitory method established that biologically active substances, neurotransmitters and amino acids had an inhibitory effect on agglutination of trypsinized rabbit erythrocytes, it was concluded that GL-I neurolectin can actively participate in the transport of neurotransmitters, amino acids and biologically active substances and their metabolism and inactivation. It was previously shown that under conditions of excess neurotransmitters in the neuron-glia-synapse region by glial cells and synaptosomes, active absorption and detoxification of acetylcholine, serotonin, dopamine, and gamma-aminobutyric acid occurs [24, 25]. It should be noted that the absorption of serotonin by glial cells and their inactivation occurs actively by conjugation with glucuronic acid, resulting in the formation of biologically inactive glucuronideserotonin [26]. The absorption of dopamine and serotonin by glial cells of the rabbit cerebral cortex also occurs in the same way.

Thus, it has been found that neurolectins are mainly presented in nerve cells, subcellular fractions, pathways, dendritic basement membranes, nerve endings, and synaptic vesicles. It is interesting that the number of neurolectins and their activity are mainly observed during mass synaptogenesis, which indicates their active participation in the formation of synapses. Therefore, naturally, the researches were begun to study the distribution and function of neurolectins at the level of synaptic vesicles, in connection with their possible role in secretion and accumulation of neurotransmitters in synaptic vesicles, which were isolated according to the method of De Robertis and his colleagues [27].

In 2002 and 2004, inositol-specific lectin was isolated from synaptic vesicles of chicken and bovine brain (BVL-I) with Triton X-100 solution [28, 29], which turned out to be a glycoprotein with a total content of 10% carbohydrates. Neurolectin showed a high sensitivity to Ca^{+2} ions, in the presence of 0.2 mM concentration of Ca^{+2} chelator of the EGTA, the activity of neurolectins was maximally inhibited. Of particular interest is the fact that the neurolectin BVL-I exhibits a specific affinity for phosphatidylinositol and phosphatidylcholine and is localized on the outer surface of the vesicular membrane, which has been experimentally proven. It has been first discovered that agglutination of trypsinated rabbit erythrocytes occurs under the influence of bovine brain native synaptic vesicles, while an agglutination induced by synaptic vesicles is inhibited by inositol [8, 20, 29]. This suggests that BVL-I is an inositol-specific neurolectin. Neurolectin is relatively rich in sulfur-containing amino acids, the amount of which is 21.4×10^4 M per 1 mg of protein.

On the other hand, neurolectins from synaptic vesicles were also seen from the brains of mature chickens. For maximum extraction of neurolectins, 0.5% Triton X-100 on 40 mM PPB (pH 7.4) was used. As a result of using this solution, specific inositol (SVL-I) and N-acetyl-D-galactosamine (SVL-NAGA) were isolated and purified on a column of immobilized Sepharose 4B N-acetyl-D-glucosamine and inositol. The lectin activity of the extracted protein increased by 2–5 times. Both lectins are glycoproteins containing 6% and 13% carbohydrates, respectively. Neurolectins are enriched with sulfur-containing amino acids, the average amount of which is 21.4×10^4 M per 1 mg of protein.

Neurolectin SVL-I is highly sensitive to Ca^{+2}, and therefore, its activity is completely inhibited by Ca^{+2} with the EGTA chelator. Its location and orientation in the vesicles were also of great interest. In order to resolve this question, it was necessary to clarify the effect of SVL-1 and SVL-NAGA on agglutination of trypsinized rabbit erythrocytes. It

was found that native synaptic vesicles agglutinate trypsinized rabbit erythrocytes. The agglutination is completely inhibited by specific hapten-inositol and phosphatidylinositol (0.03 mg/100 μl) [25, 29-32]. It becomes apparent that the neurolectins BML-I and VL-I possess essentially the same characteristics and are characterized by the same affinity for haptens, namely, inositol, inositol phospholipid and phosphatidilcholine.

Based on these data, a hypothesis is suggested that SVL-1 and SVL-NAGA participate in the secretion of neurotransmitters from synaptic vesicles [6, 8, 33,34]. According to the proposed hypothesis, neurolectin SVL-1 is fixed on the presynaptic membrane in the form of a terminal carbohydrate residue of inositol and inositol phospholipid, which is oriented towards the cytoplasm of the vesicles. At the same time, the presynaptic membrane and synaptic vesicles merge, forming a vesicular lumen, through which an active influx of Ca^{+2} ions from the synaptic cleft occurs. The formation of a similar structure was shown in the electron microscopic studies. The formation of a presynaptic lumen is accompanied by the secretion of neurotransmitters, while the stimulation of receptors activates the phospholipase C enzyme.. As a result of the action of phospholipase C, inositol phospholipid is cleaved into inositol-1,4,5-triphosphate (ITP) and diacylglycerol. The latter remains in the presynaptic membrane, while ITP in bound form with the vesicles is separated from the membrane. A part of ITP in the free state promotes an increase in the concentration of Ca^{+2} ions in the cytoplasm, and diacylglycerol activates protein kinase C. Therefore, with an increase in the concentration of cytoplasmic Ca^{+2}, the output of neurotransmitters from synaptic vesicles through the presynaptic lumen is accelerated (Fig.). This completes the secretion of the neurotransmitter and begins the stage of formation of synaptic vesicles and their filling with neurotransmitters.

As for the formation and closure of the lumen of synaptic vesicles, in accordance with the proposed scheme, lectin SVL-1, SVL-NAGA and Ca^{+2} ions are involved in it. With an increase in the concentration of Ca^{+2} ions, the inhibition of ITP binding to receptors in the cytoplasm is observed, and at the same time, the ligand-receptor bond (ITP-SVL-1) is destroyed. This process is enhanced with the splitting of ATP by neurolectins and the accumulation of phosphate around the vesicles [35-38]. It is well known that the binding of neurolectin to receptors is disrupted in an acidic environment [34]. Free-form ITP stimulates the release and accumulation of Ca^{+2} ions in the cytoplasm. As a result, an agglutination and fusion of vesicular membranes are enhanced, and neurolectin VL-NAGA is actively involved in the process. It should be noted that VL-NAGA is more sensitive to Ca^{+2} ions (3 mM) compared to VL-I (> 100 mM). Ca^{+2} ions contribute to the fusion of the ends of both vesicular and presynaptic membranes. Thus, it is concluded that after the release and loading of vesicles with neurotransmitters, fusion and union of the vesicular end membranes is ensured. It is assumed that the hypothesis needs a further development of the biochemical foundations of the individual stages, but the opinion about the participation of neurolectins in the secretion of neurotransmitters and membrane fusion, in all likelihood, will not cause doubt.

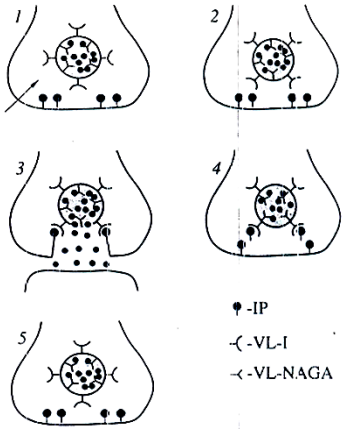

Fig. *Hypothetical scheme for the participation of the neurolectins VL-1 and VL-NAGA in the secretion of neurotransmitters.*

1 - The synapse is at rest. 2 - Antibody vesicular lectin SVL-1 on the presynaptic membrane with inositol phospholipid. 3 - The formation of presynaptic lumen and the secretion of neurotransmitters. 4 - Separation of vesicles from inositol phospholipid (IP) and restoration of the presynaptic membrane. 5 - Complete restoration of the synaptic vesicle in a relaxed state. Designations: IP - inositol-triphosphate, SVL-1 - vesicular inositol-specific neurolectin, SVL-NAGA - vesicular N-acetyl-D-galactosamine-specific neurolectin

Neurolectins from mitochondria

Since the quantitative distribution of neurolectins in the animal brain was established, it was of great interest to study the distribution of neurolectins at the level of subcellular fractions of nerve

cells. Neurolectin was extracted from brain mitochondria with a specially selected 0.5% solution of X-100 Triton and purified under conditions of 80% saturation with ammonium sulfate. It was found that agglutination with mitochondrial neurolectin is specifically inhibited by glucose, galactose, N-acetylglucosamine and N-acetyl-galactosamine hence was called BML-GluG [39]. BML-GluG was fractionated on a Sephadex G-100 column. Chromatography revealed isoforms of BML-GluG neurolectin (A, B), which showed lectin activity. BML-GluG is a glycoprotein containing carbohydrates at a concentration of 16.7 μg / mg protein. Neurolectin also contained disulfide groups. After restoration of disulfide bonds by dithiothreitol, lectin activity was maximally inhibited. BML-GluG was found to be sensitive to Ca^{+2} ions. In the presence of Ca^{+2} EGTA chelator, at a concentration of 0.2 mM, an agglutination of trypsinized rabbit erythrocytes was maximally inhibited. It was necessary to study its orientation on the mitochondrial membrane. For this purpose, it has been found that the agglutination of trypsinized rabbit erythrocytes occurs by the homogenate of native mitochondria and, therefore, using the hapten-inhibitory method, it is shown that agglutination of trypsinized rabbit erythrocytes using BML-GluG was maximally inhibited by N- acetyl-D-glucosamine. Thus, it is concluded that BML-GluG is oriented outside the mitochondrial member [39].

Nuclear neurolectins of rat brain cell

Data on nuclear neurolectins of nerve cells are quite limited. Therefore, special attention was paid to the nuclear neurolectins of the rat brain [40-42]. In order to establish the presence of neurolectins in the nuclei of rat brain nerve cells, it has been previously established that agglutination of trypsinized rabbit erythrocytes occurs with a native nuclear homogenate. In the agglutinated supernatant, the fractional composition of the proteins was studied by electrophoresis in a 7.5% polyacrylamide gel. In contrast to electrophoregrams of a non-agglutinated nuclear supernatant, a number of protein fractions were absent in the agglutinated supernatant, which indicated that as a result of the binding of nuclear proteins during agglutination with trypsinized rabbit erythrocytes, a number of protein supernatant fractions were not found. Based on these experimental data, a study was begun on the distribution of neurolectins in the cell nuclei of rat brain. For this, various composition buffers were tested, among which the buffer of the following composition turned out to be the most promising: 0.5% Triton X-100 + 0.9% NaCl + 20 mM potassium phosphate buffer (PPB), pH 5.0 [43, 44]. After homogenization of the nuclear fraction, soluble proteins were removed using PPB, the mixture was dialyzed against hemagglutination buffer, and the lectin activity of the mixture was determined. It has been shown that the specific activity of nuclear neurolectin is 104.4 units. Subsequently, the cell nucleus neurolectin was purified by the method of stepwise salting out with ammonium sulfate at a saturation of 20%, 40%, 80%. These proteins were purified by HPLC (Waters, USA) on Protein PAK-300-SW gel tandem filter columns. It was found that, when saturated, under conditions of 40% saturation with ammonium sulfate, the specific neurolectin activity increased one and a half times. After fractionation of the proteins on glutaraldehydefixed tripressed blue rabbit erythrocytes, the specific neurolectin activity of the nuclei (Nuc-L) increased 5 times and reached 534 units. It should be noted that nuclear neurolectins during gel filtration on a Protein PAK-300-SW column are divided into two protein fractions. Both fractions of proteins are characterized by lectin activity. Using the hapten-inhibitory method, it was shown that both neurolectins of the nucleus of the brain of the rat haptens were N-acetyl-D-glucosamine, D-galactose and L-fructose.

Conclusion

Quantitative and qualitative distribution of neurolectins in brain neurons, glial cells, synapses, and their subfractions (mitochondria, cell nuclei) and their proper hapten have been established in animals (rats, chickens, bulls). The possible role of neurolectins secreted by neurons, glial cells, and synaptosomes is given depending on trophic function and biologically active substance absorption and inactivation depending on the orientation of the membrane neurolectins.

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Aegilops tauschii, the D-genome donor of *Triticum* and the geographic origin of hexaploid wheat

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ABSTRACT

The place of origin of alloploid hexaploid wheat is directly connected with the distribution area of its parent species. One of them is wild plant *Aegilops tauschii,* the donor of D-genome. The distribution area of *Ae. tauschii* is stretched extensively from the western Transcaucasus (Georgia) to Central Asia, Pakistan and China. However, it is known that *Ae. tauschii* diverged into subsp. *tauschii* and subsp. *strangulata* at the very beginning of its existence as a species and each of its two subspecies - subsp. *tauschii* and subsp. *strangulata* - originated in the Caucasus. Studies of *Aegilops tauschii* provide evidence that common wheat (*T. aestivum*) was derived from single or limited number of accessions of *Ae. tauschii* and *Ae. tauschii* subsp. *strangulata* populations from its birthplace (the western Transcaucasus) were involved in the formation of common wheat, which originated in the Transcaucasus ca. 8000 BP. Natural diversity of wheat and archaeological excavations of the Neolithic sites of Georgia dated back to 8,000 BP provide evidence that the Transcaucasia is the most probable place of origin of common wheat, because: a) agriculture had been already developed and represented by all founder crops in Georgia; b) AABB-genome containing domesticated hulled and free-trashing tetraploid wheat species (*T. dicoccum, T. palaeocolchicum, T. carthlicum* and *T. durum*) had already existed in Georgia; c) hexaploid wheat species: *T. spelta, T. spaerococcum, T. compactum,* and *T. aestivum* were also present here. These three archaeological findings provide sufficient ground to propose that Ae. *tauschii* subsp. *strangulata* cross with a tetraploid wheat species could had happened here by 8000 BP.

Keywords: *Aegilops tauschii,* Subsp. *strangulata,* Hexaploid wheat, Hulled wheat, Free-threshing wheat, Western Transcaucasus.

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Introduction

Georgia is one of the most important centers of diversity of the cultivated wheat. Despite the small territory (ca 69,000 sq. km), Georgia is the only country in the world, where 15 species (*sensu strricto* classification) of wheat (*Triticum boeoticum* Boiss., *T. monococcum* L., *T. dicoccum* (Shrank) Schübl., *T. palaeocolchicum* Menabde, *T. timopheevii* (Zhuk.) Zhuk., *T. durum* Desf., *T. turgidum* L., *T. carthlicum* Nevski, *T. macha* Dekapr. & Menabde, *T. zhukovskyi* Menabde & Ericzjan, *T. turanicum* Jacubz., *T. polonicum* L., *T. spelta* L., *T. compactum* Host, *T. aestivum* L.) are present. Among them 5 species (*T. macha*, *T. palaeocolchicum*, *T. timopheevii, T. zhukovskyi, T. carthlicum*) are endemics to Georgia. Only one species – *T. carthlicum* (Georgian name 'dika') is naked and rest 4 endemics are ancient hulled wheat. Among 5 endemic species of Georgia, 4 ancient hulled wheats originated from province Lechkhumi. Lechkhumi is situated in a rather isolated valley between the mountains of the central part of west Georgia. Endemic Georgian wheat species are healthy food,

quite precious for breeders, all characterized by high resistance to fungal diseases [1].

Georgia is the only country in the world where all 7 species of domesticated hulled wheat are present (4 endemic and 3 non endemic species): *T. palaeocolchicum, T. carthlicum, T. timopheevii, T. macha, T. monococcum, T. dicoccum, T. spelta*). At the same time, Georgia is the only country in the world where all the genomes (AA, AABB, AAGG, AAGGAA, AABBDD) of the wheat can be found (AAGGAA only in Georgia). Hexaploid AABBDD wheat originated about 8,000 years ago [2, 3] by hybridization of D genome donor diploid *Aegilops tauschii* with the already domesticated tetraploid AABB wheat [4-6]. As many as four species (*T. spelta, T. spaerococcum T. compactum* and *T. aestivum*) of domesticated hexaploids (AABBDD) wheat dating back to 8,000 BP were identified in archaeological excavations in Arukhlo, Shulaveri, Khramis Didi Gora of Lower Kartli region (south eastern Georgia) [7, 8].

Origin and geographic spread of Aegilops tauschii

Aegilops tauschii Coss. (- *Aegilops squarrosa* auct. non L.) is a diploid (2n = 14) cleistogamic (self-pollinating) species of the family Poaceae. Its modern natural distribution range is from the western Transcaucasus (west Georgia) and northeastern Turkey to Central Asia, China and Pakistan. While the putative primary region of *Ae. tauschii*'s origin is the Transcaucasus [the South Caucasus] [9]. From the Caucasus, *Ae. tauschii* dispersed eastward to China across northern Iran and Central Asia and southward to central Syria [10].

Ae. tauschii divided into subsp. *tauschi*i and subsp. *strangulata* at the very beginning of its existence as a species and each of its two subspecies - subsp. *tauschii* and *subsp. strangulat*a - originated in the Caucasus. The mutation Tf(s)225b (which appeared after the origin of Ae. *tauschii* but before its subdivision into the subspecies) was retained in both subspecies (Fig. 1). Another mutation Tc(s)380 (black dots) had existed even before the origin of Ae. *tauschii* and could be found only in some local populations of subsp. strangulata, which is represented only by two accessions t9(1)s and AE929 from the Caucasus: Dagestan and Georgia, respectively [11].

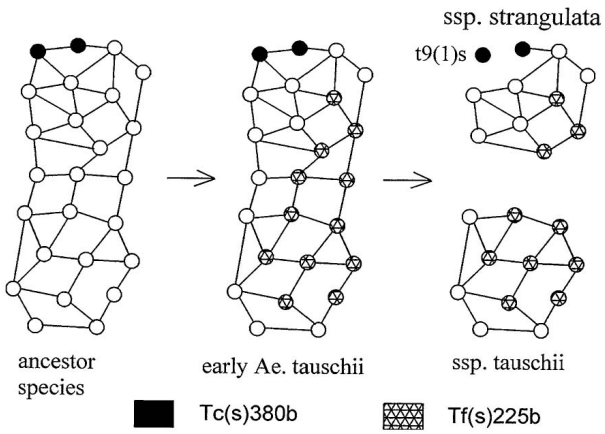

Fig. 1. *A presumed scheme of Ae. tauschii evolution. The circles present local populations in the field of gene combinations of Sewall Wright (credit of the illustration [11]).*

Subsp. *tauschii* was the first to start geographic expansion and relatively rapidly occupied a vast area from the Caucasus—eastward up to central Tien Shan and western Himalayas [11]. In contrast to subsp. *tauschii*, only some linages of subsp. *strangulata* managed to expand to the East. Therefore, the Caucasus is the only region where all the five subsp. *strangulata* lineages, Tc(s)21b, Tc(s)492b, Nf(d)237b, Tc(d)132b and Tf(s)225b were found [11].

Primary habitats of Ae. *tauschii* are patches of dwarf-shrub steppe-like formations in hilly or mountainous regions. Subsp. *strangulata* ''prefers'' the habitats with relatively warm and moist winter, while subsp. *tauschii* occupies habitats with relatively continental climate [12].

Ae. tauschii likely dispersed from the temperate desert vegetation (TDV) zone at the Last Glacial Maximum (25,000–15,000 BP)[1] in the postglacial era and the southern habitats were established as the environment experienced change to humid conditions in the Holocene [13]. Because *Ae. tauschii* has a weedy growth habit, human disturbance may have contributed to population migrations after the beginnings of agriculture 10,000 years ago.

When 205 accessions of *Ae. taushii* from the whole area of its distribution were analyzed based on chloroplast DNA variation, four intraspecific linages were identified, which were characterized by different haplogroups: HG7, HG16, HG9 and H17. It was found that the accessions of the HG7 lineage were distributed across the entire range of natural

1 It should be noted that West Georgia, the Western Transcaucasus is an important glacial refugia, where many relict plants have been preserved, and this region is rich in endemic plant species

habitats and the eastward dispersal of *Ae. tauschii* was driven by the HG7 and HG16 lineages [3]. The HG9 lineage accessions were specific to western habitats, while the distribution area of five HG17 accessions was only found in Georgia [14].

It was found that flowering time of *Ae. tauschii* varied from the earliest (144 days) to the latest (190 days) in the temperate desert vegetation zone. The eastward dispersal was driven by the HG7 and HG16 lineages (Fig. 3). HG16 diverged from HG7 in the west and independently migrated to the east. The HG9 lineage was phenotypically uniform, with 26 out of 28 accessions (92.9%) having the intermediate-flowering phenotype. All five accessions of the HG17 lineage had the late-flowering phenotype. Among the intermediate and late-flowering phenotypes, accessions of the HG7 lineage were common [3].

Ae. tauschii evolved from late flowering to early flowering and the ancient, late flowering forms (colored in blue on Fig. 3) of both subsp. *taushii* (HG7) and subsp. *strangulata* (HG9, HG17) are found only in the Caucasus [3, 14]. In contrast to subsp. *tauschii*, geographic spread of subsp. *strangulata* was a complicated, multi-stage and slow process. At the beginning of subsp. *strangulata* evolutionary history its major phylogenetic lineage for a lengthy time span had existed as a small isolated population. Several forms of subsp. *strangulata*, better adapted to relatively moister and cooler habitats, had originated [11].

In Fig. 3, HG9 (the D genome donor to hexaploid wheat) is represented by ancestral late flowering phenotypes (blue triangle) in Georgia and the South Caucasus. As it was mentioned above "geographic spread of subsp. *strangulata* was a compli-

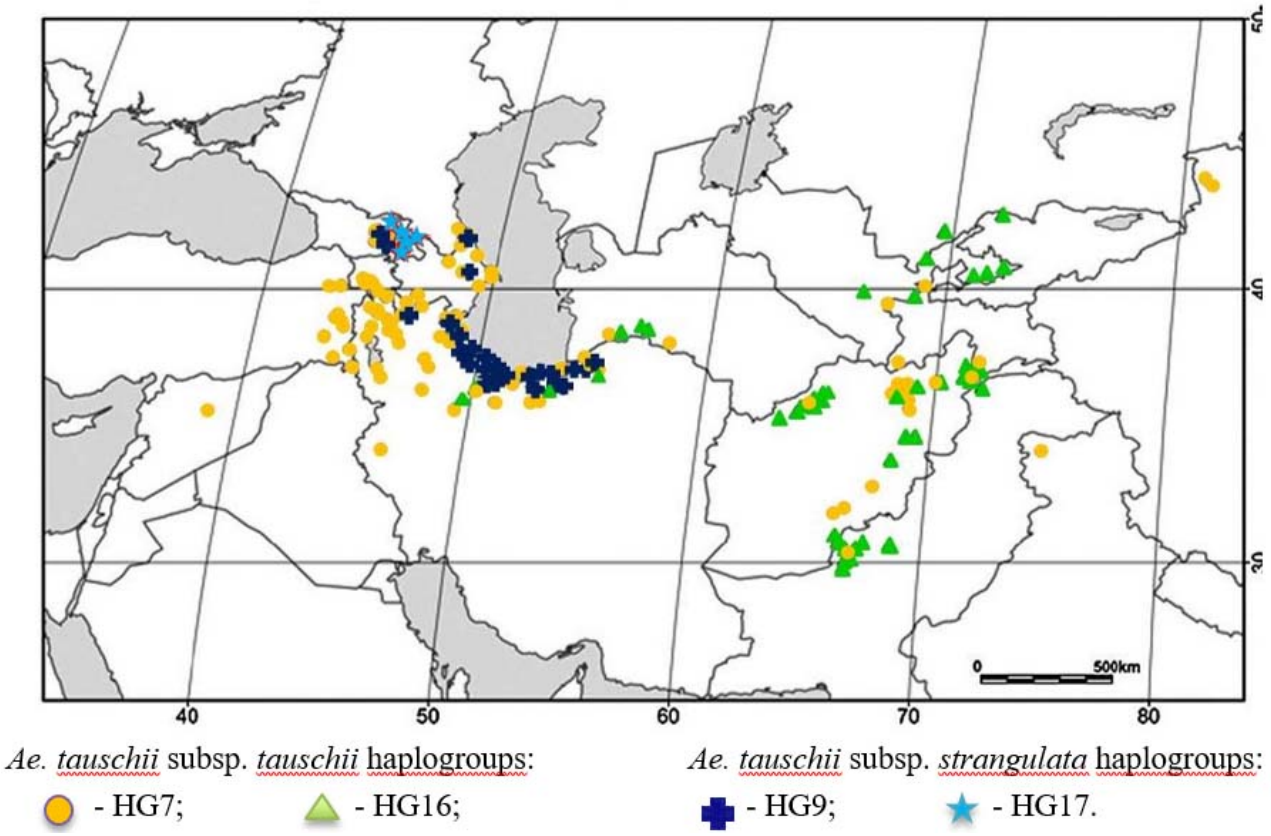

Fig. 2. *Geographical distribution of Ae. tauschii accessions. Adventive populations in the Shaanxi and Henan provinces of China are not shown. Circles, crosses, triangles, and stars indicate the HG7, HG9, HG16, and HG17 lineages, respectively (credit of the map [14]). Haplogroups HG7 and HG16 represent subsp. tauschii and while HG9 and H17 belong to subsp. strangulata.*

cated, multi-stage and slow process". i.e. in Georgia and the South Caucasus, the D genome donor subsp. *strangulata* should have existed at least 1000 years earlier before subsp. *strangulata* reached the Caspian region. Pre-Caspian Iran cannot be assumed as a place of hexaploid wheat origin because *Ae. tauschii* subsp. *strangulata* could not have been presented there earlier than 7000 BP [15].

Amplified fragment length polymorphism (AFLP) analysis was applied for a total of 122 accessions of *Ae. tauschii* to clarify the population structure of this widespread wild wheat species. Phylogenetic and principal component analyses revealed two major lineages in *Ae. tauschii*: L1 and L2. Among three major haplogroups (HG7, HG9 and HG16) previously identified in the *Ae. tauschii* population based on chloroplast variation, HG7 accessions were widely distributed to both L1 and L2, HG9 accessions were restricted to L2, and HG16 accessions belonged to L1, suggesting that HG9 and HG16 were formed from HG7 after divergence of the first two lineages of the nuclear genome [18].

Later, to identify the *Ae. tauschii* intraspecific lineage, the same 122 accessions were grouped into three intraspecific lineages: TauL1 (renamed from L1), TauL2 (renamed from L2), and TauL3 (renamed from HGL17). Geographically, the TauL1 accessions are widely spread across the species range, whereas the TauL2 and TauL3 accessions are restricted to the Transcaucasus/Middle East region and Georgia, respectively [6].

Within each TauL1 and TauL2 lineage, three sublineage groups were identified: TauL1a TauL1b and the intermediates (named TauL1x) and TauL2a, TauL2b and TauL2x (Fig.4). The chloroplast DNA haplotype evidence supported that TauL1a is ancestral to TauL1b, Similarly, TauL2a is ancestral to TauL2b. The TauL2a accessions tend to spread in the Transcaucasus, whereas TauL2b has its distribution center in the Caspian coastal region. Each TauL1a and TauL2a has the center of distribution in the Transcaucasus and represents ancestral sublineages of the species [19]. The Transcaucasus (especially Georgia) is the only region where all ancestral linages and sublineages of TauL1, TauL2 and TauL3 were found. According to Gogniashvili *et al.* [20] TauL3 and TauL1 are ancestral linages while TauL2 diverged from the Taul3.

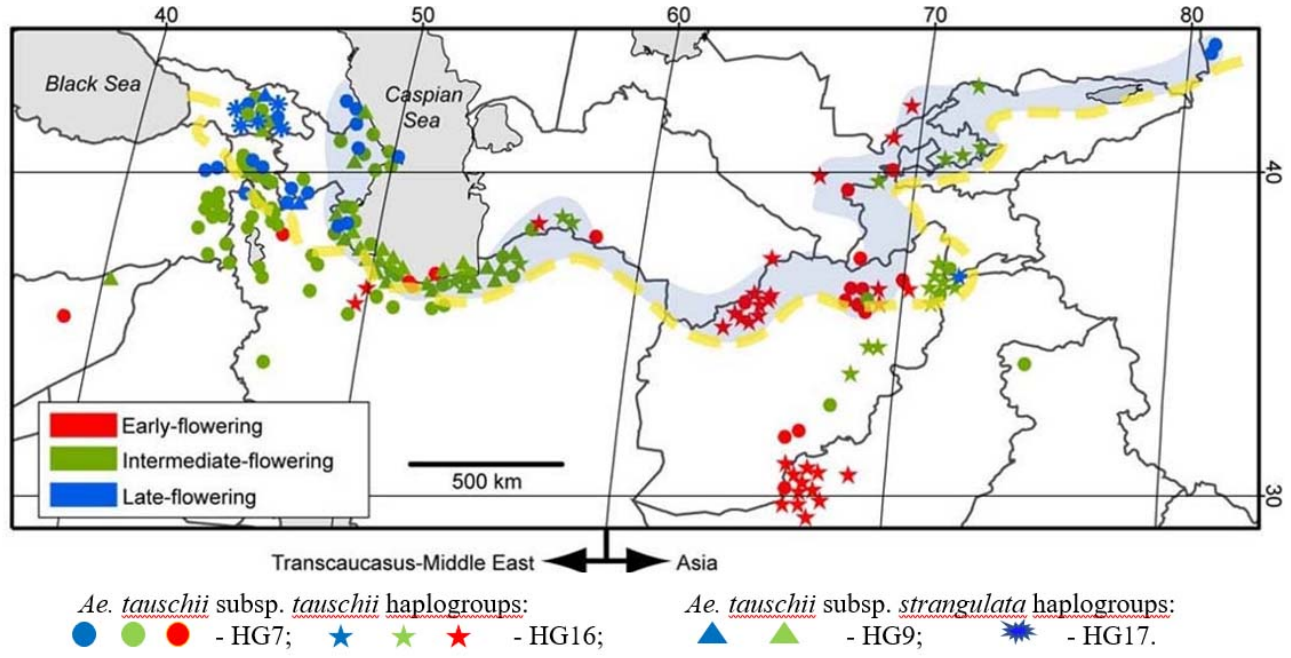

Fig. 3. *Geographic distribution of the 200 Ae. tauschii accessions. Circles, triangles, stars and asterisks denote, respectively, accessions of the HG7 lineage, HG9 lineage, HG16 lineage, and HG17 lineage. For each accession, flowering time phenotype is colored according to the key. The belt area where fairly undisturbed habitats exist is shaded [16, 17]. The dashed yellow line indicates the southern limit of the temperate desert vegetation zone at the Last Glacial Maximum. The species distribution was divided into two regions in the analyses: Transcaucasus-Middle East (longitude <60^0) and Asia (longitude >60^0) (credit of the map [3]).*

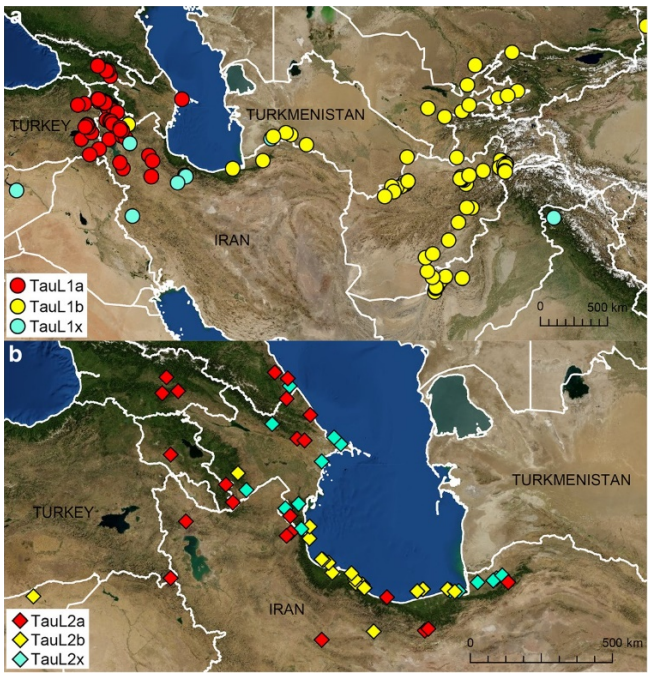

Fig. 4. *Geographic distributions of the TauL1 and TauL2 sublineages. a) The TauL1 sublineages. TauL1a, TauL1b, and TauL1x are colored red, yellow, and blue, respectively. The six accessions representing adventive populations in the Shaanxi and Henan provinces are not shown. b) The TauL2 sublineages. TauL2a. TauL2b, and TauL2x are colored red, yellow and blue, respectively (credit of the map [19]).*

TauL1 is associated with *Ae. tauschii* subsp. *tauschii*, while TauL2 and TauL3 are associated with *Ae. tauschii* subsp. *strangulata*. Subsp. *strangulata* linage TauL2 is considered to be the D-genome progenitor of hexaploid common wheat *Triticum aestivum*.

To identify the *Ae. tauschii* intraspecific lineage that is most closely related to the D genome of common wheat, Matsuoka *et al*. [6] conducted a population-level analyses using a

Diversity Arrays Technology (DArT) marker genotype dataset for 206 *Ae. tauschii* accessions that represented the entire species range and a diverse array of 188 common wheat accessions consisting of traditional and modern cultivars of the East and West and one synthetic wheat line W7984. Separation of the D genome of common wheat from *Ae. tauschii* was fully consistent with the results of restriction-fragment-length-polymorphism, microsatellite, and single-nucleotide-polymorphism studies [21-23].

Geographically, the TauL1 accessions are widely spread across the species range, whereas the TauL2 and TauL3 accessions are restricted to the Transcaucasus/Middle East region and Georgia, respectively. In contrast, almost all common wheat accessions formed a single isolated cluster that was more closely associated with TauL2 and TauL3 than TauL1 (Fig. 5). Two common wheat accessions were exceptionally closely associated with *Ae. tauschii*: the synthetic wheat line W7984 (placed near TauL2) and an Ethiopian landrace KU-9873 (placed near TauL3). The reason for the KU-9873 - *Ae. tauschii* association was not clear [6].

The PCA and Bayesian clustering, therefore, showed close genetic relationships of the TauL2

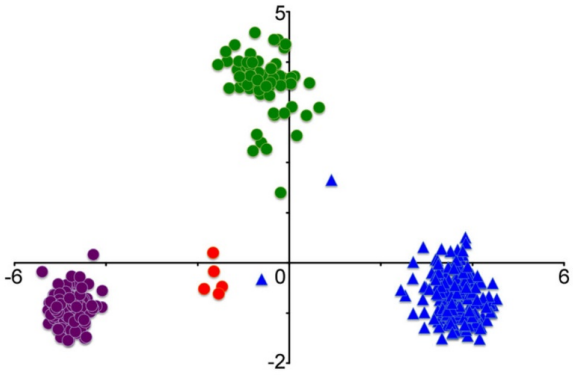

Fig. 5. *Graph of the first two axes from a PCA based on DArT marker variations. The first component (x) accounts for 41.8% and the second (y) for 8.5% of the total variance. Circles denote Ae. tauschii TauL1 (purple), TauL2 (green), and TauL3 (red), and triangles T. aestivum (credit of the chart [6]).*

and TauL3 accessions to the D genome of common wheat. Notwithstanding the observed close genetic relationship of TauL2 with the D genome of common wheat, whether the TauL2 accessions represented descendants of the *Ae. tauschii* populations that were involved in the 8,000-years-ago allopolyploid speciation of common wheat was not clear [6].

The birthplace of common wheat has been assumed to be Transcaucasia or the south coastal region of the Caspian Sea [21, 24 - 26]. Common wheat was derived from single or limited accessions of *Ae. tauschii*, and *Ae. tauschii* populations far from its birthplace were not involved in the formation of common wheat [14].

According to Nesbitt [27] the origin of bread wheat in the Near East remains as problematic as the origin of spelt in Europe. The hybridization between a tetraploid wheat and *Aegilops tauschii* could take place only once agriculture with tetraploid wheats reached the distribution zone of *Aegilops tauschii.* This extends from northern China westwards to west and southwest of the Caspian Sea, well northeast of the fertile crescent. As agriculture did not reach the Caspian Sea until after 6000 BC, the hybridization could not have occurred until then [27].

Archaeological evidence for wheat and other crops in Georgia (the south Caucasus)

The Neolithic revolution probably began in the 8th millennium B.C. [10,000 B.P.] in Georgia [28]. It is distinguished by a number of innovations in the archaeological records. Along with the diversity of tools and pottery production the remains of various domesticated plants (cereals, legumes, grape, etc.) including soft wheat, provide evidence of the high level of farming. Excavated wheat grains include transitional forms from wild to domesticated varieties.

Wheat, barley, oil-fiber crops were widely cultivated along with the vineyards and fruit gardens [29 - 31].

Fig. 5. *The Neolithic archaeological site of Arukhlo (8000 BP), Lower Kartli region of southeast Georgia*

According to McGovern *et al.* [32], the earliest evidence for grape wine in the Near East was from the early Neolithic village of Hajji Firuz Tepe in the northwestern Zagros Mountains of Iran, ca. 5,400–5,000 BC. Chemical analyses of ancient organic compounds absorbed into the pottery fabrics from sites [Gadachrili Gora and Shulaveris Gora] in Georgia in the South Caucasus region, dating to the early Neolithic period, provide the earliest biomolecular archaeological evidence for grape wine and viniculture from the Near East, at ca. 6,000–5,800 BC. The discovery of early sixth millennium BC grape wine in this region is crucial to the later history of wine in Europe and the rest of the world. Now that wine jars from as early as ca. 6,000 BC have been confirmed for Gadachrili Gora and Shulaveris Gora, preceding the Hajji Firuz jars by half a millennium [32]. Alcohol plays a central role in formation of human civilization. According to McGovern *et al.* [32] the very large-capacity jars, some of the earliest pottery made in the Near East, probably served as combination fermentation, aging, and serving vessels. They are the most numerous pottery type at many sites comprising the so-called "Shulaveri- Shomu Culture" of the Neolithic period. All these finds: the oldest grape wine, domesticated grape seeds (pips), large-capacity ceramic jars for storing wine in the early Neolithic sites of Shulaveri and Gadachrili Gora in 6,000 cal. BC shows high level of agricultural civilization in Georgia (the south Caucasus).

While not more than two species of domesticated wheat (*T. monococcum* and/or *T. dicoccum*) were identified in the majority of the Anatolian and Mesopotamian Neolithic sites contemporary with Arukhlo [28, 33]. Archeological findings from the Shulaveri – Shomu Culture suggest that a very high diversity of domesticated wheat was grown in Georgia in the early Neolithic period. Nine species of wheat including one wild (*T. baeoticum*) and eight domesticated (*T. monococcum, T. diccoccum, T. carthlicum, T. durum, T. spelta, T. spaerococcum, T. compactum,* and *T. aestivum*) were identified in archaeological excavations of Arukhlo (the end of the 7th to the beginning of the 6th millennium BC). It is the earliest appearance of the naked tetraploid (AABB) wheat (*T. carthlicum* & *T. durum*) is in the oldest layers in Arukhlo [7].

Out of 9 species of Arukhlo and Khramis Didi Gora 4 species: *T. spelta, T. spaerococcum T. compactum* and *T. aestivum,* are Hexaploids (AABBDD), three of them are free-trashing [7, 8]. As many as seven species of domesticated wheat dating back to 8,000 BP were identified in archaeological excavations in Shulaveri, Khramis Didi Gora of Lower Kartli (south eastern Georgia): *T. monococcum, T. dicoccum, T. durum, T. spelta, T. aestivum, T. compactum, T. spaerococcum* alongside with other 'founder' crops: barley (*Hordeum vulgare* and *H. distichum*), oat (*Avena sativa*), rye (*Secale cereale*), lentils (*Lens esculenta*), peas (*Pisum sativum*) and bitter vetch (*Vicia ervilia*) [7, 34, 8]. Wheat grains from Arukhlo, Khramis Didi Gora and Gadachrili Gora providing the earliest archaeological evidence of existence of hulled hexaploid *T. spelta*, as well as free-thrashing hexaploids *T. aestivum, T. compactum* and *T. spaerococcum*) in the Near East in 6,000 -5,800 cal. BC [7, 8, 35].

The South Caucasus was more diverse in terms of the wheat diversity than South Anatolia and Mesopotamia [36]. This became evident when the Shulaveri - Shomu complex was compared to its contemporary sites in northern Mesopotamia (the Halaf and Hassuna cultures) and Anatolia (Hacilar). The cereals cultivated in the South Caucasus are much more diverse than in Anatolia and Mesopotamia. The great variety of endemic wheat species in the Transcaucasus could have favored local domestication of cereals, even if these cereals were already cultivated in the Near East [36].

The origins of hulled and free-trashing hexaploid wheat in the South Caucasus

Allohexaploid common wheat (AABBDD genome) originated in the Middle East/ Transcaucasus region ca. 8,000 years ago and is derived from a natural hybrid cross between a cultivated form of *T. turgidum* (female parent) and the wild species *Ae. tauschii* (male parent) [6, 37, 38]

Archaeological work since the 1960s has confirmed van Zeist's dating of the earliest agricultural sites around the Caspian. Sites further to the south, in the Zagros mountains, such as Jarmo, Ali Kosh and Abdul Hosein have good evidence of farming (including emmer) by 6500--6400 uncal BC. In contrast, intensive surface survey around Haji Firuz Tepe and Yanik Tepe, both in Iranian Azerbaijan southwest of the Caspian Sea, failed to uncover any evidence of occupation earlier than 5500 uncal BC [27, 39].

Dvorak [5] noted that it is a widely believed hypothesis that *T. aestivum* originated by hybridization of domesticated tetraploid emmer (*T. turgidum* subsp. *dicoccon*) [38, 40, 41] with *Ae. tauschii* subsp.

strangulata. However, the belief that the tetraploid ancestor of *T. aestivum* was domesticated hulled emmer has been recently questioned [5] with a suge gestion that the tetraploid ancestor of bread wheat was free-threshing.

T. carthlicum, a free-threshing tetraploid Karthlian[2] wheat, endemic to Georgia (erroneously named as Persian wheat)[3] is considered as a subspecies of *T. turgidum* by modern *sensu lato* classifications: *Triticum turgidum* subsp. *carthlicum*, common name in Georgian - 'Dika'. This wheat has been cultivated for at least 8000 years in Georgia according to the data of the Neolithic archeological excavations [7]. 'Dika' is mentioned in the fifth cenn tury historical documents in Georgia [31].

Karthlian wheat's spike morphology resembles more the morphology of common wheat (*T. aestivum*) rather than that of other subspecies of free-threshing tetraploid wheat [42]. Moreover, it was shown that the morphology of synthetic hexaploid wheat derived from crosses between *T. turgidum* subsp. *carthlicum* and *Aegilops tauschii* resembles that of common wheat and considered subsp. *carthlicum* as a candidate for the AB genome donor of common wheat [42, 43]. With many beneficial traits and easiness of gene transferring to common wheat *T. carthlicum* has been suggested as one of the most desirable donors for bread wheat improvement [44].

There are two polyploid lineages in the genus *Triticum* [45]:

a) *T. timopheevii* - *T. zhukovskyi* lineage with AAGGAA - genome and

b) *T. turgidum* – *T. aestivum* lineage with AABBDD - genome.

Both polyploid lineages of the genus T*riticum* found only in Georgia:

1. *T. timopheevii* - *T. zhukovskyi* lineage (with domesticated AAGG tetraploid, Georgian endemic - *T. timopheevii* and AAGGAA hexaploid Georgian endemic -*T. zhukovskyi*) originate and grow only in Georgia [46].

2. *T. turgidum* – *T. aestivum* lineage with AABBDD genome represented with two sub linages:

2a –AABBDD genome hulled hexaploid represented with

2a.1. AABBDD genome hulled hexaploid endemic "Makha" linage of diploid DD (*Ae. tauschii* subsp. *strangulata*), hulled tetraploid AABB endemic (*T. palaeocolchicum)* and hulled hexaploid AABBDD endemic *T. macha* represented only in Georgia.

2a.2. AABBDD genome hulled hexaploid 'Spelta' linage of diploid DD (*Ae. tauschii* subsp. *strangulata*), hulled tetraploid AABB *T. dicoccum* and hulled AABBDD *T. spelta*

2b - AABBDD genome naked (free-threshing) hexaploid

The presence of free-threshing polyploidy lineage of diploid DD (*Ae. tauschii* subsp. *strangulata*), naked tetraploid AABB (*T. carthlicum*), naked hexaploid AABBDD (*T. aestivum/T. compactum*) in recent Georgia and in archaeological excavations of the 8000 BP in Arukhlo, Shulaveri, Khramis Didi Gora, in the same time that common wheat originated ca. 8000 years ago.

Archaeological excavations of the Neolithic sites of Georgia dated back to 8,000 BP have confirmed that both hulled and naked (free-trashing) AABB genome domesticated tetraploid wheat (*T. dicoccum, T. palaeocolchicum, T. carthlicum, T. durum*) already existed in Georgia ca. 8,000 BP and subsp. *strangulata* could have crossed with them. This is confirmed with existence of hulled hexaploid (*T. macha, T. spelta*) and naked hexaploid wheat (*T. aestivum*, *T. compactum, T. sphaerococcum*) samples in the Neolithic archaeological sites of west Georgia (Dikha Gudzuba, Anaseuli) and east Georgia (Arukhlo, Khramis Didi Gora, Gadachrili Gora) 8,000 years ago [7, 35].

2 Karthli – a province in East Georgia

3 Kartlian wheat *T. carthlicum* Nevski (common name in Georgian 'Dika' erroneously named by N. Vavilov in 1919 as *T. persicum* Vavilov [nomen provisorium]. In 1921 Zhukovskyi found it in Georgia and described as *T. persicum* Vavilov ex Zhuk. 1923. This name [*T. persicum*] is a later homonym of *Triticum persicum* (Boiss.) Aitch. & Hemsl. (*Aegilops persica* Boiss., 1846) and therefore is illegitimate (ICN Art. 53.1). Seed of this wheat was sent to Vavilov by a German private seed company under the name of Persian wheat ("Persischer Weizen"). However, the German company itself had received the seeds from Moscow, not from Iran (that time Georgia was part of the Russian Empire). According to the "International Code of Nomenclature for algae, fungi, and plants" the earliest legitimate name of this species is *Triticum carthlicum* Nevski (ICN Art. 11.4). This endemic species was widely cultivated in Georgia and is presented by 12 varieties in Georgia [47].

The great variety of endemic wheat species in the Transcaucasus could have favored local domestication of cereals, even if these cereals were already cultivated in the Near East [36].

Zhukovsky [48] mentioned that: "among 19 species of wheat in the world, 13 species represented in the Transcaucasus and the Transcaucasus (especially Georgia) is the birthplace of wheat". According to Dorofeev [49] "To the Transcaucasus - the primary center of origin of wheat belongs a leading historical role in it's domestication and dispersal. By the number of endemic species and varieties of the genus *Triticum*, this territory has no equal in the world".

As a conclusions Georgia is one of the most important centers for the diversity of cultivated wheat: 15 species of *Triticum*, out of 20 species in the world; 5 endemic wheat species (more than in any other country), all 7 cultivated hulled wheat species, as well as *Aegilops tauschii* with all 3 linages and all ancestral sub-linages represented only in Georgia. 9 species of wheat, among them 4 species AABBDD hexaploids (*T. spelta, T. aestivum, T. compactum, T. sphaerococcum*), together with all founder crops, discovered in the early Neolithic sites of Lower Kartli region (southeastern Georgia), reveals an importance of this region in wheat evolution and the origin of hexaploid wheat.

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Agro-morphological and biochemical characterization of Georgian common wheat (*T. aestivum*) – „Dolis puri" sub-varieties

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ABSTRACT

Wheat is one of the most important cereal grains in the world, which requires continuous yield and quality improvement. As one of the centers of wheat domestication, Georgia is distinguished by its diversity of local common wheat (*Triticum aestivum*) varieties. Overall, there are 40 varieties of bread wheat described in Georgia and among them, „Dolis puri" sub-varieties are one of the oldest and widely distributed throughout the different regions and altitudes of Georgia. With the accelerated genetic erosion and loss of agricultural biodiversity worldwide, conservation and characterization of genetic diversity in regional breeding pools are very urgent and important steps for their preservation. In this study, we characterized and evaluated Georgian common wheat (*T. aestivum*) – „Dolis puri" local varieties kept in different genebanks to assess their remaining genetic diversity. Complete agro-morphological and biochemical characterization of each accession were performed during 2 years of regeneration, in field and laboratory conditions. Overall, 28 traits were measured in 115 common wheat accessions, and 69 of them were taxonomically identified as 9 local sub-varieties of Dolis puri. Selected accessions were studied on seed quality (biochemical) traits and classified in core-collections according to their traditional distribution regions. Among all studied sub-varieties, white Dolis Puri sub-varieties were revealed to have better spike morphology characteristics and overall higher grain yield. The Tsiteli (red) Dolis puri sub-varieties were distinguished with higher gluten content and better grain quality traits, compared to studied white Dolis Puri sub-varieties. According to characterization data, we revealed the real value of conserved samples and increased possibilities to improve their use in research, breeding and educational programs.

Keywords: Common wheat, Dolis puri, *Triticum aestivum*, Agro-morphological, Biochemical characterization, Educational programs.

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Introduction

Wheat is one of the widely cultivated crops worldwide and the second most-produced cereal after maize [1-3]. Due to seeds composition and unique properties of gluten proteins, wheat holds a special place in the food supply of the world's population and demand for yield improvement is one of the main goals of scientists globally [4-5].

More than 90% of the worldwide produced wheat is cultivated common wheat (*Triticum aestivum* L.), also known as bread wheat [6-11]. Common wheat is an allohexaploid, combining the genomes of three ancestral diploid grass species [12], and this is undoubtedly contributing to its adaptability to different regions and climatic conditions of the world [13].

Georgia is distinguished by its great diversity of cultural plants and wild flora. These are due to a wide range of soil-climatic conditions - overall, there are described four climatic zones and 17 major soil types on the territory of Georgia [14].

Georgia is known as a hotspot for wheat diversity and endemism according to N. Vavilov, V. Menabde and their followers [15-18]. Wheat is one of the ancient and characteristic crops of Georgia. According to the findings from the various archeological sites of Georgia (Arukhlo, Khramis Gora, Shulaveri, Chikhori, Kheltubani), the cultivation of wheat in Georgia began in the VI-V century BC and continued through the whole history of the country [19-21]. So far, 27 cultivated and wild species of wheat have been described around the world out of which 14 species are found in Georgia [17, 22].

Among other domesticated wheat species present in Georgia, the great diversity of local common wheat (*T. aestivum*) is noteworthy. Many of these varieties are endemic, and a diversity of genetic variations was established during the centuries in very different natural-historical conditions. Overall, there are 40 varieties of bread wheat described in Georgia and among them, the most widespread ones are - var. *aestivum*, var. *ferrugineum*, var. *lutescens*, var. *milturum* [17, 20, 21]. All these forms are diverse according to their ecological, biological and morphological properties. Among them are varieties of comparatively dry and humid regions of East Georgia, humid regions of Western Georgia and mountainous zone of South Georgia.

Dolis puri is one of the oldest common wheat varieties in Georgia. The term "Doli" was mentioned in written documents dated back to the VIII-IX century, but I. Djavakhishvili [23] was indicating on its cultivation since much earlier times. Dolis puri used to be the principal wheat variety in Georgia during the centuries due to its high milling value, good backing property and tasty aromatic bread produced from it. Dolis puri was widely distributed throughout the different regions and altitudes of Georgia – in the high mountainous regions - Svaneti and Racha, it could grow at elevations as high as 2,000 m [24].

Like other countries worldwide, Georgia experiencing accelerated genetic erosion of agricultural biodiversity since the second half of the 20th century. It is expected soon, that this process will take an even broader scale, which will lead to large losses of local plant genetic resources *in-situ*. Modern improved higher-yielding varieties have largely replaced and have almost entirely driven away local traditional varieties from Georgian farming. Currently, problems facing plant breeders are the reduction in the diversity of the genetic material available for breeding purposes.

Nowadays *ex-situ* conservation is one of the main methods applied to preserve plant genetic resources. This method has some advantages comparing to other methods of conservation such as *in situ* or *on-farm*. Seed samples that are stored in genebank storage facilities maintain their genetic integrity. If the seed sample contains a sufficient amount of seed, it can fully represent the genetic structure of both self- and cross-pollinated plants. Seed samples are stored in appropriate conditions, they are regenerated and the chance of their loss is very low. It is easy to characterize and evaluate *ex-situ* collections, as all accessions are well-documented and it is easy to access them for research and breeding purposes [25].

Undoubtedly, conservation and characterization of genetic diversity in regional breeding pools are very urgent and important steps for their preservation.

Thus, the main goals of our study were to: (1) assess the genetic diversity of local common wheat - Dolis puri germplasm; (2) perform complete agro-morphological and biochemical characterization of each accession (3) evaluate agronomic properties and diseases and pests resistance in the field conditions; (4) identify the taxonomic classification of each accession (5) and assemble the core collection covering the complete diversity of Dolis puri sub-species ever recorded and described in Georgia [20, 21].

Materials and Methods

Plant material

The wheat accessions from the Bank of Plant Genetic Resources (Genebank) of the Agricultural University of Georgia and wheat local germplasm received from foreign genebanks, such as Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), USDA Agricultural Research Service (ARS), International Maize and Wheat Improvement Center (CIMMYT) were used for this study. This is because, that since there was not a genebank until 2004 in Georgia, a great diversity of local wheat germplasm collected on the territory of Georgia during the last century, currently is stored and protected in different international genebanks. In total, 115 common wheat accessions were regenerated and characterized during this study.

Experimental Section

Experiments were carried out at the Genebank regeneration field (Mukhrani field station of the

Agricultural University of Georgia) (N41°57′42″, E44°34′31″, altitude 549 m). The soil texture of the experimental area has an argillaceous-sandy, slightly alkaline reaction and unsalted.

Agro-morphological characterization

Local Genebank accessions and wheat samples received from the foreign genebanks were regenerated and multiplied in two-years replicated agricultural experiments. In autumn of 2017 and 2018, 20 g of each accession were sown on a 1.5-2 m^2 plot for field characterization. During the regeneration and multiplication process characterization and evaluation of each accession were performed based on traits that are indicated in the international descriptors of the crop [26].

Plant characterization data - growth class (seasonality), growth habit, pre-harvest sprouting tendency, lodging susceptibility, sowing date, flowering time and harvest date were recorded during all plant development stages – from the germination to plant maturity.

Plant height was measured by the main shoot length without a spike. The number of tillers, tillering capacity and the number of fertile tillers per plant were counted manually.

Ten mature plants from each plot were harvested manually for further characterization and phenotypic measurements in the laboratory.

In addition to plant phenotypic measurements in the field, we examined spike and grain morphology traits at the physiological maturity stage. For each accession spike data - density, awnedness, glume color, glume hairiness, number of seeds per spike, grain average number per spikelet - were recorded, also seed characteristics – seed color, size, vitreousness, and degree of shriveling - were measured. The results of measurements for each accession were averaged and means and standard deviations were calculated using the Excel program.

Measurement of grain yield components and quality traits

Usually, in small-grain cereals, spike morphology and spikelets number are crucial in determining grain yield [27-30]. Besides the tiller formation capacity and morphology of spike, grain number and grain weight are also important factors affecting grain yield [31-34].

Ten spikes from each accession were randomly selected for quantitative trait measurements. Spike length (without awn) in (cm), fertile spikelet number per spike, grain number per spike and thousand kernel weight (TKW) (g) were measured. Besides, spikelet density was determined (spikelet number per centimeter of spike length) as a ratio of spikelet number per spike to spike length [28]. Spikelet fertility (%) was identified as a ratio of fertile spikelet number per spike to total spikelet number per spike. Grain yield was calculated according to grain weight per spike and was adjusted to moisture content.

Overall, 28 traits were measured in 115 studied accessions and each was described according to their bio-morphological and agronomic traits and evaluated on grain yield.

In the selected taxonomically identified samples seed quality traits were studied. Grain's main biochemical characteristics - the content of protein, gluten, starch, fiber was measured.

Measurement of the protein, gluten, starch, and fiber content

The total protein content was measured on the Perten Inframatic 8800 NIR grain analyzer, equipped with a diode-array detector, on the wavelength range 850-1050 nm, calibrated to measure wheat parameters ten times per set. In each seed sample content of protein, wet gluten and moisture were measured in triplicate experiments and results were averaged.

The total starch content was determined via the polarimetric method specified for the determination of the starch content of native starch [35]. For the measurements, wheat grains were grounded, sifted to specific particle size and obtained flour was used for biochemical measurements. A 100 mg fraction of each sample was used to determine starch contents. Polarimeter POLAX-2L was used with an Interference filter at 589 nm and minimal indication unit: Angle of rotation 0.05°.

The crude fiber was measured following GOST 31675 [36]. This method applies to the determination of the crude fiber value in cereals and cereal products. The main principle is as follows - after boiling the sample flour with a mixture of acetic, nitric and trichloro-acetic acids, the undissolved residue is separated and ignited. The crude fiber value is calculated from the ignition loss.

Evaluation of resistance to diseases and pests

During the regeneration and field characterization, samples were evaluated on diseases and pest

susceptibility or resistance. Mainly fungal diseases and common pests for the region were evaluated according to CIMMYT's handbook on field identification of wheat diseases and pests [37]. A list of identified diseases and pests were recorded from each plot for every sample with a simultaneous reading of severity and reaction together with percent of disease spread severity according to plants Scoring Guide (IPO and CIMMYT) and scale for appraising the intensity of foliar diseases in wheat [38].

Results and Discussion

Characterization and evaluation of genebank accession is the most important procedure, which provides better access to samples to improve their use in research and breeding. Characterization implies the description of biological-morphological and agronomic traits with simple inheritance, for which international crop descriptors can be used, while the evaluation is mostly concentrated on agronomically important traits that have quantitative inheritance such as yield and quality. Characterization and evaluation of the samples enabled us to identify samples with unique traits and assemble them in core-collections to facilitate their further use.

Based on the performed complete agro-morphological characterization data, overall 69 accessions were taxonomically identified and classified in 9 local Dolis puri sub-varieties (Fig. 1). A set of bio-morphological characteristics, available etalons and photo material from the books were used to distinguish specific sub-verities of Dolis puri amongst the whole population of 115 samples [18, 19]. The identified samples were grouped according to their traditional distribution regions in Georgia - as sub-varieties of comparatively dry regions of East Georgia, the humid regions of East Georgia, the humid regions of West Georgia and the mountain zone of Southern Georgia (Table 1) (Fig. 1, Fig. 2).

Besides, plant characterization data, recorded during the regeneration, were gathered and averaged for each sub-species and compared to the literature data for each sub-varieties to ensure correct taxonomic identification of each accession (Table 2). From all studied samples plant growth class (seasonality) was mainly winter, except Tetri (white) Dolis puri, which is spring type. All samples had an upright growth habit of a young plant, naked grains, and a low pre-harvest sprouting tendency. The planting date of all samples was the same, but according to the

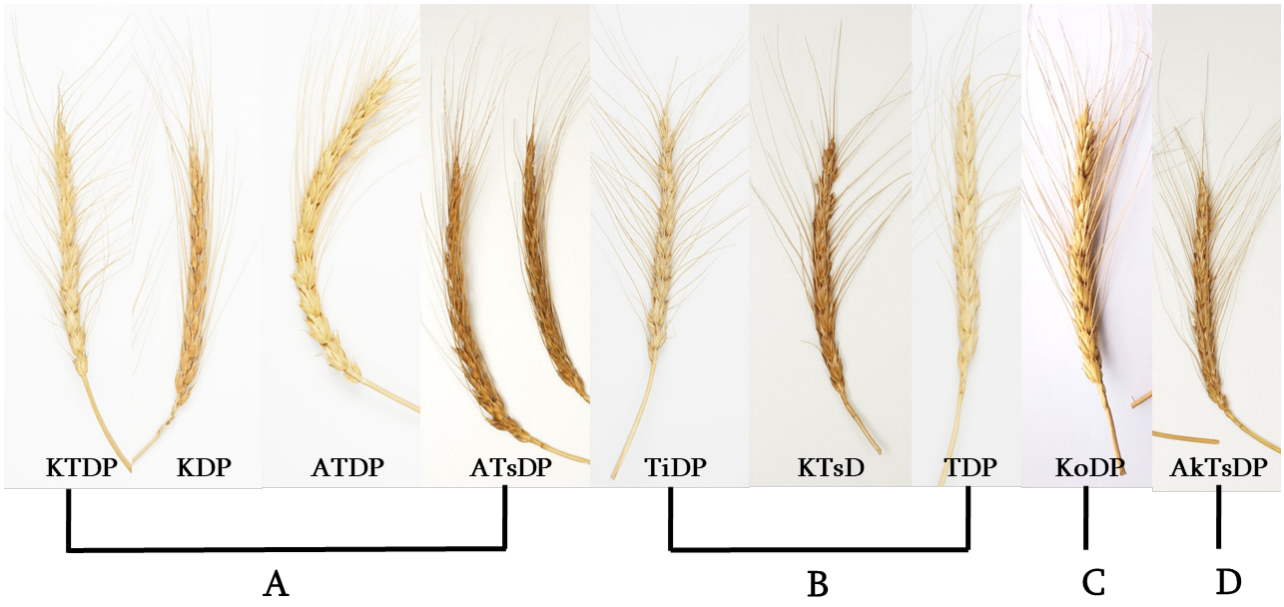

Fig. 1. *Taxonomically identified Dolis puri local varieties according to their morpho-physiological characterization data: A – sub-varieties of comparatively dry regions of East Georgia, B - sub-varieties of the humid regions of East Georgia, C - sub-varieties of the humid regions of Western Georgia, D - sub-varieties of a mountain zone of Southern Georgia. KTDP - Kartlis Tetri (White) Dolis puri, KDP - Kakhuri Dolis puri, ATDP - Adgilobrivi (local) Tetri (white) Dolis puri, ATsDP – Adgilobrivi (local) Tsiteli (red) Dolis puri, TiDP - Tianeturi Dolis puri, KTsD - Kartlis Tsiteli (red) Dolis puri, TDP - Tetri (white) Dolis puri, KoDP - Korboulis Dolis puri, AkTsDP - Akhaltsikhis tsiteli (red) Dolis puri.*

Table 1. *The list of the identified Dolis puri local varieties according to the morpho-physiological characterization data. The Dolis puri local names: Kartlis, Kakhuri, Tianeturi, Akhaltsikhis, Korboulis means respective regions - Kartli, Kakheti and Tianeti, district Akhaltsikhe and also village Korbouli in Georgia. In Georgian „Tetri" means white, „Tsiteli" - red and „Adgilobrivi" - local.*

	Local names	Accession Number #	Scientific name	Abbreviation
1. Sub-varieties of comparatively dry regions of East Georgia				
1	Kartlis Tetri (White) Dolis puri	GEO3062	*Triticum aestivum* var. *aestivum* (var. *erythrospermum* Korn)	KTDP
2	Kakhuri Dolis puri	GEO3066, GEO3067, GEO3068, GEO3069, GEO3070, GEO3071, GEO3072, GEO3073	*Triticum aestivum* var. *aestivum* (var. *erythrospermum* Korn)	KDP
3	Adgilobrivi Tetri (white) Dolis puri	GEO0055, GEO3079, GEO3081, GEO3082, GEO3083, GEO3084, GEO3085, GEO3086, GEO3087, GEO3088, GEO3089, GEO3090, GEO3091	*Triticum aestivum* var. *aestivum* (var. *erythrospermum* Korn)	ATDP
4	Adgilobrivi Tsiteli (red) Dolis puri	GEO0156, GEO3107, GEO3108, GEO3109, GEO3110, GEO3111	*Triticum aestivum* L. var. *ferrugineum* (Alef.) Mansf.	ATsDP
2. Sub-varieties of the humid regions of East Georgia				
5	Tianeturi Dolis puri	GEO3063, GEO3064, GEO3065	*Triticum aestivum* var. *aestivum* (var. *erythrospermum* Korn)	TiDP
6	Kartlis Tsiteli (red) Dolis puri	GEO0053, GEO0054, GEO3095, GEO3096, GEO3097, GEO3098, GEO3099	*Triticum aestivum* L. var. *ferrugineum* (Alef.) Mansf.	KTsD
7	Tetri (white) Dolis puri	GEO3046, GEO3047, GEO3048, GEO3049, GEO3050, GEO3051, GEO3052, GEO3053, GEO3054, GEO3055, GEO3056, GEO3057, GEO3058, GEO3059, GEO3060, GEO3061	*Triticum aestivum* var. *aestivum* (var. *erythrospermum* Korn)	TDP
3. Sub-varieties of the humid regions of Western Georgia				
8	Korboulis Dolis puri	GEO0056, GEO0057, GEO0058, GEO3092	*Triticum aestivum* var. *aestivum* (var. *erythrospermum* Korn)	KoDP
Sub-varieties of a mountain zone of Southern Georgia				
9	Akhaltsikhis Tsiteli (red) Dolis puri (Meskhuri)	GEO0050, GEO0051, GEO0052, GEO0492, GEO3100, GEO3101, GEO3102, GEO3103, GEO3104, GEO3105, GEO3106	*Triticum aestivum* L. var. *ferrugineum* (Alef.) Mansf.	AkTsDP

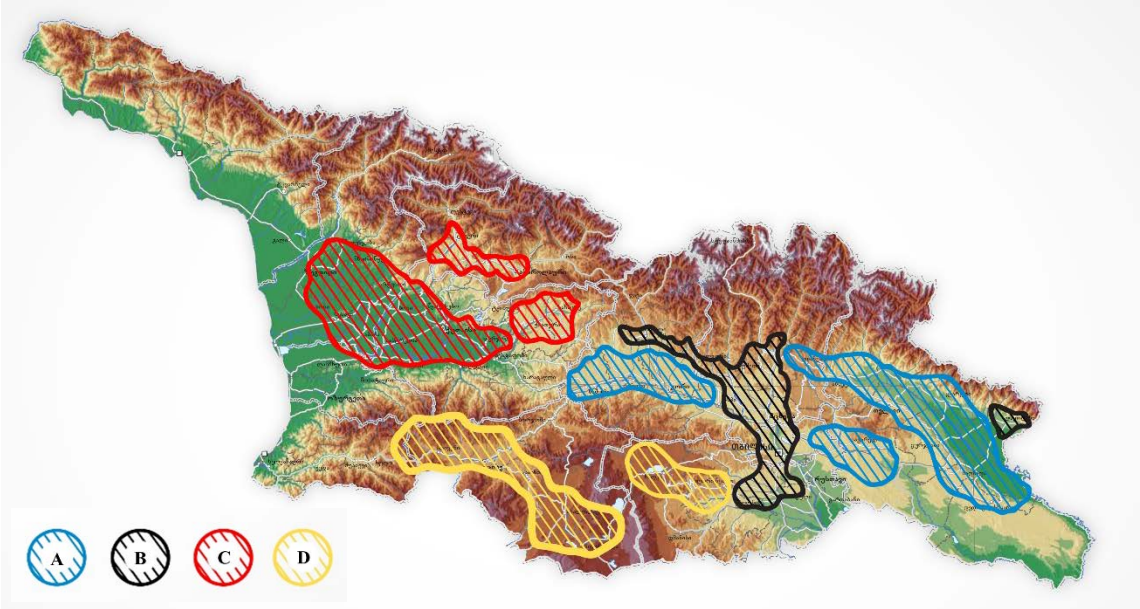

Fig. 2. *Main regions of Georgia with the spread of local Dolis Puri sub-varieties: A – Comparatively dry regions of East Georgia (Kakheti, Kartli and Mtskheta-Mtianeti regions); B - Relatively humid regions of East Georgia (Shida Kartli region); C - The humid regions of Western Georgia (Imereti, Racha-Lechkhumi, Samegrelo and Svaneti Regions); D - Mountain zone of Southern Georgia (Samtskhe-Javakheti region). Modified from the Atlas of natural hazards and risks of Georgia, Tbilisi, Georgia, 2018. Map source: ASTER GDEM is a product of METI and NASA; CENN/IT (p.11); Climate data was used from the Climate Atlas (p.17), Source: Atlas of Georgia, 1964; Data on the distribution of Dolis puri's sub-varieties are presented from Naskidashvili et. al. 1983; Samadashvili et. al. 2019 [18, 19, 39].*

Table 2. *Field characterization data of studied Dolis puri accessions: growth class (seasonality), the growth habit of young plant, pre-harvest sprouting tendency, sowing date, days to flower, harvest date. The abbreviation is explained in table 1.*

#	Variety	Growth class	Growth habit of young plant	Pre-harvest sprouting tendency	Sowing date	Days to flower	Harvest date	Grain hull	Lodging susceptibility
1	KTDP	Winter	Erect	Low	28.10.18	212.5	05.08.19	Naked	Yes
2	KDP	Winter	Erect	Low	28.10.18	214	30.07.19	Naked	Yes
3	ATDP	Winter	Erect	Low	28.10.18	216	25.07.19	Naked	Yes
4	ATsDP	Winter	Erect	Low	28.10.18	214.5	30.07.19	Naked	No
5	TiDP	Winter	Erect	Low	28.10.18	219.5	30.07.19	Naked	Yes
6	KTsD	Winter	Erect	Low	28.10.18	222	30.07.19	Naked	Yes
7	TDP	Spring	Erect	Low	28.10.18	221.5	30.07.19	Naked	Yes
8	KoDP	Winter	Erect	Low	28.10.18	217	30.07.19	Naked	No
9	AkTsDP	Winter	Erect	Low	28.10.18	220	30.07.19	Naked	Yes

Table 3. *Plant, spike and grain characterization data of studied Dolis puri accessions: plant height (cm), tillering capacity (number of fertile tillers), spike density, awnedness, glume color, glume hairiness, number of spikelets per spike, number of seeds per spike, grain number per spikelet. The abbreviation is explained in table 1.*

Variety	KTDP	KDP	ATDP	ATsDP	TiDP	KTsD	TDP	KoDP	AkTsDP
#	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Plant height (cm)	86.6±14.6	105.7±9.6	98.9±9.8	106.2±10.8	103.3±8.7	108.3±5.3	105.8±8.5	107.0±10.6	105.4±13.7
Tillering capacity	4.70±1.42	7.71±2.35	7.98±1.99	8.32±2.23	9.25±1.57	6.00±2.04	8.35±2.09	7.40±3.37	6.88±2.35
Spike density	Interm.	Interm.	Dense	Interm.	Interm.	Dense	Interm.	Interm.	Dense
Awnedness	Awned	Awned	Awned	Awnletted	Awned	Awned	Awned	Awned	Awnletted
Glume color	White	Red-brown	White	Red-brown	White	Red-brown	White	White	Red-brown
Glume hairiness	Absent	Absent	Absent	Low	Absent	Absent	Absent	Absent	Low
Grain number per spikelet	2-3	2-3	2-3	2-3	2-3	2-3	2-3	2-3	2-3
Seed colour	Red	Red	Red	Purple	Red	Purple	Red	Purple	Red
Seed size	Interm.	Interm.	Interm.	Interm.	Interm.	Interm.	Interm.	Interm.	Interm.
Seed vitreousness	Partly vitreous	Not vitreous	Not vitreous	Vitreous	Not vitreous	Not vitreous	Not vitreous	Not vitreous	Not vitreous
Degree of seed shriveling	Plump	Plump	Plump	Plump	Plump	Plump	Plump	Plump	Plump

harvest date, Kartlis Tetri (White) Dolis puri needed more time until maturity. Most of the samples were susceptible to lodging, except for Adgilobrivi Tsiteli (red) Dolis puri and Korboulis Dolis puri.

Characterization of the Dolis Puri sub-varieties revealed quite differences between studied varieties even within the distribution region. Sub-varieties of East Georgia, both – comparatively dry and humid regions were characterized by higher tillering capacity. Adgilobrivi Tetri (white) Dolis puri, Adgilobrivi Tsiteli (red) Dolis puri, Tianeturi Dolis puri, Kakhuri Dolis puri and Tetri (white) Dolis puri have a high number of fertile tillers (7-9) comparing to other studied varieties. Among them, the highest tillering number was recorded in the case of Tianeturi Dolis puri. The Kartlis Tetri (White) Dolis puri was revealed to have the lowest tillering capacity among all studied varieties (Table 3).

As a result of our study, Korboulis Dolis puri was revealed to have the highest spike length, grain number per spike, grain weight per spike, and spikelet number per spike compared to other studied varieties. All these spike components undoubtedly contributed to the final grain yield per spike and resulted in the highest grain weight per spike and in overall, grain yield observed in the case of Korboulis Dolis puri (Table 4). Adgilobrivi Tetri (white) Dolis puri revealed to have also profound spike and grain characteristics and consequently high grain yield.

Table 4. *Grain yield components of studied Dolis puri accessions: spike length, spikelet number per spike, grain number per spike, thousand kernel weight (TKW), spikelet fertility, spikelet density, grain weight per spike, grain yield. The abbreviation is explained in table 1.*

#	Variety	Spike length (cm)	Spikelet number per spike	Grain number per spike	Thousand kernel weight (g)	Spikelet fertility (%)	Spikelet density	Grain weight per spike (g)	Grain yield (g)
1	KTDP	11.08±0.17	21.33±1.15	50.00±9.00	23.55±0.73	87.18±5.32	1.94±0.10	1.17±0.18	1.05±0.18
2	KDP	12.50±0.50	22.00±2.00	40.33±2.52	37.90±2.71	86.47±7.55	1.76±0.09	1.53±0.14	1.37±0.14
3	ATDP	12.65±0.35	20.67±0.58	58.50±9.19	39.93±1.45	85.17±8.05	1.68±0.10	2.33±0.28	2.08±0.28
4	ATsDP	11.55±1.02	18.40±1.58	35.20±7.45	31.04±2.76	-	1.60±0.08	1.09±0.26	0.98±0.26
5	TiDP	10.67±0.58	20.00±2.00	55.67±1.53	28.17±0.94	86.23±8.51	1.87±0.11	1.57±0.06	1.41±0.06
6	KTsD	11.26±2.86	18.94±1.80	26.36±5.38	33.41±7.13	-	1.74±0.29	0.89±0.21	0.80±0.21
7	TDP	12.53±0.15	22.33±2.08	35.00±2.83	39.72±0.47	83.63±6.60	1.76±0.23	1.39±0.13	1.25±0.13
8	KoDP	14.50±0.71	22.33±0.58	68.50±0.71	41.58±1.41	89.79±3.53	1.52±0.07	2.89±0.07	2.59±0.07
9	AkTsDP	10.83±1.20	18.03±1.64	32.08±8.43	26.53±8.23	88.12±5.44	1.69±0.13	0.86±0.32	0.77±0.32

Table 5. *Biochemical characteristics: protein, gluten, starch and crude fiber content in seeds of selected samples. The abbreviation is explained in table 1.*

Variety	KTDP	KDP	ATDP	ATsDP	TiDP	KTsD	TDP	KoDP	AkTsDP
#	1	2	3	4	5	6	7	8	9
Protein, DW* (%)	16.13±0.21	18.03±0.21	16.47±0.15	19.77±0.21	18.97±0.12	19.53±0.25	17.90±0.10	19.13±0.23	19.60±0.10
Gluten, FW** (%)	34.73±0.35	38.20±0.53	33.70±0.35	42.10±0.30	40.23±0.42	41.83±0.42	37.67±0.42	40.83±0.67	42.40±0.46
Starch, DW (%)	66.73±0.39	64.39±0.19	67.34±0.08	65.82±1.16	64.39±0.78	65.74±0.06	60.78±0.39	66.08±0.39	65.18±0.59
Crude Fiber, DW (%)	4.26	4.44	3.95	2.97	4.86	3.43	5.01	3.90	4.02
Moisture (%)	10.60±0.00	10.53±0.06	10.60±0.00	10.20±0.00	10.40±0.00	10.37±0.06	10.40±0.00	10.30±0.00	10.37±0.06

DW* - Dry weight
FW ** - Fresh weight

Among Dolis puri sub-varieties, Tsiteli Doli (Triticum aestivum var. ferrugineum), or "red wheat," is a landrace of winter bearded (or awned) soft wheat. There are several local varieties of Tsiteli Doli, one of which is from Akhaltsikhe, in the southern Georgian region of Samtskhe-Javakheti (Fig. 2). They are well known for their grain quality traits and the high quality of bread produced from them. According to our study, spike characteristics of the Tsiteli Dolis Puri sub-varieties are less profound, but taking into account their tillers formation capacity and grain quality traits, they are valuable varieties for Georgian agriculture (Table 4, Fig. 1).

Grain quality traits

Selected Dolis puri varieties' seed biochemical composition is given in Table 5. Tsiteli (red) Dolis puri sub-varieties turned out to have an incredibly high amount of protein content – more than 19 % per seed dry weight (Table 5, Fig. 1). From Tetri (white) Dolis puri sub-varieties - Korboulis, Tianeturi, and Kakhuri Dolis puri revealed to have high protein content, 19.13 %, 18.97 %, and 18.03 %, respectively. Consequently, the Tsiteli (red) Dolis puri sub-varieties were distinguished with higher gluten content compared to white Dolis Puri sub-varieties (Table 5).

However, Tetri (white) Dolis Puri sub-varieties demonstrated higher starch content in the grain. Kartlis Tetri Dolis puri, Adgilobrivi Tetri Dolis puri, and Korboulis Dolis puri are distinguished with high starch content in the seed, — 66.82 %, 66.2%, and 66.08% respectively. The crude fiber content in studied varieties was ranging from 2.97 to 5.01.

Evaluation of diseases and pests

According to field evaluation data, most of the studied samples are expressing moderate resistance (5-10%) to the leaf rust (Table 6). For Fusarium head blight of wheat (FHB), also called head scab, which is caused by the fungus *Fusarium graminearum*, from studied Dolis Puri sub-varieties Korboulis Dolis puri was least resistant to this infection (10%). Dolis Puri sub-varieties of East Georgia revealed higher resistance to Fusarium head blight of wheat.

Aphids infestation was rarely observed, only in the case of Adgilobrivi Tetri Dolis Puri it was identified.

Conclusion

The field experiments and agro-morphological characterization of the studied accessions revealed differences between all identified Dolis puri local sub-varieties. Korboulis Dolis puri was revealed to have better spike morphology traits, compared to other studied varieties and as a result, the highest grain weight per spike and overall grain yield. Also, Adgilobrivi Tetri (white) Dolis Puri revealed to have profound spike and grain characteristics and consequently higher grain yield.

Among Dolis puri sub-varieties, Tsiteli (red) Dolis puri (*Triticum aestivum* var. *ferrugineum*) sub-varieties have less profound spike and grain characteristics, but good tillers formation capacity and excellent protein content. The Tsiteli (red) Dolis puri sub-varieties were also distinguished with higher gluten content and other grain quality traits, compared to studied white Dolis Puri sub-varieties,

Table 6. *Evaluation of diseases and pests: susceptibility to leaf rust, Fusarium head blight of wheat (FHB) (Fusarium graminearum) and Aphids (Diuraphis noxia). The abbreviation is explained in table 1. Disease reactions were recorded as - no visible infection (0), resistant (R), moderately resistant (MR); reading of severity and reaction was recorded together with percent of the severity of the infection.*

	Variety	Susceptibility to leaf rust	Susceptibility to Fusarium head blight	Aphids infestation
1	KTDP	5 MR	0	-
2	KDP	5 MR	1 R	-
3	ATDP	10 MR	5 MR	yes
4	ATsDP	5 MR	1 R	-
5	TIDP	5 MR	0	-
6	KTsD	5 MR	0	-
7	TDP	10 MR	5 MR	-
8	KoDP	5 MR	10 MR	-
9	AkTsDP	5 MR	5 MR	-

as it was supposedly because they are traditionally known for their perfect bread baking characteristics.

According to bio-morphological characterization and evaluation data, we are assembling the core collection covering the complete diversity of Dolis puri varieties ever recorded and described in Georgia. As a result, we revealed the real value of conserved samples and increased possibilities to improve their use in research and breeding to facilitate variety improvement, as well as in phylogenetic studies and educational programs.

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Food consumption influenced by television advertisements among generation-Y young consumers living in Budapest

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A B S T R A C T

Considering Generation-Y, television has become part of their lives from early childhood. Television provides a possibility of recreation, with the aim to entertain or to provide everyday information. Consumers are surrounded by advertisements that appear on television. Advertisement professionals are aware that consumers do not watch television primarily to see advertisements, therefore they must create interesting, clever advertisements for companies that grab the viewers' attention. Several feelings appear in advertisements for foods: emphasis is on the liveliness of youth, on friendships, family ties and other idyllic emotional states. Young people would like to resemble to the persons in the advertisements for the given foods. In our study, we examin the attitudes of Generation-Y youths in Budapest towards television advertisements, and we try to establish how traditional TV advertisement spots can influence them beside other advertising communication tools.

Keywords: Influencing, Generation-Y, Food consumption, Television advertisements, Consumers, Buying food.

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1. Introduction

Consumers are surrounded by a constant flow of advertisements whether they like it or not, but the evaluation of advertisements varies. Consumers in various age groups have different attitudes towards these ads. Young persons are the main targets for various TV spots or advertisements present on the internet in social media, as these youths would like to be the way presented to them by these ads. When buying food products, they make decisions based on their positive and negative experiences. However, advertisements can have a distorting effect on young people as well.

Television as an advertising medium, along with BTL tools, still has a significant role – reaches a great number of people, cost effective, draws viewers' attention, has an effect on consumers' senses. Kárpáti and Lehota differentiates between 3 advertising goals: providing information, convincing and reminding. Messages of the ads can be moral, rational and emotional [1].

In our study, we were curious to know the attitudes of Generation-Y towards food advertisements traditionally appearing on TV besides the advertising techniques of various marketing tools.

The study involved a questionnaire that was used with Generation-Y youths in Budapest.

2. Food consumption of Generation-Y today

According to Tari young people who belong to Generation-Y were born between 1980 and 1995, and are often referred to as millenial or first digital generation. They have been exposed to the influence of advertisements from their childhood. They are the children of Generation-X, being part of a transition from the world of offline to online communication tools. Young people of this generation are characterized by flexibility and independence, but they also manifest a hunger for information and an urge to fulfill there needs immediately [2]. Young

consumers today are typically material, critical and they glorify products. Based on a study in Hungary there are significant differences in consumer attitudes in the country and in the capital [3]. Although the difference has existed before, now it is clearly divided regarding the way of thinking and the attitude towards new things, as well as regarding consumption. By today, product brands of companies not only provide a guarantee for quality, but they have become a symbol of status, and they appear as an entity linked to young consumers [4-7].

Researching the factors that determine food consumption in relevant literature, we can come across numerous findings [8, 9]. Food consumption megatrends include recreational values, comfort values, health values, ethical values. Part of the recreational value trend is mood food – food that makes you feel better, wellness food – food that supports well-being for body and soul, sensual food – food that stimulates various senses, slow food – traditional food, ethnic food – foods of other nations, and retro food – food that is nostalgic [10, 11]. In several countries of the world, like in East- or Southeast Asia, changing lifestyle, increasing purchasing power and international openness brought about not only growing demand but also significant changes in the food consumption habits, like demand for protein-rich foodstuffs (e.g. meat products) has significantly grown [32, 33]. Comfort trend incorporates foods that are easy to carry and to store, cheap foods, foods of higher and lower quality [12] . Health appears as a trend, made up of foods with beneficiary effects and functional foods, allergic-free and curative foods [13]. Along with those mentioned, foods representing environmental and social responsibility also appear [14, 15].

According to Lehota the most important motivational types of food consumption can be:

- Nutritional motivations: consumption of fat, carbo-hydrates, protein and vitamins based on gender, age, climate and nature of work
- Health motivations: evaluating the combination of nutrients based on their effect on one's health
- Recreational motivations: it tastes good, it is enjoyable, delicious
- Comfort motivations: storage life, length of preparation
- Safety motivations: evaluating risks and safety of foods
- Pleasing certain social groups: snob effect, acceptance of group behavior
- Motivations of prestige: expressing one's place in society by food consumption
- Natural and environmental motivations: waste handling and protection natural resources
- Political motives: emotions against producers and processers or against commercial groups [16]

3. The relationship between consumers and advertisements

Advertisement is a form of communication during which signal transfer occurs between the giver and the receiver. Looking at it from the consumer's side, advertisement in itself means an input of information, and the communication message can reach them through the media that carries any advertisement. The incoming advertisement message is decoded by individuals according to their own memories, earlier experiences and values. Advertisements affect the consumers' senses through diverse communicational channels with written words, pictures, sounds and combination of these. Based on advertising media, we can differentiate between:

- Visual advertisement (printed press)
- Auditive advertisement (radio)
- Audio-visual advertisement (TV, internet)

Reception of the advertising message may be distorted by several sources of noise, moreover, reception may be influenced by the consumers personal motive just as well [17]. Today, the content and the form of the advertisement is shaped by the vendor, the customer, and other persons in the environment all together. On top of all this, an advertisement is also a cultural phenomenon that infiltrates consumers' everyday life, is part of the news, of mass media and of everyday speech. Various advertising slogans become a part of common talk. The advertisement as a communicational message finds consumers in different situations. The message of the advertisement itself may have various reception with the consumers, depending on whether the consumer is open to the information or is just experiencing it as general noise. Consumers are exposed to a great number of impulses and stimuli, out of which selection is made by a characteristic mechanism. The information given out by the advertisement results in a change of behavior either directly, or by changing the attitude. This can be explained by the fact that the person receiving the ad consciously researches information regarding foods, which may form their attitude towards food, and may change their relationship to food. On the other hand, shaping

and changing the attitude may be possible without thoroughly deliberating the advantages and disadvantages of product characteristics. In this case, the key words of advertised foods triggering association will do the convincing, or factors such as music, visual effects and humor. These factors are tied to the affective structure and contribute to attaining attraction towards foods [18].

3.1. The influencing effect of advertisements

Tools of marketing communication can be basically divided into two groups: ATL (above the line), and BTL (below the line) tools. The ATL group is characterized by the use of classical tools that reach great masses of people and employ different mass media. They usually carry a message advertising a product or a service. On the other hand, BTL tools include everything that ATL does not. Characteristically, they do not address great masses and they hit a more personal tone. Today, among the ATL tools television is still able to reach a great spectrum of consumers [19].

Influencing consumers can be divided into six factors that aid and increase the power of convincing:

1. Reciprocity: advertisements ask for consumers' time and attention, and in return they offer an opportunity, feeling, happiness.
2. Commitment: consumers stick with their own choices, because they would appear to be inconsistent should it turn out that they made a mistake with their previous decision.
3. Social reinforcement: they have a strong desire to be accepted by society, and this is how they try to fulfill it.
4. Professional expertise, authority: they are obedient to different convincing expert opinions appearing in advertisement.
5. Attractions: products advertised by well-known people, celebrities, influencers will become attractive to consumers.
6. Scarcity: consumers' fear of missing out by not being able to purchase the given product [20].

The success of the advertisement lies in promising experiences and desires in a convincing way to potential consumers, so that they actually believe that by purchasing the advertised product, they can get these emotions along with it as well. This success requires showing patterns that reflect all of these emotions. Groups or persons are represented in food adverts with whom consumers can identify with and would like to become like them [21].

3.1.1. Characteristics of television advertisements

The advantage of TV as an advertising medium is that all consumer groups can be reached by it and it affects different senses at the same time such as the hearing and vision of the consumer. In TV ads, desired lifestyle patterns appear, with which consumers wish to identify [22]. The primary objective of advertisements is to influence consumers' attitudes towards food and thus to convince them that selecting the given brand of food was a suitable decision for them. Their blatant goal is to convince consumers to switch between brands and buy the food advertised by them. This effect of the advertisement must be achieved in a couple of minutes [23]. Convincing in TV ads is conscious, since consumers share the culture, so it is easier to convince them to purchase the foods Knowing the brands of advertised foods makes purchasing them more attractive, because the emotions seen in the ad in a purchasing situation induce different reactions in consumers [24]. Television advertising spots should always aim for grabbing the attention of viewers [25].

Inflicting emotions is the central strategy of televised advertisements. Makers of the ads offer different combinations of emotions to TV viewers, promising that, if they purchase their products, they will feel the same way as people in the ad [26]. Advertisements not only create needs, desires, but also a life philosophy among young people. However, many times advertisements show idealized pictures to youths. And while others promise happiness by purchasing the product, so they will buy these advertised foods so that they can catch up with them by food consumption [27]. Music appearing in TV spots, besides grabbing attention, also has an emotional effect on consumers [28].

Research by Olsson and Larsson sheds light on the importance of a sense of humor in advertisements. A sense of humor makes the TV ad worthy of attention for young consumers, and will urge them to stay in front of the TV to watch the ads. Thanks to a sense of humor, the advertisement imprints into the memory of young consumers, and thus has a positive effect regarding the advertised foods [29,30].

Today, the media have become a most important socializing tool that shapes young people's attitudes along with that of other social groups. The material attitude of Generation-Y is also influenced by TV spots. As research by Opree et al. proves, the more

advertisements young people are exposed to, the more material they become [31].

Old and new media exist together, and this must be taken into consideration by companies that plan to engage in advertising activities. Using BTL marketing tools, the greatest scene of advertisements will be social media and video sharing portals.

4. Objectives and methods

In our research, our primary objective was to examine the effect of food advertisements through the communication channel of television on Generation-Y youths in Budapest. We conducted primary data collection based on a non-representative sample, with the central element of asking Generation-Y youths in Budapest. The collected data were analysed and evaluated by statistical methods.

In our study, we conducted a survey research, using random sampling, with the city of Budapest as sampling point. Generation-Y young people were interviewed in Budapest using random sampling. During random sampling, the choice of individuals one after the other without repetition, n<N in number, in which case at each selection from the pool, selecting an already existing element has the same probability. Our questionnaire was filled out by 188 persons, so the sample size to be statistically analysed is 188.

During statistical data processing we did a cross table processing with the aid of SPSS program package. We have done the correlational study of dependent and independent variables.

We have put forward two hypotheses in our research. According to our first hypothesis, out of the communication channels affecting food consumption, TV will have a greater influence than other communicational advertising tool in the case of young people in Budapest whose highest education is elementary school. In our other hypothesis we suppose that the more income Generation-Y youths in Budapest have, proportionately the advertisements channelled through the television will have a lesser effect among all factors influencing food consumption.

5. Results and analysis

In order to prove the first hypothesis presented above, we have conducted a cross table examination in the SPSS system, which contains the highest education of young people interviewed, and also the advertising effect of different communicational channels on food consumption. In the table below we are examining the possible correlations between these two factors.

Based on the data represented in Table 1 we can state that there is a significant correlation between the examined data and questions, thus the results can be evaluated. (The level of significance is of a value lower than 0.001, which is lower than 0.005). Cramer's V value is 0.484, which shows a weak-medium strength correlation. The value of Gamma is 0.753, which indicates a medium-strength, one-direction correlation.

Table 1 shows that most young people in Budapest whose level of education does not surpass the 8th grade, 58.1% of them believes that TV ads have the most influence on them when shopping for food. 64.4% of those who finished elementary school,

Table 1. *Correlation between advertisements of communicational channels affecting food consumption and highest education*

Crosstab-analysis						
			Impact of advertising communication channels			
			TV	Internet, social sites	Acquaintanceship, friends, dietitian	Total
What is your highest level of education?	Less than elementary school education	Count	18	13	0	31
		Percent	58,1%	41,9%	0,0%	100,0%
	Elementary school	Count	16	29	0	45
		Percent	35,6%	64,4%	0,0%	100,0%
	Vocational worker, vocational school	Count	6	47	8	61
		Percent	9,8%	77,0%	13,1%	100,0%
	Graduation and College/University degree	Count	4	19	28	51
		Percent	7,8%	37,3%	54,9%	100,0%

Symmetric Measures

		Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Nominal by Nominal	Phi	,684			,000
	Cramer's V	,484			,000
Ordinal by Ordinal	Gamma	,753	,057	9,583	0,000
N of Valid Cases		188			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	87,966[a]	6	,000
Likelihood Ratio	89,497	6	,000
Linear-by-Linear Association	62,572	1	,000
N of Valid Cases	188		

a. 0 cells (0,0%) have expected count less than 5. The

Source: our own research (N=188), 2020

and 77% of graduates of vocational and technical schools believe that advertisements of the internet and of different social media have an effect on them in their food purchases. Out of those with at least a secondary education judged that testimonials of their acquaintances, friends and dieteticians have a great impact on them, and in 37.3% the advertisements of the internet and of different social media.

Based on the information gathered, our hypothesis can be said to have been proven right, as the data above reflect well that TV as an advertising tool influencing food consumption is most effective in the case of young people of Budapest with the highest education as elementary school.

In testing our other hypothesis, we also used a cross-table examination. During the cross-table analysis we examined whether there is a correlation, and if there is, what is the direction of it between the income of Budapest youths and the influential effect of TV spots. The data from the results of the cross-table analysis derived with the help of the SPSS program are shown in Table 2.

Table 2. *Correlations between monthly income and food advertisements seen on TV*

Crosstab-analysis						
			When you buy food, do the ads you see on TV affect you?			
			Yes	Neutral	No	Total
What category would you classify your monthly income?	Under 150 000 Ft	Count	7	10	7	24
		Percent	29,2%	41,7%	29,2%	100,0%
	Between 150 000 Ft and 200 000 Ft	Count	0	28	61	89
		Percent	0,0%	31,5%	68,5%	100,0%
	Between 200 000 Ft and 250 000 Ft	Count	4	12	37	53
		Percent	7,5%	22,6%	69,8%	100,0%
	Over 250 000 Ft	Count	0	4	18	22
		Percent	0,0%	18,2%	81,8%	100,0%

Symmetric Measures

		Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Nominal by Nominal	Phi	,451			,000
	Cramer's	,319			,000
Ordinal by Ordinal	Gamma	,379	,111	3,209	,001
N of Valid Cases		188			

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	38,233[a]	6	,000
Likelihood	34,907	6	,000
Linear-by-Linear Association	13,237	1	,000
N of Valid	188		

Source: our own research (N=188), 2020

Based on the data in Table 2, it can be stated that there is a significant correlation between the examined data and questions, so the correlational study can be evaluated. (The level of significance value is lower than 0.001, which is lower than 0.005) The value of Cramer's V is 0.319, which indicates a weak correlation, and the value of Gamma is 0.379, which shows a weak, one-direction correlation.

Based on the data in Table 2, it becomes obvious that, among young people in Budapest having a monthly income higher than HUF250,000, 81.8% believes their purchasing decisions are not influenced by different food advertisements seen on TV. Among those Budapest, Generation-Y youths with a monthly income between HUF200,000 and HUF250,000, 69.8% also believes that they do not make their decisions relying on advertisements when buying food. The figures are similar in the case of interviewees with a monthly income between HUF150,000 and HUF200,000 – 68.5% of them answered „no" to the question regarding the influence of advertisements.

According to the findings above, we can conclude that our hypothesis was not proven right, because, except for the youths interviewed with the lowest monthly income, in further income categories we cannot observe the significant influencing effect of TV as a communications channel. It is clearly visible from the data obtained that, apart from the lowest income category, in all the other income categories TV spots were not indicated as a tool to influence food consumption by the interviewees. So food consumption of participants in the survey is not influenced by advertisements communicated on TV.

6. Conclusion

We can see that our sample was not representative, so no general and important conclusions can be drawn. However, this study may serve as a suitable basis for the extended application of a quantitative research later on. Our first hypothesis supposing that of the communications channels affecting food consumption, TV has a more influential effect than other advertising tools among young Budapest people with 8th grade as highest education, has been proven right, as TV spots have the most influence on them when purchasing food. Generation-Y uses different technical tools daily, so advertisements

on the internet and on social media have a great effect on them in their food purchases. An interesting finding was that, Generation-Y youths in Budapest with at least a secondary education rely more on the opinions of acquaintances and friends regarding different foods than on the advertisements of the internet. In our other hypothesis we assumed that as the income of Generation-Y youths in Budapest increases, advertisements on TV among all other channels of advertisement influencing food consumption will have a decreasing effect on them proportionately. This hypothesis could not be proven right, as only with Generation-Y youths in Budapest with the lowest monthly income showed to be influenced significantly by ads on TV. The food consumption of those of all other income categories was not significantly influenced by ads communicated through the television. Reason being that today's world is characterized by turning over to the internet, and youths preferring BTL marketing tools as opposed to ATL marketing tools.

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Molecular characterization and phylogenetic relationships of *Globodera rostochiensis* isolates in two geographically distinct regions (Samckhet-Javakheti and Svaneti) of Georgia

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ABSTRACT

Molecular characterization of *Globodera rostochiensi* was performed by the amplification and sequencing D3 expansion region of the large subunit ribosomal gene (28S rDNA).Molecular analysis of *G. rostochiensis* had not been previously performed in Georgia. Sequence analyses of D3 expansion region of the rRNA using computer program BLASTN shown (94-99%) identity with D3 expansion region to all known isolates of *G. rostochiensis*. Based on analysis of *G. rostochiensis* isolates D3 expansion regions are grouped in three major clades (A, B and C) on the phylogenetic tree.Clade A are divided in three subclades, clade C are divided in two subclades. Isolates from Samtckhet-javakheti population are in subclade 1 of clade A and in subclade 1 of clade C. Isolates from Svaneti populations are in subclade 2 of clade A and in clad B. In Clade C subclade 2 is presented by three isolates from Svaneti and by one isolates from Samckhet-Javakheti. Our investigation showed of high genetic variation of D3 region of rDNA of the isolates of *G. rostochiensis* in two geographically distinct origins (Samckhet-Javakheti and Svaneti) of Georgia.

Keywords: *Globodera rostochiensis*, Molecular characterization, Phylogenetic tree, rDNA, D3 expansion region, Polymerase Chain reaction (PCR).

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Introduction

Potato is one of the main agricultural crops in Georgia. Georgia produces early and late potato varieties in almost all regions. Potato production area equals to 25,000 ha and its average yield is 20-25 t/ha. In traditional potato growing areas (Svaneti, Samckhet javaheti and Kvemo Kartli) the yeld is higher than 30-35 t/ha.

Among the plant-pests that limit potato production and quality, the potato cyst nematodes (PCN) are the harmful around the world. Yield losses caused by PCN are estimated up to 30% [1,2] .Two species of PCN – *Globodera pallida* [3,4] and *Globodera rostochiensis* [4,5] are recognized as plant quarantine pests and are added to the EPPO A2 list [6]. *G. rostochiensis* is present in all EU countries.It is globally recognized as one of the most important factors of yield loss [5]. Cyst nematodes live on the roots of host plants and can damage them to the extent of causing growth retardation, water st ress, nutrient deficiency, early wither and ultimately yield loss [7].

PCN are among the most difficult plant pests to control. Cysts protected by the durable wall can survive for over 30 years [8]. Control measures for cyst nematodes are: use of healthy planting material, crop rotation, chemicals, solarisation, bio-fumigation, weed removal. Currently, the most reliable control method against the cyst nematodes is breeding of resistant potato cultivars [9]. Council Directive 2007/33 /EEC, which establishes the measures against populations of PCN in order to determine their distribution and prevent their spread (Council Directive 2007/33 /EC), regulates control of PCN

(*G. pallida* and *G. rostochiensis*). There was no legislative regulation of these pests before 2016 in Georgia. The Government Resolution #302 from July 1, 2016, developed within the action plan of DCFTA (Deep and Comprehensive Free Trade Area) Agreement for legal acts approximation to EU .

Our rout surveys conducted in two geographically distinct regions of Georgia producing potatoes - Samtskhe - Javakheti and Svaneti revealed potato cyst nematodes (PCN). Morphological, morphometric and molecular analyses of PCN confirmed that belongs to *Globodera rostochiensis* [10,11].

Two genomic regions have been routinely characterized among nematode taxa: the ribosomal RNA array and the mitochondrial genome. The ribosomal RNA genes and their intervening sequences are the best-characterized gene regions in Nematoda [12].

In Georgia, is not information on the molecular characteristics of cyst nematodes. Therefore, the aims of this study were to molecular characterization and phylogenetic analysis of isolates of *Globodera rostochiens*is within the Georgian population (Svaneti and Samckhet Javakheti) using sequence of D3 expansion regions of 28S rRNA ribosomal nuclear RNA gene.

Materials and Methods

DNA Extraction and detection *Globodera rostochiensis* by multiplex PCR

DNA isolation from individual cysts was conducted using the Nematode DNA extraction& purification kits (Clear Detection) according to manufacturer's protocol. A multiplex PCR test were used for molecular identification. PCR was performed by the universal ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3) and cyst nematodes' (*G. pallida, G. rostochiensis*) spe[13] cific primers (PITSp4: 5′-ACAACAGCAATCGTCGAG-3'; PITSr3: 5′-AGCGCAGACATGCCGCAA-3) [13].

PCR was conducted in thermocycler (SimpliAmp™ Thermal Cycler _Applied Biosystems").

PCR mixture in a total volume of 25µl contained 25-50 ng DNA, and 0.25 µM ITS5, PITSp4R and PITSp4 primers. Positive controls contained G. pallida and G. rostochiensis from the Cheech collection of bank of Plant Pathogens DNA as matrices. Negative control contained PCR grade water instead of DNA Cycling conditions were: one cycle 95^{0}C for 5 min, 35 reaction cycles of 95^{0}C for 1 min, 55^{0}C for 30 s min, 72^{0}C for 1 min, followed by a final extension of 10 min at 72˚C and 4˚C hold.

Analyses of obtained PCR fragments were conducted by horizontal electrophoresis on 1,5% agarose gel containing ethidium bromide (0.2µg/ml) in TAE buffer (40mM Tris acetate, pH 8.0, 2mM EDTA-Na2) [14]. Visualization of fragments was done on trans-illuminator Benchtop UV. The sizes of the PCR products were determined by comparing the bands with a 100bp DNA ladder (Biolabs).

Amplification DNA fragments for phylogeny

D3 expansion region was amplified using primer pairs: D3A (5' GACCCCTCTTGAAACACGGA-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3' [15].

PCR condition were: 94°C for 5 min s; 30 cycles (94°C for 1 min 62°C for 1 min

and 72°C for 1 min); final extension 5 min at 72 °C.

PCR products of the were cleaned up and sequenced using an ABI 3500xL Genetic Analyze

Sequence and Phylogenetic Analysis

Obtained sequencing results were analyzed by computer program BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cg).

Phylogenetic analyses to resolve the relationships between the isolates were conducted in MEGA7 [16] using both distance- and character-based methods.

The evolutionary history was inferred using the UPGMA method [17]. The bootstrap consensus tree inferred from 400 replicates is taken to represent the evolutionary history of the taxa analyzed [18]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Tamura-Nei method [19] and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated. There were a total of 248 positions in the final dataset.

Results and discussion

Nematode identification

Identification of all the samples from the two Globodera populations (Samtskhe - Javakheti and Svaneti) i.e. *G. rostochiensis* (20 isolates) were con-

firmed by conventional multiplex PCR with ITS 5 universal and PITSp4 , PITSr3 specific primers of the cyst nematodes' (G. pallida, *G. rostochiensis*). Size of PCR fragment 434 bp confirms that PCN samples from two populations Samtskhe- Javakheti and Svaneti belongs to *G. rostochiensi* (Fig. 1).

Molecular characterization and sequence analysis

Molecular characterization of *Globodera rostochiensis* was performed by the amplification and sequencing D3 expansion region of the 28S rRNA.

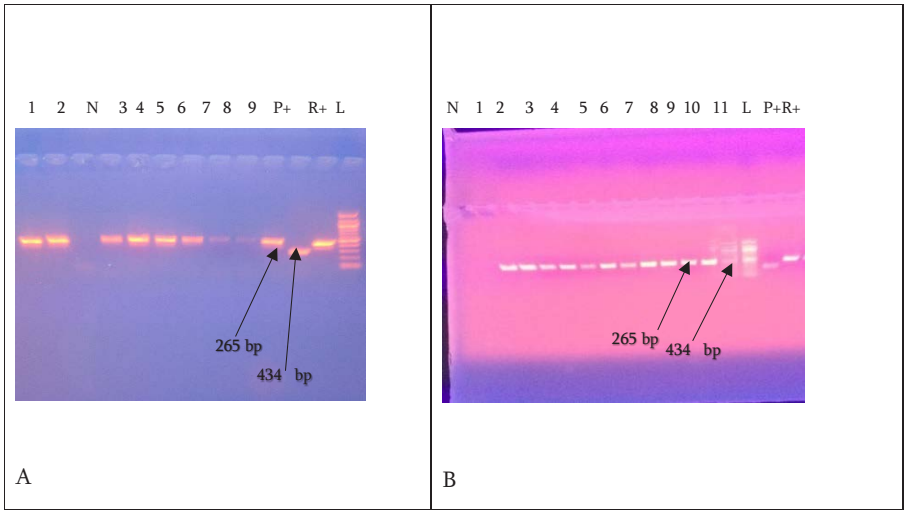

Fig. 1. *Multiplex polymerase chain reaction (PCR) of G. rostochiensis isolates using primers PITSt4, PITSr3, and ITS5. A. 1-9 (GL10, GL11, GL12, GL13, GL14, GL15, GL16, GL17, GL18,) G.rostochiensis isolates from Samckhet Javakheti; P+ G.pallida DNA (positive control)); R+G.rostochiensis DNA (positive control); L--100bp DNA ladder; N-negative control (nuclease free water); B.1-11 (GL1, GL4, GL5, GL8, GL9, GL19, GL20, GL21. GL22, GL23, GL24) G.rostochiensis isolates from Svaneti); R+G.rostochiensis DNA (positive control); N-negative control (nuclease free water); L--100bp DNA ladder; P+ G.pallida DNA (positive control).*

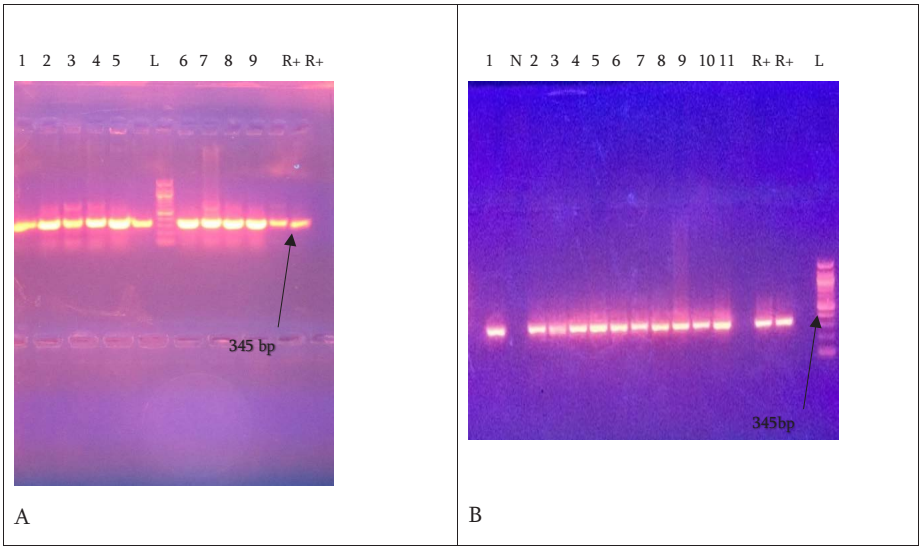

Fig. 2. *Amplified D3 expansion region of 28sRNA of Globodera rostochiensis izolates . A. 1-9 (GL10, GL11, GL12, GL13, GL14, GL15, GL16, GL17, GL18) G.rostochiensis isolates from Samckhet Javakheti; R+G.rostochiensis DNA (positive control); L--100bp DNA ladder. B.1-11 (Gl1, GL4, GL5, GL8, GL9, GL19, GL20, GL21GL22, GL23, GL24)) G.rostochiensis isolates from Svaneti); R+ G.rostochiensis DNA (positive control); N-negative control (nuclease free water); L--100bp DNA ladder.*

Amplicon size of the D3 region is 345 bp (Fig. 2).

Sequence date of D3 expansion region of the rRNA of *Globodera rostochiensis* were compared to those referenced in the NCBI database of other populations using computer program BLASTN. . High identities (94-99%) of the D3 rRNA sequences indicates that Georgian isolates of *G. rostochiensis* are similar to all known isolates of *G. rostochiensis* .

The sequences of D3 expansion regions were aligned using CLUSTAL V [20] The sequence lengths and frequencies of nucleotide distribution for the different isolate of *Globodera rostochiensis* are shown in Table 1.

Pairwise distance of D3 expansion region of *Globodera rostochiensis* isolates are presented in Fig. 3. The number of base substitutions per site

Table 1. *Sequence lengths and nucleotide composition of ITS1-5,8S region of G. rostochiensis isolates from Samckhet Javakheti and Svaneti*

Isolates of Globodera Rostochiensis	D3 expention region bp	T(U)%	C%	A%	G%
GL1	302,0	20,5	23,2	24,2	32,1
GL4	304,0	20,7	23,0	24,0	32,2
GL5	304,0	20,7	23,0	24,3	31,9
GL8	304,0	20,7	23,0	24,0	32,2
GL9	305,0	21,0	23,0	23,9	32,1
GL10	299,0	21,1	23,4	23,4	32,1
GL11	299,0	20,1	23,4	23,7	32,8
GL12	302,0	20,9	23,2	23,8	32,1
GL13	303,0	21,5	23,1	23,4	32,0
GL14	304,0	21,4	23,0	23,4	32,2
GL15	307,0	20,2	22,8	24,8	32,2
GL16	304,0	20,7	23,4	24,7	31,3
GL17	310,0	14,8	30,0	20,3	34,8
GL18	301,0	20,6	23,3	25,2	30,9
GL19	272,0	22,8	23,5	24,3	29,4
GL20	305,0	20,3	23,0	24,3	32,5
GL21	306,0	20,9	22,9	24,2	32,0
GL22	290,0	21,4	23,4	24,5	30,7
GL23	305,0	21,3	23,0	24,6	31,1
GL24	306,0	20,9	22,9	24,2	32,0
avg	301,6	20,6	23,5	24,0	32,0

The average nucleotide composition is as follows: 20,6% T(U), 23.5% C, 24,0% A and 32% G% (Table1).

from between sequences according to the Tamura-Neil model is shown below the diagonal. The number of base differences per sequence from between sequences is shown above.

Pierwase distance of D3 expansion region between Georgian *G.rostochiensis* isolates show that GL16 (Samckhet-javakheti) differs from GL17 (Samckhet-javakheti) and GL19 (Svaneti); base differences per sequence =0,5, 0,1 respectively; number of nucleotide substitutions per site =0.0 (Fig. 3). GL17 (Samckhet-javakheti) differs from GL18 (Samckhet-Javakheti) and GL19, GL20 , Gl21, Gl22, GL23, GL24 (Svaneti); base differences per sequence =0.5, 0. 3, 0.5, 0.5, 0.5, 0.5, 0.5 respectively; number of nucleotide substitutions per site =1.8. GL19 (Svaneti)) differs from GL20, GL21, GL22, GL23, GL24 (Svaneti) - base differences per sequence =0.1. GL18 (Samckhet-javakheti) differs from GL19 (Svaneti) base differences per sequence =0.1 (Fig. 3).

Phylogenetic analysis

Based on analysis of *G.rostochiensis* isolate`s D3 expansion regions are grouped in three major clades (A, B and C) on the phylogenetic tree (Fig. 4).. Clade A are divided in three subclades, clade C are divided in two subclades. Isolates (GL13, GL14, GL16, GL18) from Samtckhet-javakheti population are in subclade 1 of clade A and isolates (GL10, GL15) in subclade 1 of clade C. Isolates (Gl 11, GL 12, GL24, GL4) from Svaneti populations are in subclade 2 of clade A and isolates (GL1, GL20, GL5, GL8) in clad B. In Clade C subclade two is presented by three isolates (GL22, GL23, GL19) from Svaneti and by one isolates (GL17) from Samckhet-Javakheti.

Our investigation showed of high genetic variation of D3 region of rDNA of the isolates of G. rostochiensis from different geographic origins (Svameti, Samckhet-Javakheti) of Georgia.

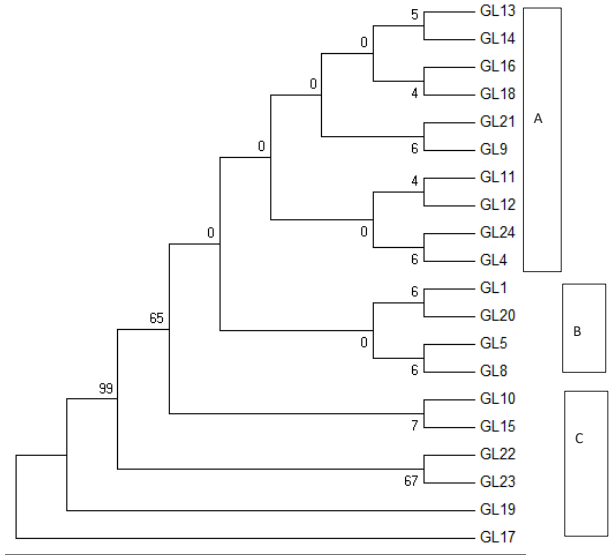

Fig. 4. *Phylogenetic relationships between isolates of G. rostochiensis from different geographic origins (Svameti, Samckhet-Javakheti) of Georgia.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. GL1		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
2. GL4	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
3. GL5	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
4. GL8	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
5. GL9	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
5. GL10	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
7. GL11	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
3. GL12	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
9. GL13	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
10. GL14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
11. GL15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
12. GL16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
13. GL17	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8		0.5	0.3	0.5	0.5	0.5	0.5	0.5
14. GL18	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8		0.1	0.0	0.0	0.0	0.0	0.0
15. GL19	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	1.3	0.2		0.1	0.1	0.1	0.1	0.1
16. GL20	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8	0.0	0.2		0.0	0.0	0.0	0.0
17. GL21	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8	0.0	0.2	0.0		0.0	0.0	0.0
18. GL22	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.2	0.0	0.0		0.0	0.0
19. GL23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.2	0.0	0.0	0.0		0.0
20. GL24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8	0.0	0.2	0.0	0.0	0.0	0.0	

Fig. 3. *Pairwise distances between D3 regions of of Globodera rostochiensis isolates.*

The evolutionary relationships between the potato cyst nematodes (G. rostochiensis and G.pallida) found in Georgia and studed in other countries will be shown by the phylogenetic analyses, which enhances the knowledge in the evolutionary genetics.

Molecular characterization and phylogenetic analysis of PCN by rDNA fragment sequencing methods is important as far as similar studies have not been held in Georgia yet.

Ribosomal RNA genes encoding 5.8S, small subunit 1 8S and large subunit 28S have been widely used to inter phylogenetic relationships among closely and distantly related taxonomic lineages [21].

D3 expansion segments of the 28S ribosomal RNA molecule have been used as meaningful genetic markers for resolving phylogenetic relationship at lower and higher taxonomic levels and developing species- specific primers [15, 22-27].

Nucleotide sequences of the D3 expansion region of 28S rDNA corresponding to positions 3304-3648 are available for some nematode species [28, 29]. This region was successfully amplified from formalin-fixed nematodes and could be useful for nematode identification [29]. Although this region is specific at the species level in the genus Pratylenchus, for example, and may be used for species identification [15, 21]. it is unlikely to be useful for the identification of Globodera species parasitizing in solanaceous plants as it is highly conserved [22].

Acknowledgement

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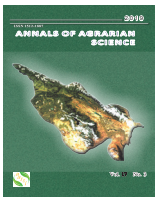

Prospects for processing low-grade rebellious copper ores of the Madneuli deposit by heap bioleaching

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ABSTRACT

Over 40 years of copper mining and processing in Madneuli Mine, millions of tonnes of overburden rocks, substandard ores and enrichment waste have been accumulated in mine dumps, which contains a significant amounts of useful components. Let alone the fact that these wastes occupy large areas of land, they are also hazardous to environment due to their sulfide content. The article presents the results of a study carried out as a laboratory simulation of the heap bacteriological- chemical leaching process. The test object was a low-grade rebellious sulfide and oxidized copper ore extracted from the mine dumps. The experiment was performed using acidophilic iron and sulfur-oxidizing bacteria isolated and activated from the Madneuli ore field microflora. In parallel with the process of ore heap bacteriological-chemical leaching, an acid leaching with diluted sulfuric acid solution was carried out under the same conditions. The studies have demonstrated that the method of copper heap bioleaching in ores of such type is advantageous in comparison with acid leaching. This method gives 27% higher copper recovery compared to the chemical leaching method.

Keywords: Bacterial, Heap, Leaching, Copper, Pyrite, Oxidation.

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Introduction

Recently, in all mining countries worldwide, special attention has been paid to the technogenic mineral raw material (solid minerals deposits) obtained in the process of ore extraction and processing. The rising prices for precious and nonferrous metals and the decrease in the number of rich, free-milling ores containing these metals have led to the actualization of the issue of exploiting technogenic ores.

A mining facility is a huge source of industrial waste, leading to the exponential increase in environmental pollution.

In environmental terms, sulfide-containing wastes fall into particularly hazardous category. In the process of mining and processing of ferrous ores, the residual sulfides, along with ferric sulphides undergo an oxidation process over time; Heavy metals are transformed into water-soluble salts (sulfates) followed by their leaching. Hypergenic changes in technogenic products occur much faster than in natural geological conditions. One of the important factors in the intensification of processes is the increase of activation of the surface of sulfides in the braking and granulation process.

The terms “acid mine drainage” (AMD) and “acid rock drainage” (ARD) are used in foreign scientific literature, which refers to the problem of water contamination by the operation of mining industries. The process of sulfide oxidation is determined by several factors:

1. PH of the solutions which come in contact with sulfide minerals.
2. Surface and morphology of minerals.
3. Concentration of oxygen and trivalent iron in the solution.
4. Temperature.
5. Galvanic effect during the contact between different sulfides.
6. Bacterial exposure [1-3].

As far as century and a half ago, Louis Pasteur discovered the special role that microorganisms play in the process of substance exchange in environment. He was the first to prove the microbes are involved in the transformation of different elements. Louis Pasteur's researches laid the foundation for a new science - microbiology, which evolved in various directions, including technical and geological microbiology.

Elements such as sulfur, iron, copper, manganese, calcium, etc. fell into the scope of interests of Geological microbiology. For 70 years geomicrobiology was considered to be a purely theoretical discipline, but it was becoming increasingly apparent that a number of processes involving microbes were proceeding at such a pace that their implementation and management were actually feasible. Foremost, it concerned the microbiological oxidation of sulfides.

Bacterial processes in sulfide deposits can be used to intensify the hydrometallurgical processing of sulfide ores.

Thus the connection between geological and technical microbiology has developed and a new field of microbe use emerged, which made possible to apply this process in mining industry for the extraction of metals, primarily copper [4].

One of the modern alternative methods that does not involve the disadvantages of traditional pyro- and hydrometallurgical methods for sulfide ore processing is biogeotechnology. The use of biotechnology in non-ferrous metallurgy allows us to find new approach in solving such complex problems as processing of low-grade materials with particularly difficult enrichment properties (including dumps and substandard ores). However, the technological process is simplified to a significant degree, since instead of using roasting, acid leaching and other environmentally harmful processing methods, oxidative leaching can be carried out in environmentally friendly conditions [5, 6].

The method of metal bioleaching is based on the use of acidophilic chemolithotropic microorganisms, which in the process of evolution have acquired the ability to use the energy released in the process of oxidation of inorganic substances (divalent iron, elemental sulfur, etc.) for their vital activity, and act as catalysts in chemical reactions. For the synthesis of proteic substances, these microorganisms use carbon dioxide or inorganic carbonate compounds for autotrophic nutrition [7-9].

Acidithiobacillus ferrooxidans play the leading role among mesophilic acidophilic chemolithotropic microorganisms used in biohydrometallurgy. Its use accelerates oxidation rate of divalent iron to trivalent iron by 100,000 and more. In the process of bacteria-catalyzed iron oxidation, which can be presented in a simplified form as shown below, energy is released (Gibbs energy change at pH = 2 is 33 kJ /mol).

$$4Fe^{2+}+O_2+4H^{+}\text{--}\longrightarrow 4Fe^{3+}+2H_2O \quad \text{(Bacterial oxidation)}$$

Microbiological oxidation of divalent iron produces a bioreagent - an oxidant, which is a compound of trivalent iron and bacteria-synthesized exopolysaccharides, the oxidation potential of which in sulfuric acid solutions is 80-120 mV higher compared to the pure ferric oxide sulfate under the same conditions [1, 2]. Thus, in accordance with modern views minerals are predominantly oxidized by bioreagents. The share of cellular enzymes interactions with minerals is negligible [10–15].

Oxidation reaction of sulfide minerals in simplified form:

$$MeS+Fe^{3+}\longrightarrow Fe^{2+}+S^0+Me^{2+}$$

Oxidation of sulfide minerals with oxygen and acids proceeds very slowly with the following reaction:

$$MeS+1/2O_2+2H^{+}\longrightarrow Me^{2+}+S^0+H_2O$$

The elemental sulfur produced by the oxidation of sulfides is oxidized by microorganisms to sulfuric acid:

$$2S^0+3O_2+2H_2O\longrightarrow 2H_2SO_4$$

With the decrease in the acidity of the solution, the hydrolysis of $Fe_2(SO_4)_3$ results in the precipitation of ferric hydroxide and the release of protons, which makes it possible to control the concentration of ferric iron and the pH in the solution.

$$Fe^{3+}+3H_2O\longrightarrow Fe(OH)_3\downarrow+3H^{+}$$

A. th. ferrooxidans and A.th. thiooxidans are aerobic bacteria, therefore they develop only in the presence of oxygen needed for endogenous respiration and iron oxidation. Oxygen and carbon dioxide concentrations in solutions are one of the main parameters determining the activity, growth and rate of the biooxidation process in bacteria.

0.14 kg of oxygen is required for oxidation of Fe^{2+} to Fe^{3+} per kg of iron, and 2 kg of O_2 for oxidation of 1 kg S to SO_4. Likewise, 1 kg of chalco-

pyrite requires 0,74kg of oxygen, that is, 2.13 kg of oxygen is required to extract 1 kg of copper from chalcopyrite. In 20^0C water O_2 is $8 \cdot 10^{-4}$ gr/l , e.i., 6,4 kg per 1000 m^3 which is not enough, thus the role of aeration is important.

The role of the bacterial factor in the oxidation of sulfides was first studied in 1947 by Colmer and Hinkle. They found that Acidithiobacillus ferrooxidans caused the oxidation of the divalent iron to trivalent in acidic quarry waters of the Bingham pyrite-containing coal ore field. Trivalent iron is one of the most powerful oxidizers of sulfides and bacterial regeneration of trivalent iron is very important in the oxidation of sulfides and leaching of ferrous metals.

Extraction of metals from low-grade ores, overburden and dump rocks by heap bacterial leaching is cost-effective, as sulfide oxidizing reagents are continuously regenerated by microorganisms. Heap bacterial leaching of copper ore began in 1958, after the US company Kennecot Mining patented the use of iron oxidizing bacterium A.thiobacilus ferrooxidans for copper extraction and applied this method to process low-grade ores of the Bingham Canyon Mine (USA) [16,17]. In 1982, in Chile, Sociedad Mineral Pudahuel first applied biotechnology to extract copper from Lo Agurre's low-grade ore deposits, and the SX-EW technology to recover copper from the solution.

Currently, the heap bacterial method accounts for 25% of the world's annual copper production, which is carried out at plants of Chile, Australia, the USA, Mexico, Peru, Bulgaria, Burma, China and other countries. In addition, several plants in Australia, Finland, China, Turkey apply this technology in the processing of copper-zinc ores. Recently, China has been actively conducting researches on the application of heap bacterial leaching method in copper-zinc ore processing, and developed technologies are instantly introduced.

Foreign hydrometallurgical practices clearly show the prospects of heap bacterial leaching method, especially for the extraction of copper, uranium and gold in low-grade ores, mining and enrichment plant wastes.

The heap bacterial leaching technology is as follows: Solutions containing sulfuric acid, oxidants (oxygen, Fe (III), etc.) and microorganisms (Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans, Leptospizilum ferroxidans, etc.) are introduced onto the surface of the heap or into the heap. Different methods are used for the process of solution distribution. The major part of solution coming out of heap, which is enriched with nonferrous metals, is collected and ferrous metals are recovered out of it with the use of various methods (cementation, extraction, etc.).

In world practice, copper heap leaching technology is mainly based on the leaching of secondary copper sulfides - chalcocite (Cu_2S) and covelline (CuS) in ores. Heap bacterial leaching of primary copper mineral chalcopyrite (SuFeS) is often commercially restricted due to the residual products attributed to passivation of minerals during the oxidation process.

The issue of utilization of technogenic waste is relevant for Georgia too.

The largest copper deposit of Georgia is in the Madneuli sulfide ore field, where a quarry mining method was first introduced in 1958 and it has been operational since 1975 (Picture 1 shows a view of the mine quarry).

Copper ores in the mine are represented in the form of vein-type and disseminated deposits. Com-

Fig. 1. *Madneuli Gold-Copper-Polymetallic Ore Quarry*

position rocks - secondary quartzites, silicified tuffs, are main ore minerals: Primary - chalcopyrite, pyrite, rare sphalerite; Secondary- covelline, chalcosine, bornite, cuprite, insignificant amount of copper sulfates and carbonates. According to the mineralogical composition and technological characteristics, three types of copper are distinguished:

Chalcopyrite-pyrite - good enrichment properties

Chalcopyrite-covelline-chalcosine-pyrite - moderate enrichment properties

Covelline-chalcosine-pyrite - difficult enrichment properties

The average copper content in ores over the years indicates that (1975 - 1.2%; 1985 - 1.0%; 1995 - 0.83%; 2005 - 0.7%; 2015 - 0.65% and currently 0.50%) the deposit is gradually becoming poorer.

The ore deposits of the central and northeastern part of the Madneuli quarry are characterized by a complex geological genesis. In particular, the mineralogical composition of copper ores is heterogeneous, with the significant amounts of secondary copper sulfides (covelline, chalcosine, bornite), copper is also found in oxidized form (10-30%). Compound mineral rock formations are represented in hydrothermal alterations (silicification, kaolinisation, chloritization) of tuffs and tuffaceous aleurites. Ores of such areas are characterized by: containment of nonmetallic silt-forming minerals (kaolinite, sericite, alunite, chlorite, jarosite) and frequent tectonic faults, which makes them difficult to enrich.

At present, only a small portion of free-milling copper ores remains, and the demand for copper concentrates has not changed. There is a large amount of low-grade rebellious copper ores in the vicinity of the ore deposit (Table 1).

Their processing by traditional methods (gravity, flotation) is unviable, as it is impossible to obtain saleable copper concentrates. The copper content in the concentrate varies from 6.0 to 12.0%, which is an indicator of sub-standard concentrates and the low coefficient of economic parameters. According to today's market values, if copper is the major useful element in the concentrate, then its mineral content should be 14.0%, otherwise its market price is nonreimbursable. Therefore, there is a need to develop and implement other modern and innovative technologies for the processing of these ores, which will both expand the raw material base for copper production and allow rational use of non-renewable natural resources.

The purpose of the study was to determine the viability of copper extraction in low-grade rebellious cooper ores by bacterial-chemical leaching in laboratory conditions.

Objects and methods of the study

The test object was a low-grade rebellious sulfide and oxidized copper ore extracted from the mine dumps.

A low-cost method of processing poor copper-containing raw materials is application of heap leaching method.

Airlift percolators allow conducting studies of the bacterial leaching process in passive filtration (solid mass) mode, this is a laboratory model of ore heap leaching. The studies used the association of iron and sulfur-oxidizing bacterial cultures of Acidithiobacillus ferrooxidans (A.th.ferrooxidans) and Acidithiobacillus thiooxidans (A.th. Thiooxidans), separated and activated from the acidic quarry waters of Madneuli Mine.

For the preparation of nutrient media, we used the textbook of G. I. Karavaiko [5].

As the criterion of oxidizing activity of bacteria, we take the level of divalent iron's oxidation to the trivalent state in the 9K nutrient medium. We performed cultivation of bacteria on the laboratory shaker (180 r/min) on the 9K nutrient medium with the temperature of 28-30^{0}C, solid-to-liquid=1:10.

pH and Eh was determined by means of electrometric method on the laboratory pH-meter (pH-

Table 1. *Information on Dumps for 2018*

Dump N	Area (Ha)	Volume (m3)	State
1	76.5	32,657,500	Operational
2	78.0	52,801,800	Closed
3	90.0	31,826,800	Operational
4	60.5	16,469,900	Operational

340). We applied complexometric titration method with Trilon B to determine divalent and trivalent iron content in the solutions; copper content in solutions was determined by tittration using sodium thiosulfate solution; and for the determination of copper content in the solid mass, we applied atomic absorption method (Perkin Elmer; AAnalist 200).

Results and review

The substance content of the technological sample was determined by chemical, deportment, mineralogical, gravimetric analysis. The results of the tests are given in Tables 2, 3, 4.

The physical characteristics of the research subject were determined by volume weight - 2.47 g/cm^3, relative density - 2.74 g/cm^3, bulk density 1.25 g/cm^3, 3.6% moisture absorption; Porosity (true) - 9.85%.

Deportment and mineralogical analysis have clearly shown that chalcosine is represented by a total group of minerals that develop at the expense of chalcopyrite and are found in a form of pure monolayers of pyrite and chalcopyrite grains.

Chalcopyrite-free grains are rarely found solely in small class and silts (0.015-0.08 mm).

Micro vein-type fractures in pyrite grains are filled with minerals of the chalcosine group, pyrite embedded with quartz is also coated with chalcosine-covelline layer. In thin class, most of the pyrite grains are covered by the monolayer resulting from the oxidation of chalcopyrite. The results of the physical analysis of the sample are shown in table ...

Such chalcosine-pyrite-oxidized copper ores belong to the rebellious ores, in which pyrite of the third generation, in contrast to the pyrite created at the earlier mineralization stage, intergrows with covelline and chalcosine into 0.001-0.01 mm smallest dense aggregates.

The above-mentioned dense aggregate of intergrown pyrite and secondary copper sulfides (covelline, bornite, chalcosine) significantly complicates the complete dissolution of minerals during the milling process, which results in a sharp decrease in the amount of copper recovered and the quality of concentrate.

A solution of 9K of nutrient medium has been added into the percolator containing a -10 + 0 mm

Table 2. *Chemical composition of rebellious copper sulfide ores*

Chemical composition, %, g/t													
Humidity	Heating loss	SiO_2	Fe_2O_3	MgO	CaO	P_2O_5	Al_2O_3	SO_3	S”	Na_2O	K_2O	Cu	Au
0.76	5.1	78.7	8.61	1.1	1.3	0.1	6.3	8.94	4.01	0.7	1.0	0.64	0.4

Table 3. *Deportment analyses of rebellious copper sulfide ore*

Total composition of copper, %	Copper sulfides, %		Oxidized copper, %
	Primary	Secondary	
0.64	-	0.49	0.15
100.0	-	77.2	22.8

Table 4. *Gravimetric analyses of 10mm crushed rebellious copper sulfide ore*

Class, mm	Yield, %	Au, g/t	Extraction, %	Cu, %	Extraction, %
-10+5	52.9	0.33	48.5	0.58	47.9
-5.0+2.5	23.0	0.32	22.1	0.62	22.2
-2,5+1,25	10.2	0.29	8.9	0.73	9.9
-1.25+0.63	4.0	0.39	4.6	0.91	4.8
-0.63+0.315	3.3	0.39	3.9	1.04	4.5
-0.315+0.16	2.1	0.4	2.5	1.14	3.2
-0.16+0.08	1.4	0.47	2.0	1.23	2.7
-0.08+0	3.1	0.71	6.6	1.31	5.3
	100.0	0.36	100.0	0.64	100.0

thick ore. Nutrient medium consisted of: Ammonium sulfate - 0.15 g/L; Potassium chloride - 0.05 g/L; Magnesium sulfate - 0.5 g/L; One substituted potassium phosphoric acid - 0,1 g/L; Calcium nitrate - 0.01 g/L pH = 2.5. The nutrient medium contained 10% bacterial culture mixture. Bacterial titer 10^4 cell/ml.

Air was continuously supplied to the system by a compressor; the amount of air was regulated by special clamps. Such a system ensured multiple percolation of the solution and enrichment of the system by O_2 and CO_2, which is essential for the microorganisms' vitality.

After each 7-day cycle, the solutions were drained off. The amounts of Cu2 +, Fe3 +, and Fe2 + in the solution were determined. The pH of the solution was checked daily. If necessary, adjustments were made by adding sulfuric acid to 1.5-2.2 value.

The experiment was carried out at a temperature of 25-30^{0}C (Fig.2).

Based on a series of tests performed in small (h-280 mm, d-25 mm) percolators, the optimal parameters for the process were determined. The impact of various factors on the copper leaching process has been studied: solid:liquid ratio, pH of solution; downtime between the active percolations.

Factors such as solid-to-liquid ratio are of great importance during the leaching process. The leaching process was studied at solid:liquid ratios of 1: 2; 1: 3,5 and 1: 5. The results of the tests are given in Table 5.

In sterile control (bacteria-free nutrient medium), copper extraction was 2.5% over the same period of time.

It is known that for the development of iron- and sulfur-oxidizer thiobacterium A.th.ferrooxidans and A.th. thiooxidans an acidic medium of pH = 1.5-2.5 is required. During the oxidation of sulfide minerals, especially pyrite, sulfuric acid is formed causing a decrease of the pH value. If the bacteria are not adapted to such conditions, then their activity decreases. Therefore, the impact of pH on bacterial activity was studied in two variants - pH = 1.5 and pH = 2.0; solid: liquid = 1: 5 and solid: liquid= 1: 2. The results of the tests are given in Table 6.

The effect of downtime between the active percolations on the quality of copper leaching.

In order to study this parameter of the leaching process, the experiment was conducted at pH = 2.0-2.1, since previous experiments have shown that the maximum copper extraction reaches its peak on the 49th day, and then decreases during the following days, we considered 1 full cycle for 49 days and chose this period as the duration of the trial. The downtime between the percolations varied: 1 day, 2 days, 3 days and 4 days. Solid:liquid ratio 1:2 and 1:5 The results of the trial are given in Table 7.

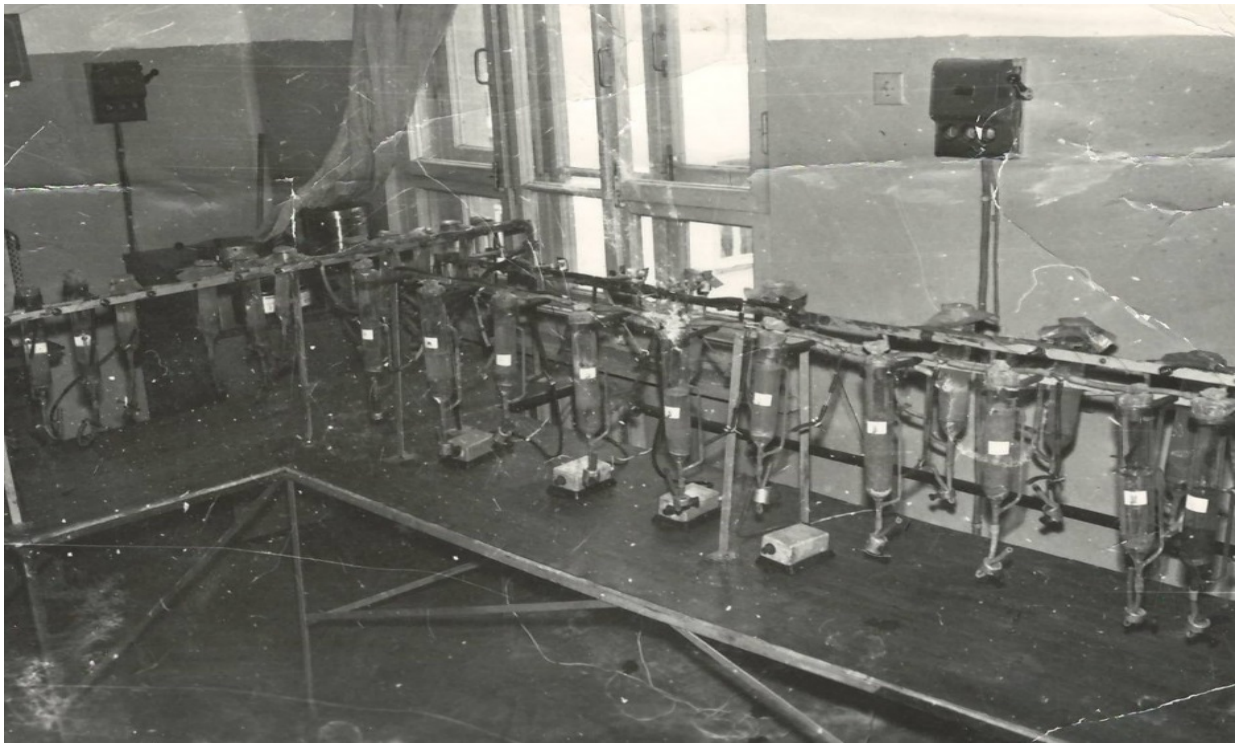

Fig. 2. *Small airlift percolators*

Table 5. *Effect of solid-to-liquid ratio on the quality of copper leaching by percolation in low-grade copper ore*

№	Percolator №	Solid-to-liquid ratio	Nutrient medium volume, ml	Inoculant volume, ml	Number of samples, g	Bacterium pH		Copper extraction in 7 days, %												Average daily copper extraction, %
								7	14	21	28	35	42	49	56	63	70	77	84	91	
1	1	1:2	180	20	100.0	A. th.ferrooxidans, A.th. thiooxidans	2.14	3.3	7.0	10.3	14.3	20.1	26.8	34.4	38.1	42.3	45.3	48.4	51.1	53.1	0.41
2	2	1:3,5	160.0	18	50.0		2.2	4.0	8.2	12.7	17.6	25.3	32.5	40.1	46.8	52.0	57.8	60.1	62.5	64.8	0.50
3	3	1:5	250	25	50.0		2.1	5.3	8.8	13.2	20.0	28.1	38.2	50.2	56.0	59.3	63.0	68.1	70.0	72.3	0.556
4	K	1:1	100	-	100.0		2.2	0.3	0.6	0.8	0.9	1.0	1.2	1.6	1.73	1.81	1.84	1.92	1.97	2.5	0.03

Table 6. *Effect of pH on the quality of copper leaching by percolation in copper ore*

№	Percolator №	Solid-to-liquid ratio	Nutrient medium volume, ml	Inoculant volume, ml	Sample quantity, g	Culture used	pH	Copper extraction in 7 days, %												Average daily copper extraction, %
								7	14	21	28	35	42	49	56	63	70	77	84	91	
1	5	1:2	180.0	20.0	100.0	A. th.ferrooxidans, A.th. thiooxidans	2.1	3.3	7.0	10.3	14.3	20.1	25.8	34.4	38.1	42.3	45.3	48.4	51.1	53.1	0.41
2	6	1:2	180.0	20.0	100.0		1.5	4.1	9.1	12.0	16.2	22.4	30.3	38.5	44.5	48.9	52.5	53.7	56.8	58.0	0.45
3	7	1:5	225.0	25.0	50.0		2.1	5.3	8.8	13.2	20.0	28.1	38.2	50.2	56.0	59.3	63.0	68.1	70.0	72.3	0.56
4	8	1:5	225.0	25.0	50.0		1.5	5.2	9.0	14.7	22.6	32.0	42.4	54.1	59.1	63.8	67.4	72.2	74.1	75.5	0.58

Table 7. *The effect of downtime between the active percolations on the quality of copper leaching*

№	Percolator №	Number of tailings, in g	Nutrient medium volume, ml		Microorganisms used	Solid:liquid	pH	Cycle duration, days	Copper extraction in 7 days, %							Average daily copper extraction, %
									I	II	III	IV	V	VI	VII	
1	10	100.0	180.0		A. th.ferrooxidans, A.th. thiooxidans	1:2	2.1	7+1+7	3.7	8.1	14.3	20.1	27.3	32.0	40.1	0.82
2	11	100.0	180.0			1:2	2.0	7+2+7	4.3	11.5	17.5	25.0	35.1	40.0	47.3	0.97
3	12	100.0	180.0			1:2	2.0	7+3+7	4.2	12.8	18.5	24.1	33.3	41.3	51.8	1.06
4	13	100.0	180.0			1:2	2.1	7+4+7	4.3	13.1	20.8	28.5	26.1	43.3	52.5	1.07
5	14	50.0	225.0			1:5	2.0	7+4+7	7.2	18.8	29.5	38.2	46.3	55.6	65.3	133

For comparison purposes, the test subject underwent a heap chemical leaching with a diluted sulfuric acid solution (2.53 g/L) under the following conditions: Solid-to-liquid ratio 1:5, pH-2,1, trail period 91 days, cycle - 7 days. The results of the test are given in Table 8.

Thus, the experiment demonstrated the viability of bacterial-chemical leaching of low-grade rebellious sulfide and oxidized copper ores in Madneuli deposit, as well as its advantage compared to the acid heap leaching method. The experiment has demonstrated that under the same conditions, the amount of copper transferred to solution by bacterial-chemical leaching was 27.0% higher than by pure chemical leaching.

Conclusion

The composition of medium technological samples taken from the Madneuli copper ore deposit dumps is studied by chemical, mineralogical, deportment, gravimetric analysis. This sample consists of: Cu – 0,64%; S_{sulf} - 4,01%; SO_3 – 0,1%; SO_3 – 8,94%; Fe – 8,6%; SiO_2 – 78,7%

Copper is represented in the forms of secondary sulfides (covelline, chalcosine) and as oxidized sulfide, 77.2% and 22.8%, respectively.

The viability of copper extraction in low-grade rebellious cooper ores by bacterial-chemical leaching in laboratory conditions has been established. In airlift percolators, under different conditions (solid

Table 8. *The results of copper ore heap bacterial and chemical leaching*

Copper mineral state	Copper content in ore, %		Leaching type	Copper extraction in solution, %
	Initial	Final		
Oxidized	0.15	0.02	Bacterial-chemical	85.0
Secondary	0.48	0.16		66.6
Primary	-	-		-
Total	0.64	0.18		72.3
Oxidized	0.15	0.06	Chemical (sulphuric acid 5g/L)	60.0
Secondary	0.48	0.29		39.7
Primary	-	-		-
Total	0.64	0.35		45.3

and liquid phase ratio, pH of solution, downtime between the active percolations), during the 91 days period, with 1: 5 solid-to-liquid ratio and pH = 2.1, the amount of copper transferred from the ore to the solution is 72.3% (of which 66.6% is from secondary sulfides and 85.0% - from oxidized minerals).

The amount of copper transferred to the solution by acid leaching method is 27.0% lower]]than the amount achieved with the bioleaching method, performed under the same condition (60.0% from oxidized minerals, and 39.7% - from secondary sulfides) - 45.3%.

In the bioleaching process, a mix of acidophilic iron and sulfur-oxidizing bacteria A.th.ferrooxidans and A.th. thiooxidans, isolated from the acidic quarry waters of the Madneuli deposit was used.

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Production of *Staphylococcal anatoxin* and development of a diagnostic test system

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A B S T R A C T

In three small series a non-toxic, immunogenic form of staphylococcal alpha-hemolysin (α-toxin) was obtained. The purified anatoxin was precipitated and a diagnostics test system developed. Its activity, specificity, and other characteristics were studied. The test system is designed for rapid serological diagnosis of staphylococcal infection, as well as for the post-vaccination evaluation and quantification of antitoxic antibodies. In passive (indirect) hemagglutination reaction the normal titer of *Staphylococcal antitoxic* antibodies (IgG) in human serum varied among different individuals between 1:5 – 1:40. In clinical material the titer of antitoxic antibodies for infections caused by *Staphylococcus aureus* ranged from 1:160 to 1:1280. In animals, this titer is normally 1:40 - 1:160.

Keywords: Non-toxic, Immunogenic form, Infections, *Staphylococcal anatoxin*, Clinical material, Rapid serological diagnosis.

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Introduction

A staphylococcal infection is a complex pathological process that may result in an asymptomatic carrier state, severe intoxication, or formation of purulent inflammatory foci. Staphylococci cause both nosocomial and community-acquired diseases [1]. According to the World Health Organization (WHO), *Staphylococcus aureus* tops the list of bacteria that most commonly cause infections in healthcare facilities. *S. aureus* accounts for 31% of all nosocomial infections. Due to the rise of antimicrobial resistance (AMR), staphylococcal infections have become one of the greatest problems of modern medicine [2, 3]. In Georgia, according to the Vakhtang Bochorishvili Clinic, more people die from staphylococcal infections than from diphtheria, salmonellosis, hepatitis, and typhoid fever combined [4].

The virulence of *S. aureus* is associated with its adhesion to receptors of sensitive cells [5]. *S. aureus* synthesizes a number of toxins that differ by their mechanisms of action. The toxic substances are divided into three groups: (1) PTSAgs, superantigens that induce toxic shock syndrome (TSS); (2) exfoliative toxins (EF) implicated in staphylococcal scalded-skin syndrome (SSSS), which occurs most commonly in infants and young children; and (3) staphylococcal toxins that act on cell membranes and include alpha-toxins and several bi-component toxins (*e.g.,* PV leukocidin associated with severe necrotizing pneumonia in children [6, 7]. Of these toxins, the staphylococcal alpha-toxin (α-hemolysin) is attracting much attention for its role in disease pathogenesis.

This study is aimed at obtaining the staphylococcal alpha-toxin, purifying the anatoxin, and developing a diagnostic test system for the evaluation and quantification of staphylococcal antitoxic antibodies in human and animal blood serum.

Materials and Methods

Obtaining and purifying the staphylococcal anatoxin involved bacteriological, biochemical, and serological methods. The following microbiological growth media were used: brain-heart broth and agar, nutrient agar, blood agar (Biolife, Milano, Ita-

ly; Eliava Bacteriological Media Production, Tbilisi, Georgia).

To obtain the alpha-toxin (α-hemolysin), a known producer strain, *S. aureus* 0-15 was selected. Particular attention was paid to the preparation of a casein enriched nutrient medium (broth and agar). For this purpose, we prepared our own yeast extract (100 ml distilled water added to 25 g dry yeast powder, boiled for 30 min, filtered through double filter papers, and stabilized by the addition of 1% chloroform), casein hydrolysate (20 g casein powder (Sigma, USA) dissolved in 1 L distilled water and stored at 4 °C), and wheat bran extract (200 g wheat bran added to 1 L distilled water with 1% chloroform, incubated at 45 °C for 12-14 h, and filtered through 0.8-μm filter). The final medium contained 20 g casein, 120 g wheat bran, and 5 ml yeast extract in 1 L distilled water. It was autoclaved at 110 °C for 30 min.

The alpha-toxin producing staphylococcal strain was grown overnight in culture tubes. The inoculum was transferred into 5-liter flasks containing 1 liter of the casein medium and incubated at 37 °C for 5 days. At the end of the growth period, the suspension was treated with formaldehyde for 10 days, and the anatoxin purified in 3 steps. Purity of the anatoxin was checked *via* electrophoresis.

The anatoxin was tested for safety and toxicity. Safety testing aimed to determine the general and local reaction of vaccinated animals in accordance with existing technical regulations. The experiment was performed on 3 clinically healthy chinchilla rabbits with a live weight of 1.5–2.0 kg. The animals were provided with proper nutrition and care under zoological hygiene conditions. The study drug was administered intravenously in the amount of 5 ml. We observed the experimental animals for 10 days. The anatoxin was deemed harmless if all three animals remained alive and clinically healthy for 10 days.

Toxicity of the purified staphylococcal anatoxin was studied according to the European Pharmacopoeia (European Pharmacopoeia, 5.0; 01/2005:20609, p. 153). We tested 5 clinically healthy white mice with a live weight of 17-22 g. One hundred microliters of anatoxin (warmed to 28–30 °C) were administered intravenously and the animals observed for 24 h. According to Pharmacopoeia, the animals should remain healthy without lethality during the observation period.

To increase the stability and shelf-life of the developed diagnostic test system, we lyophilized the drug in a gelatin-sucrose protective medium (7 % sucrose and 5 % gelatin). The semi-finished product was frozen at -40 °C and then freeze-dried. Testing the lyophilized drug no change was observed in 4 months in humidity, rehydration time, specificity, and sensitivity compared to the initial levels. The products will be retested after 1 year and 2 years.

Results and Discussion

Staphylococcal anatoxin has been successfully used in Ukraine and Russia for preventive (*e.g.,* during a pre-operative period) and therapeutic (furunculosis, hordeolum, or other relatively mild infectious diseases) purposes. The drug is not produced in Georgia. Actually, it is included in the list of deficient drugs. Imported anatoxin is expensive. Therefore, it is extremely important to improve the technology and introduce the drug in healthcare and veterinary practices.

For anatoxin production, selection of nutrients and the proper culture conditions for the producer strain, *S. aureus* s 0-15 are critically important. For culturing, we used the enriched casein hydrolysate medium (broth or agar) with an amine nitrogen concentration of 1.3-1.5 mg/ml, determined as the amount of alkali consumed (about 0.5 ml of 0.2 N NaOH). Once the amine nitrogen was determined, the medium was heated to boiling point. The mixture then was let to cool, and the pH adjusted to 7.2 -7.4. Then, 0.05 % Na_2HPO_4 and 0.05 % KH_2PO_4 were added and the mixture filtered through a 0.45-μm Millipore filter. One liter of medium was poured into a 5-liter container and sterilized in an autoclave at 112 °C for 30 min.

It is also important to select the clone with the highest hemolytic activity. Cells of strain 0-15 were grown for 18–20 h and then transferred onto blood agar to obtain isolated colonies. After an additional 20–22 h, colonies with the largest hemolysis zone (diameter 7–8 mm) were selected and grown in culture tubes containing 8-10 ml of casein broth for 18–20 h. The seed culture was transferred to a 5-liter container with 1 L of pre-filled and sterilized casein medium and incubated at 37 °C for 5 days. Twice a day, in the morning and in the evening, the culture was aerated through sterile filters with 10-12 L of oxygen (O_2) for 5-6 min and 2 L of carbon dioxide (CO_2) for 1 min. After 5 days, the cloudy suspension was separated by centrifugation at 3,000 x *g* for 20 min in a refrigerated centrifuge. The toxin-containing supernatant was sterile filtered through a 0.22-μm filter. The crude alpha-toxin was tested for

activity. In the hemolysis reaction, the minimum hemolytic dose of the toxin was determined to be 1:100.

To obtain the harmless form of the anatoxin, we added 0.4 % of formalin to the sterile liquid and stored it in a thermostat for 10 days. Then, the solution was kept for 5 more days at room temperature to convert the toxin into the anatoxin. To further purify the anatoxin, predetermined amounts of a 25 % sodium chloride solution and a 1 N trichloroacetic acid solution were added to the native anatoxin mixture. After holding for 1 h in a refrigerator, the mixture was centrifuged at 2-5 °C for 15 min. The resulting precipitate was dissolved in 100 ml of sterile saline (pH 6.8-7.0). The final step of anatoxin purification was ethanol precipitation with 2.5 volumes of pre-cooled (-20 °C) 96 % alcohol. The mixture was centrifuged at 2–3 °C. The resulting precipitate was dissolved in ice-cold saline (pH 6.8-7.0), filter sterilized (0.22 μm), and filled into ampoules.

To quantify the activity of our product, we determined its antibody-binding capacity (ABC). Results showed that the specific activity of anatoxin was 11 ABC, *i.e.,* it met the requirements of a standard drug. Similar result was obtained by examining the imported staphylococcal anatoxin (Medgamal, Moscow, Russia, 2019; anatoxin staphylococcal purified, C 174-0316, 1 ml; 11 ABC).

A second goal of the study was the development of a test system for post-vaccination immune response evaluation and rapid diagnosis of a staphylococcal infection in healthcare and veterinary practices. The passive (indirect) hemagglutination reaction is often used in diagnostic practices in Georgia and abroad [8-13]. It is easy to use and provides the test results in 1.5-2 h, but it requires expensive equipment.

One important step in the preparation of a highly sensitive and active diagnostic test system is the selection of the adsorbent (erythrocyte) stabilization method. Mostly, human Type I, sheep or goat erythrocytes, or latex particles are used. Formaldehyde was used to produce standard erythrocytes earlier [13]. The optimal number of formalin-treated erythrocytes in a 2.5 % suspension was 6 x 10^6 cells/ml. Erythrocytes were washed three times with saline, re-suspended in saline (pH 7.0-7.2), and counted. To increase the sensitivity and specificity of the diagnostic system (*i.e.,* to remove proteins, polysaccharides, and other impurities from the erythrocyte membrane surfaces), we treated in triplicates 5 ml of a 2.5% erythrocyte suspension with potassium dichromate ($K_2Cr_2O_7$) in different dilutions (1:1,000; 1:5,000; 1:20,000), and incubated these tubes at 37 °C for 20 min. Erythrocytes treated with potassium dichromate were washed with saline and the 2.5% suspension reestablished.

To further increase the sensitivity of the test system, 5 ml of the erythrocyte suspension were added to 5 ml of tannic acid (0,05 g/ml). The mixture was incubated at 37 °C for 20 min, washed twice with 1/15 M phosphate buffer (pH 7.2) and once with 1/15 M phosphate buffer (pH 6.4). After treatment, the erythrocytes were sensitized with the purified staphylococcal anatoxin. In terms of specific activity, the most sensitive test system was obtained by treating the erythrocytes with the 1:5,000 dilution of potassium dichromate. The optimal sensitizing dose of staphylococcal anatoxin is shown in Diagram 1 and Fig. 1.

Serial production of a sensitive test system depends on the optimal dose of anatoxin. This optimal dose is experimentally determined as the amount of anatoxin that detects antitoxic antibodies at a maximum dilution, while the control test is still negative. Table 1 shows that 30 μg of anatoxin detected low number of antibodies (11.42 ± 2.37). High dos-

Table 1. *Determination of optimal diagnostic dose for staphylococcal anatoxin*

№	Number of formalinized erythrocytes	Anatoxin dose [μg/ml]	Antibody titer range	Obtained result in PHAR (M±m) *	Probability of difference (P)
1	5.5x10^5	30	5–20	11.42±2.37	
2	5.5x10^5	80	16–640	457.1±88.7	<0.01
3	5.5x10^5	150	160–640	434.3±97.2	

** – Horse staphylococcal anti-toxin immunoglobulin (100 IU/ml) was used in the passive hemagglutination reaction.*

es resulted in higher titers, and the controls tended to be positive, risking over-diagnosis. The optimal amount of anatoxin was 80 μg/ml, which provided high titers of antibodies. Using the optimal dose, we prepared 3 small series of lyophilized drug, each in the amount of 35 - 40 ampoules (Fig. 2). This anatoxin diagnostic test system may now be used by experts for monitoring anti-staphylococcal immunoglobulin concentrations.

The activity of anti-staphylococcal serum or immunoglobulin in the reaction (Passive Hemagglutination Reaction; PHAR) may be expressed in international units (IU/ml), as proposed by Varlakova and co-workers [14]. We used the reference immunoglobulin (26 IU/ml, Microgen, Moscow, Russian Federation) and the test serum diluted 1:10. We calculated the test serum activity in international units (IU/ml) with the following formula:

$$X = C \times B/A \quad \text{(Equation 1)},$$

where X is the staphylococcal antitoxic antibody activity in test serum in IU/ml; A indicates the reference serum back titer PHAR; B is the test antitoxic serum PHAR; and C is the reference serum activity in IU/ml.

Additionally, the test system detects in a passive hemagglutination reaction the minimum number of antitoxic antibodies in human or animal serum, and converts the titer (1:10 – 1:40) detected in healthy people into international units:

$$X = C \times B/A = 26 \times 40/1{,}280 = 0.8 \text{ IU/ml} \quad \text{(Equation 2)}.$$

This PHAR number is 10 or more times greater than the Lymes-hemolysis data calculated through the currently accepted method for antitoxic antibody determination. The advantage of PHAR is that results were obtained in 1.5–2.0 h and the test does not require significant material and labor costs. In a Passive Hemagglutination Reaction, unlike in other ELISA tests, hemolyzed serum can be examined.

In short, the diagnostic test system developed and adopted uses lyophilized antigen (anatoxin) and sensitized erythrocytes to detect and quantify staphylococcal antitoxic antibodies in human and animal blood serum. The test system can be used not only in research but also in healthcare and veterinary practices to diagnose staphylococcal infections, as well as to evaluate post-vaccination immunity.

The specificity of the proposed antigenic (anatoxin) diagnostics was tested against sera from clinically healthy humans, patients with a staphylococcal infection (complicated furunculosis and post-operative abscesses), other bacteriologically confirmed toxigenic strains, a commercially available immunoglobulin (Tetanus Gama 250 IU/ml; N 022488062, Paskoli, Barga, Lukka, Italy), and healthy bovine sera (heifers and cows; age 1.5–3 years). Conventionally, diagnostic antitoxic antibody titers in humans correspond to dilutions of 1:80 and higher. Results of this study are shown in Table 2.

The table shows that the number of antitoxic antibodies in healthy human serum is significantly smaller compared to the numbers in infected humans.

Results for healthy human serum antibody titers ranging from 1:5 to 1:20 were confirmed in an earlier study [13]. The titer for normal serum antitoxic antibodies in the blood of unvaccinated animals (cattle) was 1:40 - 1:160 in PHAR. Test antibodies were within the normal range against the anti-tetanus IgG (1:20), in spite that the amount of anti-tet-

Table 2. *Indicators of staphylococcal anatoxin diagnostic test system specificity*

№	Test serum	Number of sera	Titer range	Antibody titer (M±m)	Probability of difference (P)
1	Normal human serum	9	5–20	12.7±2.6	
2	Human serum with staphylococcal infection	17	40-320	98.82±10.41	<0.05
3	Tetanus Gama [250 IU/ml]	1	1:20		
4	Normal bovine serum	11	20–160	74.5±14.36	

anus antibodies was 250 IU (if converted in PHAR, it is equivalent to 1:200,000 dilution). The titer of anti-alpha-toxin antibodies in people infected with staphylococci was higher than normal; the difference is statistically significant.

We also tested the system specificity in the Passive Hemagglutination Inhibition Reaction. Purified anatoxin (0.25 ml; activity 10 ABC) was added to antitoxic serum applied in a polystyrene microtiter plate, and incubated at 37 °C for 20 min. Subsequently, the diagnostic drug was dripped on it. We placed the plate again in the thermostat for 40 min and finally, left it for 20 min at room temperature. The obtained results showed that the reaction in the last 5 wells was negative, which indicated inhibition of the hemagglutination reaction and its specificity.

Conclusion

1. Staphylococcal anatoxin (harmless, immunogenic form of alpha hemolysin) was produced and studied. Its activity (11 ABC) meets the required standard.
2. The staphylococcal anatoxin is a treatment and prevention drug, which may be used in healthcare and veterinary practices.
3. A rapid diagnostic test system for staphylococcal infection (formalin- and tannic acid-treated sheep erythrocytes sensitized with staphylococcal anatoxin) was designed and produced. The diagnostic system is used in a Passive (Indirect) Hemagglutination Reaction (PHAR). Its sensitivity is equivalent to a standard ELISA test.
4. The test system is designed to detect and quantify antitoxic antibodies in human and animal sera, as well as to assess the post-vaccination immunity.

Acknowledgement

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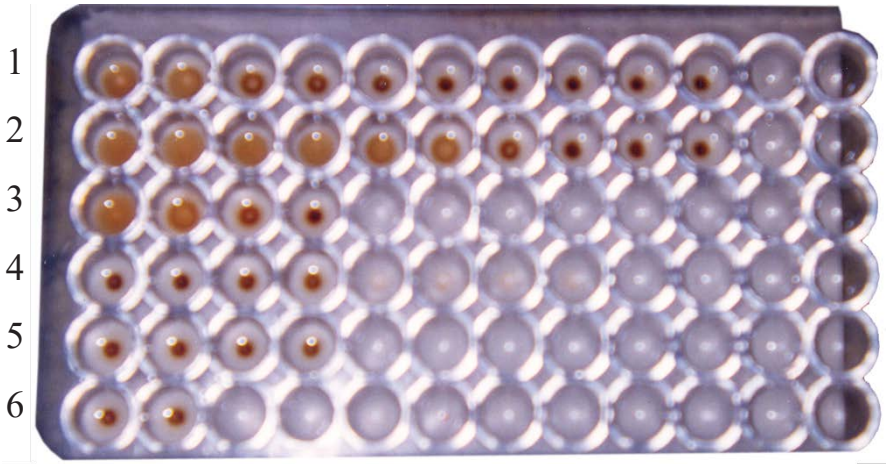

Fig. 1. *Determination of staphylococcal antitoxin antibodies in a Passive Hemagglutination Reaction (PHR). (Row 1: healthy cattle serum; Row 2: serum of a human with a staphylococcal infection; Row 3: healthy human serum; Rows 4, 5, and 6 are controls)*

Fig. 2. *Ampuls with the diagnostic test system*

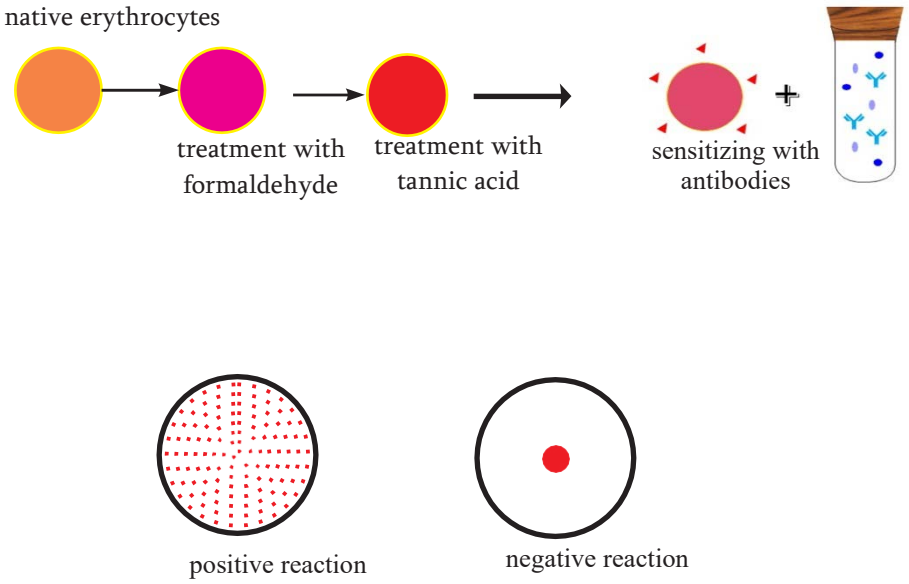

Diagram 1. *Passive (indirect) hemagglutination reaction*

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Comparative analysis of the three Caucasian oak taxa in Georgia (South Caucasus) based on leaf macromorphological variation

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ABSTRACT

Comprative analysis of three oak taxa (*Q. petraea* subsp. *iberica, Quercus robur* subsp. *imeretina*, and *Q. robur* subsp. *pedunculiflora*) based on the leaf morphological and morphometric analysis have been done. Fifteen statistically significant variables out of the original 24 leaf characters were identified by PCA. The ANOVA *F*-statistics together with Tukey's post hoc range test and PCA analysis allowed the identification of some leaf traits with ability to partly differentiate all three studied taxa from each other. Particularly, the leaf traits related to leaf petiole size and number of lobes and intercalary veins are much higher in *Q. petraea* subsp. *iberica* individuals compared to *Q. robur* subsp. *imeretina* samples, while in *Q. robur* subsp. *pedunculiflora* they are intermediate between these two taxa. Linear regression analysis reveals that statistically significant leaf traits combined in the first and second principal components negatively or positively, but significantly weakly or moderately influenced by ecological factors related to different annual and growth season moisture and temperature conditions.

Keywords: Caucasian oak taxa, Leaf morphometry, Principal Components Analysis, Regression Analysis, Ecological Factors, Leaf traits.

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Introduction

There are more than 500 different oaks species in the worldwide [1]. Among 18 oak species distributed in the Caucasus 7 grows in Georgia. *Pedunculatae* oaks presented by *Q. hartwissiana* Steven, *Q. robur* subsp. *imeretina* (Steven ex Woronow) Menitsky, *Q. robur* subsp. *pedunculifllora* (K. Koch) Menitsky), and *sessiliflorae* oaks with *Q. petraea* subsp. *dshorochensis* (K. Koch) Menitsky, *Q. macranthera* Fisch & C. A. Mey, ex Hohen, *Q. petraea* subsp. *iberica* (Steven ex M. Bieb.) Krasilln. and *Q. pontica* K. Koch.

In the economical and ecological point of view oak in the Georgian forest is considered as one of the most significant species. Oak stands are the most diverse and rich in terms of the floristic composition. Nowadays, they cover 10.5% of the forest area, which is about 241,000 hectares [2]. *Q. petraea* subsp. *iberica* occupies the largest area among the species in the oak genus. It is widely distributed in the forest of Georgia and very like to the most widespread species in Europe - rock oak (*Q. petraea* (Matt.) Liebl.), which replaces our species in the mountains of the North Caucasus [3, 4]. *Q. robur* subsp. *pedunculifllora* occurs at alluvial plains of the river basins of Eastern Georgia [5]. In the western Georgia it is replaced by *Q. robur* subsp. *imeretina*. Both of them belongs to be the group of Q. robur L. [6].

Extensive taxonomy study of oak was made by Krasilnikov [7, 8]. Based on the research he developed the interspecies systematics of some species, where subspecies is the main unit. The monograph of Menitsky „Oaks of Asia" also is important for the phylogenetic studies of the oak genus [9]. The issues of variability, evolution and taxonomy of the roburoid and galiferal Caucasian oaks are widely discussed here.

Hybridization and the presence of hybrid zones is a common fact among the oak species [9-11], which facilitates the high level of phenotypic plasticity and genetic diversity. Therefore, classification of oak genus, assessment of genetic differentiation between species and determination of population structure is quite difficult and a number of studies have been implemented [12-15]. High polymorphism of oak species is a result of introgressive hybridization processes and widespread in the area of coexistence of two or more species, facilitates conditions for conducting of studies for identification of morphological and genetical units and biodiversity on species and population levels [16-18].

Previous implemented studies in Georgia is important for identification of taxonomy data for oak species. Until now most molecular-genetic and morphological studies performed on European and American species [19-21] and there is relatively few information about those species occcured in Georgia [15, 22-24].

So, there is the less information about Caucasus oak, and there is a need to conduct studies for clarification of its taxonomical status and presented article is partly addressed to this issue. Therefore, research aim is to conduct a comparative analysis of three Caucasian oak taxa (*Quercus robur* subsp. *imeretina, Q. robur* subsp. *pedunculiflora and Q. petraea* subsp. *iberica*) based on leaf morphometric study.

Materials and Methods

Study Object and Study Area

The research object of the presented article are: *Q. robur* subsp. *imeretina*, *Q. robur* subsp. *pedunculiflora* and *Q. petraea* subsp. *iberica*. The field surveys were conducted in 2017-2019 years in the three regions of Georgia; Imereti (Western Georgia), Shida Kartli and Kakheti (Eastern Georgia). Only natural stands were included in the sampling.

Individuals of *Q. robur* subsp. *imeretina* presented by 10 locations and 80 trees were collected within the range of heir distribution in Imereti region (western Georgia), while the samples of *Q. petraea* subsp. *iberica* (9 locations, 85 trees) and *Q. robur* subsp. *pedunculiflora* (5 locations, 65 trees) were collected in different regions of eastern Georgia (Table 1). 5 healthy leaves were collected from each tree and the distance between them was at least 50 m. The total number of investigated leaves taken only from adult trees was 1150.

Slope declination, exposition, forest type, stand density, height and diameter of oak trees were scored for each study site (data not shown) according to forest taxation protocols [25-27]. The locations of investigated oak tax were presented in Fig. 1.

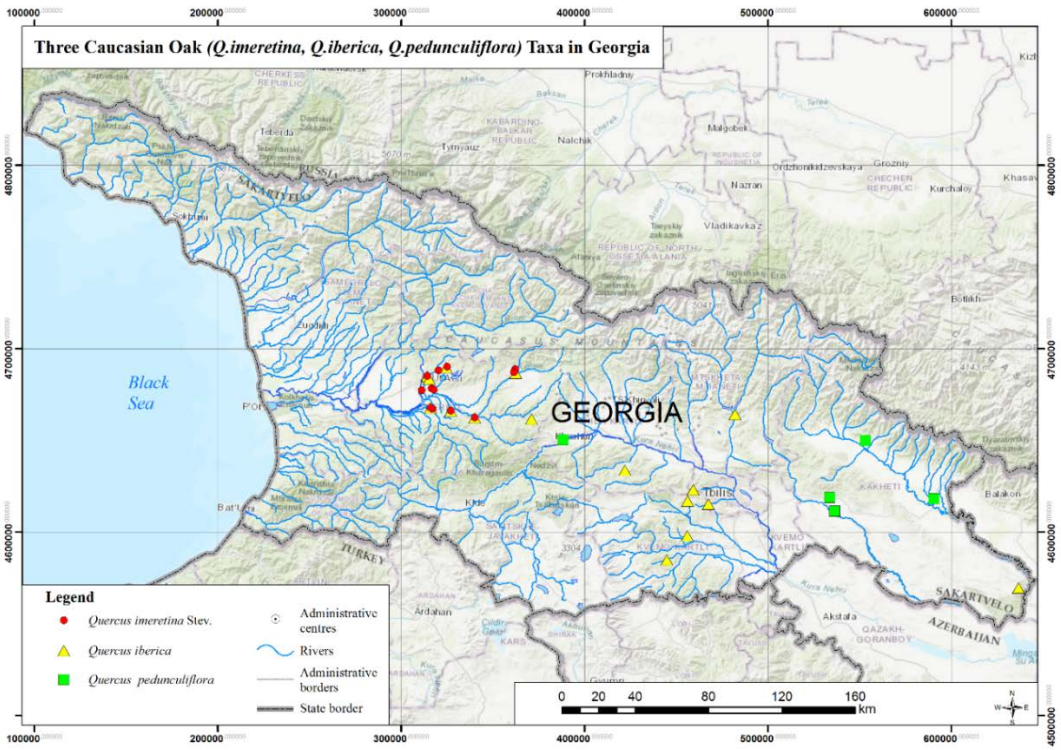

Fig. 1. *Ampuls with the diagnostic test system*

Climatic data

Ecological characterization of investigated oak taxa according to study sites was done in table 1. Climatic data for the last 34 years (1985-2019) of hourly weather model simulation for each selected localities were obtained from 'Meteoblue' (2019) (https://www.meteoblue.com/en/weather/historyclimate/climatemodelled/) that have a spatial resolution of approximately 30 km. We used annual and growth season precipitation amount (mm), sum of mean daily maximum temperatures (° C), number of sunny, partly cloudy and precipitation days for both periods, and mean daily maximum temperatures (° C) for each month of growth season to characterize the climatic heterogeneity of the environments in different locations of the Caucasian oak taxa in Georgia (South Caucasus) within the range of distribution. Growth season includes climatic data corresponding to May, June, July, August and September, respectively.

Table 1. *Ecological characterization of three Caucasian oak taxa in Georgia (South Caucasus) within the range of distribution. Data were obtained from the 'Meteoblue' (2019) (https://www.meteoblue.com/en/weather/historyclimate/climatemodelled/) for the last 34 years (1985-2019). * In case of Q. robur subsp. imeretina for the study location from Ajameti Managed Reserve (Imereti, western Georgia) samples were taken from different altitudes from 144 m to 205 m a.s.l.; accordingly, all ecological data were done for each altitude in the corresponding column.*

Abbreviation	Population	Altitude (m a.s.l.)	Latitude/ Longitude	Annual precipitation (mm) (mm)	Sum of annual mean daily maximum t (°C)	Annual sunny days	Annual partly cloudy days	Annual precipitation days	Growth season precipitation (mm)	Sum of growth season mean daily maximum t (°C)	Growth season sunny days	Growth season partly cloudy days	Growth season precipitation days	April mean daily maximum t (°C)	May mean daily maximum t (°C)	June mean daily maximum t (°C)	July mean daily maximum t (°C)	August mean daily maximum t (°C)
Q. petraea* subsp. *iberica																		
1	Zestaponi (Sviri), western Georgia	205	42°1291'/ 42°9084'	815	229	86.1	148.1	168.7	315	153	42.3	90.4	84.3	20	23	26	29	29
2	Chiatura (Darkveti), western Georgia	533	42°3217/ 43°3312'	815	187	86.1	148.1	168.7	315	132	42.3	90.4	84.3	16	20	23	25	26
3	Mount. „Shavi Mta", Kakheti, eastern Georgia	813	41°2671'/ 46°6303'	279	191	152.4	148.6	73.2	128	143	92.3	73.2	36.7	17	23	18	31	30
4	Vicinity of Betania Monastery, southwest of Tbilisi, eastern Georgia	835	41°6899'/ 44°6089'	262	185	124.1	159.5	84.7	134	138	70	86.7	43.6	16	20	25	28	27
5	Mtskheta (Vicinity of vil. Didgori), eastern Georgia	1600	41°7589'/ 44°9088'	229.6	125	124.1	159.5	84.7	101	108	70	86.7	43.6	11	15	20	23	22
Q. robur* subsp. *imeretina																		
1	Ajameti Managed Reserve, western Georgia	144	42°7745'/ 42°' 14165	1193	222	89.7	131.9	179	449	146	42.5	80.6	91	19	22	25	27	28
		171	42°7871'/ 42°13584'	1193	222	89.7	131.9	179	449	146	42.5	80.6	91	19	22	25	27	28
		205	42°1291'/ 42°9084'	815	229	86.1	148.1	168.7	315	153	42.3	90.4	84.3	20	23	26	29	29
2	Terjola (Vicinity of Brolis-kedi), western Georgia	159	42°2257'/ 42°79093'	1584	214	106.2	128.1	175.6	555	147	55.3	75.9	85.5	19	23	25	27	28
3	Zestaponi (vil. Chognari), western Georgia	168	42°236'/ 42°7759'	1584	213	106.2	128.1	175.6	555	147	55.3	75.9	85.5	19	23	25	27	28

4	Kutaisi (Saghorie Forest), western Georgia	173	42°2257'/ 42°7118'	1584	213	106.2	128.1	175.6	555	147	55.3	75.9	85.5	19	23	25	27	28
5	Tkibuli (Vicinity of vil. Orpiri), western Georgia	318	42°3250'/ 42°8190'	1584	199	106.2	128.1	175.6	555	147	55.3	75.9	85.5	18	22	24	26	26
6	Tkibuli (Vicinity of Khresili), western Georgia	392	42°3447/ 42°8759'	1594	193	181	128.1	175.6	565	137	55.3	75.9	85.5	17	22	24	25	26
7	Chiatura (Vicinity of vil. Zodi), western Georgia	604	42°3239'/ 43°3184'	1178	192	99.1	144.8	175.3	548	141	53.9	83.5	90.1	17	22	25	27	27
Q. robur* subsp. *pedunculiflora																		
1	Lagodekhi (vil. Heretiskari), eastern Georgia	225	41°7102/ 46°0874'	204	192	141.2	156.2	62.1	83	55	81	82.9	27.2	16	21	26	29	29
2	Kvareli (vil. Gremi), eastern Georgia	401	41°9999/ 45°6425'	983	225	88	158.1	170.8	535	160	53.9	90.9	91.6	19	24	29	31	31
3	Sagarejo (Vicinity of vil. Manavi), eastern Georgia	542	41°6549/ 45°437'	270	211	150.5	148.7	73.9	150	156	88.5	75.7	38.2	18	23	28	31	31
4	Khashuri (vil.Osiauri), eastern Georgia	670	41°9982/ 43°6523′	432	181	67.7	183	142	179	133	38.6	106	68.3	16	20	23	26	26

Leaf parameters

In leaf morphological characterization was based on descriptor list for Oak species (Fig. 2) according to Kremer et al. [28]. All leaves were digitized; morphometric parameters were measured using the program ImageJ1.47v (https://imagej.net/Plugins). Material taken from the field was processed into herbarium specimens.

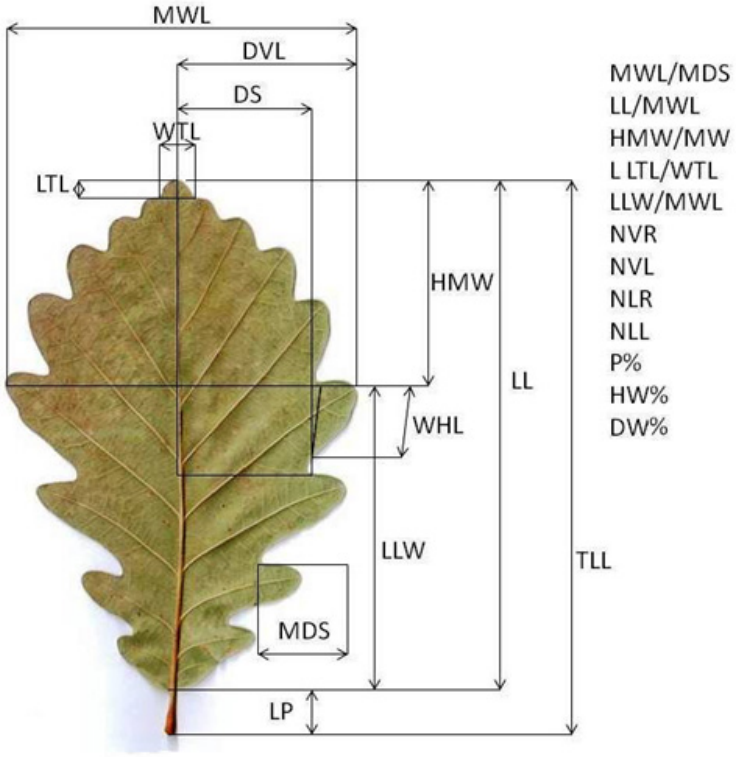

Fig. 2. *Leaf parameters*

Data analysis

Analysis of variance (ANOVA) together with Tukey's HSD post hoc range test (P< 0.001) was used to analyze leaf macromorphological differences in the investigated Caucasian oak taxa. At first, a principal component analysis (PCA) was used to remove highly correlated variables and replace the entire data file with a smaller number of uncorrelated variables. PCA was then carried out with the selected variables, and these were reduced to three principal components representing most of the information in the original dataset. A principal component (PC) solution was determined based on scree plot and Kaiser criterion (all eigenvalues greater than 1). Regression analysis were conducted to evaluate the correlation between principal components (PCs) and ecological factors (r) and proportion of the variance (r^2, %) for a dependent variables (PCs) that explained by independent variables or ecological factors in the regression model.

The statistical package SPSS ver. 21.0 (https://www-01.ibm.com/) was used for ANOVA with multiple comparisons, while PCA and regression analysis were performed by software PAST [29].

Results

Leaf trait variability comparison among the three Caucasian oak taxa

15 statistically significant variables out of the original 24 macromorphological leaf traits were identified by PCA. The ANOVA *F*-statistics together with Tukey's post hoc range test (p <0.001; Table 3) allowed the identification of some studied leaf traits with ability to differentiate all three studied taxa from each other. Particularly, mean values of the leaf petiole length (LP), number of intercalary veins on both sides (NVR, NVL) and percentage of leaf petiole in total leaf length (P, %) (Table 2) are much higher in *Q. petraea* subsp. *iberica* individu-

Table 2. *Means (AV), Standard Deviations (SD) and F values of leaf traits in three Caucasian oak taxa. Comparisons among oak taxa were done using one-way ANOVA analyses with Tukey's HSD test; asterisks indicate overall significance of the F-statistics (* – p < 0.05; ** – p < 0.01, *** – p<0.001), and the letters indicate significant differences among the means at p<0.001 according to Tukey's HSD test.*

Leaf traits	Oak taxa			*F*
	Q. petraea subsp. *iberica*	*Q. robur* subsp. *imeretina*	*Q. robur* subsp. *pedunculiflora*	
LP (Length of petiole)	1.44±0.4[a]	0.29±0.14[b]	0.94±0.28[c]	682.14***
HMW (Height of maximal width)	4.82±1.04[a]	3.86±0.95[b]	4.13±0.17[b]	31.78***
MDS (Maximal depth of sinus)	1.42±0.54[a]	1.72±0.51[b]	1.6±0.49[ab]	11.48***
WHL (Width of the most handing lobe)	1.5±0.35[a]	1.75±0.42[b]	1.77±0.38[b]	16.09***
DVL (Distance of the principal vein to top of the most handing lobe)	3.03±0.85[a]	2.72±0.58[b]	2.88±0.61[ab]	7.49***
WTL (width of the terminal lobe)	0.61±0.24[a]	0.89±0.46[b]	0.5±0.36[a]	25.41***
LTL (length of the terminal lobe)	0.95±0.33[a]	1.16±0.37[b]	0.82±0.4[a]	31.29***
NLR (Number of lobes on the right side)	7.75±1.48[a]	5.68±1.3[b]	6.43±1.75[b]	150.81***
NLL (Number of lobes on the left side)	7.7±1.56[a]	5.68±1.23[b]	6.2±1.6[b]	143.32***
NVR (Number of intercalary on the right side)	8.17±1.48[a]	4.5±1.18[b]	6.69±1.31[c]	298.22***
NVL (Number of intercalary on the left side)	8.12±1.52[a]	4.56±1.06[b]	6.33±1.48[c]	278.76***
TLL (Total leaf length (LL+LP))	11.31±1.63[a]	9.46±1.33[b]	10.33±1.7[c]	55.41***
P (Length of petiole x 100/ total leaf length)	12.85±3.69[a]	3.12±1.33[b]	9.03±2.15[c]	632.55***
HMW/MWL	0.83±0.2[a]	0.71±0.19[b]	0.73±0.22[ab]	12.81***
LLW/MWL	0.11±0.05[a]	0.17±0.1[b]	0.09±0.07[a]	30.81***

als compared to *Q. robur* subsp. *imeretina* samples, while in *Q. robur* subsp. *pedunculiflora* they are intermediate between these two taxa. Additionally, Tukey's post hoc multiple comparisons at $p<0.0001$ identified some traits shared between *iberica-pedunculiflora* and *imeretina-pedunculiflora* sample pairs. The mean differences of sizes in terminal lobes (WTL, LTL) and ratios between dimensions of terminal lobe and lamina (HMW/MWL, LLW/MWL) are not significantly different between *iberica-pedunculiflora* sample pairs, while traits related to the height of maximal width and the most handing lobe (HMW, WHL), and number of lobes on the both sides (NLR, NLL) do not show significant differences among *imeretina* and *pedunculiflora* individuals (Table 2).

The PCA gave congruent results and revealed that the first three principal components account for 74.55% of the total variation in the dataset (40.27, 18.11, and 16.17%, respectively; Table 3). The first PCA is mainly influenced by variables expressing leaf size and lobes and veins number and mostly differentiates *Q. petraea* subsp. *iberica* and *Q. robur* subsp. *imeretina* samples with partly overlapping of individuals from *Q. robur* subsp. pedunculiflora and *Q. robur* subsp. *imeretina* group in the middle part of the plot (Fig.1). The second PCA is influenced dimensions related to maximal depth of sinus and width of the most handing lobe, while the third PCA is related to sizes in terminal lobes, and ratio between the length and width of lamina (Table 3) but both provided no further subdivision.Linear re-

Table 3. *Eigenvalues, percentages of explained variance and cumulative percentage of explained variance, contribution of the variables to the first three principal components values of each leaf character in 3 Caucasian oak taxa. Redundant variables: LL (Length of lamina); MWL (Maximal width of lamina); DS (Distance of the principal vein to the sinus); HW (height of maximal width x 100/ total leaf length); LLW (Length of lamina from base to the widest part (LL-HMW); DW (Height of maximal width x 100/ total leaf length); HMW/MWL; LTL/WTL; LLW/MWL*

Total variance explained	**PC1**	**PC2**	**PC3**
Eigenvalues	6.04	2.72	2.43
Percentage of explained variance	40.27	18.11	16.17
Cumulative percentage of variance	40.27	58.38	74.55
Leaf character	**Eigenvalues**		
LP (Length of petiole**)**	0.31		
HMW (Height of maximal width**)**			0.5
MDS (Maximal depth of sinus**)**		0.5	
WHL (Width of the most handing lobe**)**		0.47	
DVL (Distance of the principal vein to top of the most handing lobe**)**		0.48	
WTL (width of the terminal lobe)			0.34
LTL (length of the terminal lobe)			0.4
NLR (Number of lobes on the right side**)**	0.36		
NLL (Number of lobes on the left side**)**	0.36		
NVR (Number of intercalary on the right side**)**	0.34		
NVL (Number of intercalary on the left side**)**	0.36		
TLL (Total leaf length (LL+LP)**)**	0.26	0.27	
P (Length of petiole x 100/ total leaf length**)**	0.29		
HMW/MWL			0.4
LLW/MWL			0.33

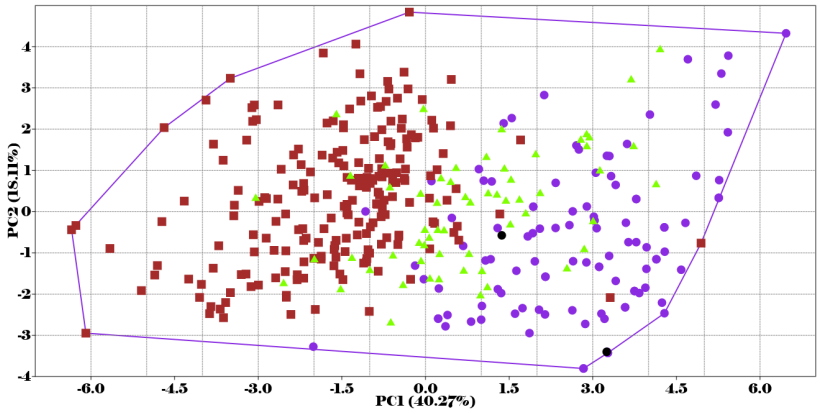

Fig. 3. *Principal component analysis (PCA) plot based on studied leaf traits of the three Caucasian oak taxa individuals in Georgia within the range of distribution with different moisture conditions and temperatures. Violet circles indicate samples of Q. petraea subsp. iberica; dark brown squares - Q. robur subsp. imeretina and light green triangles – Q. robur subsp. pedunculiflora.*

gression analysis based on ecological factors related to different annual and growth season moisture and temperature conditions allowed to identify statistically significant linear influence on differentiation of the leaf trait values ($p < 0.0001$, Table 4) between the investigated Caucasian oak taxa in Georgia. All the trait values explained by PCs (PC1, PC2) negatively or positively but significantly weakly or moderately ($r \leq 0.3$-0.6) correlate with all ecological factors included in the presented study. Among temperature conditions, the highest negatively significant influence was accounted for mean daily maximum t (°C) in April (r=-0.48 for PC1, r=-0.33 for PC2, Table 4), and significantly positive influence

Table 4. *Results of regression analysis (r^2 – coefficient of determination of the proportion of variance for the dependent first two principal components (PC1, PC2) that explained by independent ecological factors in the regression model; r_{xy} – coefficient of correlation between principal components (PC1, PC2) and ecological factors; ^{ns} not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).*

Trait	PC1		PC2	
	r^2 (%)	r_{xy}	r^2	r_{xy}
Latitude	21	-0.46 ***	13	-0.37 ***
Longitude	26	0.51 ***	7	0.27 ***
Altitude (m a,s,l,)	13	0.37 ***	8	0.29 ***
Annual Precipitation	35	-0.60 ***	17	-0.41 ***
Sum of annual mean daily maximum t (°C)	6	-0.23 ***	2	-0.15 **
Annual sunny days	1	0.03^{ns}	1	0.12 *
Annual partly cloudy days	31	0.55 ***	6	0.25 ***
Annual precipitation days	24	-0.49 ***	13	-0.36 ***
Growth season precipitation (mm)	32	-0.57 ***	18	-0.42 ***
Sum of growth season mean daily maximum t (°C)	1	-0.11 *	1	-0.09^{ns}
Growth season sunny days	13	-0.36 ***	6	-0.24 ***
Growth season partly cloudy days	0	0.06^{ns}	0	0.008^{ns}
Growth season precipitation days	23	-0.48 ***	11	-0.33 ***
April mean daily maximum t (°C)	23	-0.48 ***	12	-0.34 ***
May mean daily maximum t (°C)	9	-0.29 ***	3	-0.17 *
June mean daily maximum t (°C)	6	-0.25 ***	3	-0.17 ***
July mean daily maximum t (°C)	1	-0.12 *	3	-0.19 ***
August mean daily maximum t (°C)	5	0.22 ***	3	0.16 ***

was shown for August (r=0.22 for PC1, r=0.16 for PC2, Table 4), respectively. Furthermore, the significantly negative influence of annual sum of mean daily maximum t (°C) on leaf trait variability was relatively weakly higher than for the growth season (Table 4). Site (latitude, longitude, altitude) and moisture conditions, and number of partly cloudy and precipitation days moderately (r ≥0.3-0.6, p<0.0001, Table4) influenced on leaf trait variation. Among them, the most influenced were annual (r=-0.6*** for PC1 and r=-0.41, *** for PC2, Table 4) and growth season (r=-0.57*** for PC1 and r=-0.42, *** for PC2, Table 4) precipitation; they explained 32-35 % (Table 4) of the proportion of variance for the leaf trait variation related to leaf size and lobes and veins number. Particularly, with the increase of precipitation these parameters in the investigated oak taxa were decreased. Additionally, it was shown that growth season sunny days' number (r=-0.36 for PC1, r=-0.24 for PC2, Table4) was negatively and moderately significantly influenced on leaf trait variation compared to the number of annual sunny days (not significant for PC1 and significantly weak for PC2, Table 4), respectively.

Discussion

Our univariate (ANOVA with Tukey's multiple comparisons) and multivariate (PCA) analysis allowed the identification some leaf traits with ability to partly differentiate studied taxa from each other. Particularly, the leaf traits related to leaf petiole size and number of lobes and intercalary veins are much higher in *Q. petraea* subsp. *iberica* individuals compared to *Q. robur* subsp. *imeretina* samples, while in *Q. robur* subsp. *pedunculiflora* they are intermediate between these two taxa and share more pronounce similarity with the first one. The sharing/convergence of leaf traits between *pedunculatae* and *sessiliflorae* oaks is not an occasional phenomenon. As in the case of other sympatric and closely related oak species, this could be explained with the incomplete reproductive isolation that characterize oaks in general [13, 30] and overlapping morphological variation due to ecological adaptation [15, 31, 32]. Conversely, *Q. petraea* s.l. and *Q. robur* s.l. generally grow in different ecological niches and areas in the Caucasus, with the second species occurring exclusively in lowland, mesophylous forests. The obtained morphological differences in leaf sizes related to petiole size together with number of lobes and veins recorded between the *Q. petraea* subsp. *iberica* and *Q. robur* subsp. *imeretina* samples might therefore reflect isolation and strong ecological adaptation to different environments [15, 28]. As frequently mentioned, no absolute diagnostic character discriminating between oak taxa could be detected. As the authors [33] noted, *Q. robur* s. l. and *Q. petraea* s. l. offspring can be discriminated in experimental or natural conditions, whenever shaded or not by surrounding adult trees, and preferably using leaves from the first flush. Masking the species differences could result from non-random mating in small, fragmented woodland populations. Hybridization and introgression between the species could also have played a significant role [34].

Furthermore, according to our results there is a linear dependence between ecological factors (annual and growth season precipitation and temperature conditions) and the leaf trait values. There is a correlation between macromorphological features explained by first two principal components and studied ecological factors. Leaf trait variability obtained in our study negatively or positively but significantly weakly or moderately correlate with all ecological factors. Particularly, with the increase of precipitation, leaf size and number of veins decrease. It is also important that the number of sunny days during the growing season, negatively and moderately significantly influenced on leaf trait variation. Based on the obtained data we can assume, that the investigated oak taxa (*Quercus robur* subsp. *imeretina*, *Q. robur* subsp. *pedunculiflora*, *Q. petraea* subsp. *iberica)* in the different climatic conditions give us different results depending on the humidity indicators and the ecotope where they are distributed.

More extensive investigations with additional morphological descriptors (e.g., flower organs, cupule scales, pubescence, trichome shape) together with molecular markers, the self-ecology of each taxon are needed to clearly assess the true taxonomic status of these oaks and understand the existent oak biodiversity in the Caucasus to assist conservation of this important biome.

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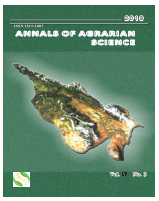

A new population of *Zelkova carpinifolia* (Pall.) K. Koch in environs of Tbilisi – characteristics of the species growth, regeneration and soil conditions of the site

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ABSTRACT

A new population of a Tertiary relict tree - Caucasian elm - *Zelkova carpinifolia* (Pall.) K. Koch was discovered in September of 2019 by the researchers of the National Botanical Garden of Georgia (NBGG) during the field studies of woody species, growing in Tbilisi environs, planned within the scope of the research project of the Department of Woody Plants Collections.

A new site of occurrence of *Zelkova carpinifolia*, located in the mostly semiarid zone, near the Jvari Monastery, was unknown so far. Zelkova stands in Georgia have been recorded in humid areas, where total annual precipitation makes: 1157 mm - in Ajameti Reserve (western Georgia) and 900-1100 mm - in Babaneuri State Reserve (eastern Georgia). Total annual precipitation in the study area, which formerly was a southern part of Tbilisi National Park, does not exceed 400-560 mm. On the study area Caucasian elm occurs as a pure stand as well as in a mixed stand with hornbeam – *Carpinus orientalis* and Georgian oak – *Quercus iberica*. The newly described population is of mixed age – growing individuals as well as trees of 20-95 years age occur here. Natural regeneration of Zelkova is represented around the individual trees by root offsprings of different age, diameter and height, emerged around the individual trees, which number varies between 6-15 and depends on the density level of hornbeam and oak cover. Propagation of Zelkova by seed seems to be low. It occurs here mostly on the forest clearings and natural meadows as single individuals of different age and height. The studied Zelkova stands are developed on a cinnamonic soils, which are characterized by the nut-granular structure with a light clayey mechanical composition, with neutral or slightly alkaline pH. The herbarium samples from the newly described population of *Zelkova carpinifolia*, dated by 11 June, 2020 are deposited in the National Herbarium of Georgia (TBI) under the barcodes TBI 1060 195 and TBI 1060 196. Discovery of a new population of *Zelkova carpinifolia* in environs of Tbilisi is of great interest for the study of biogeography and history of this ancient species.

Keywords: *Zelkova carpinifolia*, Tertiary relict, Pure stand, Longitudinal growth, Annual increment, Root sucker.

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Introduction

In September of 2019 in the course of one of planned fieldwork expeditions in Tbilisi environs within the scope of the research project "Reasons of drying of woody cultures planted in Tbilisi environs at different times, choosing of steady woody species for the afforestation and development of agricultural recommendations for planting", aimed at studying growth and development of woody plants, on the territory, located north-west of Jvari Monastery, (which is adjacent to the former southern part of the Tbilisi National Park), the researchers of the National Botanical Garden of Georgia have discovered a new stand of Caucasian elm - *Zelkova carpinifolia* (Pall) C. Koch which was unknown so far. According to the existing literature Caucasian elm was reported from the west Georgia – Samegrelo and Imereti, though in East Georgia it was recorded by V. Mirzashvili [1] and I. Abashidze [2] only on the 747 ha area of the Babaneuri State reserve, where it grows whether as a pure stand or is mixed with Georgian oak (*Quercus iberica*) and field maple (*Acer campestre*).

Species united in this genus are represented by tall or medium height trees. They are distributed in South Caucasus, in Northern part of Iran and Japan.

According to the data by G. Kozlovski and J. Gratzfeld [3] two of six species, united in Genus Zelkova - *Z. sicula* and *Z. abelicea* are circumscribed to the Mediterranean basin*;* one - *Z. carpinifolia* - to the west Eurasia and South Caucasus region; *Z. serrata, Z. schneideriana* and *Z. sinica* to the East Asia. By the data of G. Kozlowski, et al. [4], genus Zelkova is distributed in 12 countries, and among them *Z. sicula* is endemic to the Sicily and *Zelkova abelicia* is endemic of Greece.

Stem of Zelkova is of greyish color or has a rusty coloration, where an old skin is peeling off, giving it appearance of a rusty iron column. Trees grow up to 20-30 m height and the girth does not exceed one and half meter.

Stem is void of lateral branches to a quite big height and develops as an erect tree. Small branches are developed upper, close to the top. Because of this the stem has a small number of branches and it is straight. Young shoots are of greyish-brown color. Buds are sessile and alternately arranged, as well as leaves. Leaves coarse, leathery, venation palmate, serrate, dark-green from above, lustrous. Two types of flowers develop – bisexual and male. Perianth bell-shaped, 4-5 lobed, film-like and dry. Number of pollen 4-5. Ovary sessile, indirectly ovate, smooth, single-lobed, with a single seed. Stigma 2, fruit dry, convex ovate nutlet, with a single seed, cotyledons convex, double-lobed at the base and the top.

Only one species of the genus Zelkova - *Zelkova carpinifolia* is spread on the territory of Georgia. Mostly tall trees with greysih brown stem occur, annual shoots reddish-brown, tomentose, peeling up with rusty colour. Bent outwards leaves oblong or oblong-elliptic, cuneate or blunt. Cordate at a base. Teeth blunt, dark green from above, light green on the lower surface. Flowers greenish-white or brown. Flowering takes place in March, together with leaf emergence. Fruit ripens in August-September. Fruit is nut(let), bare or slightly tomentose. Seed is of brown colour.

Latin name of the genus originates from merging of two Georgian words – dzeli (pillar) and kva (stone) and reflectes the properties of its wood – hard like a stone and endured. Wood of Zelkova is distinguished with high technical characteristics. It is of light color, very dense, elastic, but is subject to the mechanical processing and can be well polished. By the endurance it holds one of primary positions among different kinds of wood. In the west Georgia, where wood decays easily and in a short period due to high temperature, high relative humidity and abundant precipitation, wood of Zelkova persists undamaged for centuries. It is also hardly damaged by pests. Because of this it has a quite wide application and is an unrivalled material for the construction. Exactly these properties caused decrease in its stocks in the distribution range. The species is included in the Red List of Georgia with the category VU (vulnerable) (The Decree of the Government of Georgia #190, 20 February, 2014) [5] and the Global Red Red list of Zelkova [4, 6]. A series of works dedicated to Zelkova have been published recently [7-9].

Object of Study and Methodology

A new stand of Zelkova carpinifolia has been described from the site, which has not been reported so far. It is located north of Jvari Monastery, on the south facing slope of 10-15˚ inclination, coordinates near the outer boundary are the following: Lat. N 41.84264 Long. E 044.73967 H=541 m; Lat. N 41.84333 Long. E044.74028, H=590 m; Lat. N 41.84264 Long. E0 44.739670 H=603.

On the studied site stand of Zelkova carpinifolia is represented as a pure stand and also is mixed with Georgian oak and Hornbeam at a share 7zlk+2hrnb+1oak. As calculated using GPS, a total area occupied by Zelkova attains 17.674 ha.

The herbarium samples from the newly described population of Zelkova carpnifolia were collected on 11 June, 2020 and are deposited in the National Herbarium of Georgia (TBI) under the barcodes TBI 1060 195 and TBI 1060 196.

Under the Zelkova carpinifolia stand the cinnamomic soils of different depth, depending on the landscape, have been described. Their mechanical composition was studied using the pyrophosphate method [10]. pH was determined according to [11], content of carbonates - according to Geisler-Maksimyuk method [10].

Sample plots have been identified on the study area according to the method by V. Gulissashvili [12], where the working platforms of 2x2 m size have been delimited, where natural regeneration of Zelkova was studied. Height of plants in (m), diameter at the taxation height (1.3 m), and age of trees was determined using the Pressler's borer. Character of Zelkova longitudinal and diameter growth was determined using a complex analysis, accepted in Forest taxation.

Fig. 1. *View on a newly described stand of Zelkova carpinifolia situated near the Jvari Monastery*

Results and Discussion

A newly described stand of Zelkova grows on the territory, located north of Jvari Monastery. The study area is located in the zone of thin arid forests or light woodland, which according to V. Gulissashvili [12] and N. Ketskhoveli [13] starts from the western part of Tbilisi and spreads on the slopes of left and right banks of river Mtkvari at 400-600m ASL.

Summer is hot and winter is warm in the zone of the study area. Mean temperature in January fluctuates near 0°C, though temperature of the hottest months – July and August is within the range of 23.3-27.7°C. Maximum precipitation falls in May and the first half of June, and minimum - in January. June and July are distinguished by especially scarce precipitation. Exactly in these months is recorded the lowest index of air relative humidity (15-18%) and a complete drying of the fertile layer of soil, due to which the trees are forced to pass into the dormant state, the leaves are deformed, then become yellow and fall down. Also early drying of the grass cover takes place. Similar process was described by Tselniker [14], whose long term observations have proved that periodical drying of soil below the maximum index of hygroscopic humidity causes development of special adaptive mechanism to these extreme conditions, like complete turning to yellow of leaves of the lower part of woody plants crone and their abscission, as well as passing of woody plants into the state of forced dormancy.

Cinnamonic soils of different depth are described on the study area, which lower boundary passes through the cinnamomic meadow soils. The mentioned soils are developed on loess like sediments and sandstone of different levels of exhaustion. Genesis of the typical cinnamonic soils of Georgia, their physical and chemical properties are comprehensively studied by T. Urushadze [15]. Thus here we present a general characterization of the soil underlying the *Zelkova carpinifolia* stand. The studied soils have the following build of the profile: A-AB-BC-CD. Soil, presented on the study object is almost identical, they differ only by the strength of horizons. Due to uneven relief the depth of soil layer on the study object varies from 25-38 cm to 52 cm. A and AB horizons of the soil are characterized by dark brown colour, fine grained structure, clay-like mechanical composition of loose texture and high concentration of tree roots. The soil is characterized by the formation of clayey minerals in the middle layers of the profile, being expressed by the maximum content of the fraction of physical clay and its decrease along with the soil depth. As

to the pH of the soil, it fluctuates from the neutral (in the horizon A) to slightly alkaline (in the lower layers of the soil). Neutral pH of the upper layer of the soil indicates acidifying effect of the litter and detritus. By the content of humus soils under *Zelkova carpinifolia* stands are attributed to moderately fertile soils. Content of humus in upper soil horizon varies within the range of 3.10-3.22% and significantly decreases with the soil depth. Content of carbonates in the upper horizon is not detected, but it increases along with the depth and is conditioned by the influence of the mother rock.

For the studies of the main forest forming species in Georgia, the scale of natural regeneration, developed by V. Gulissashvli [12] for mountainous forests is accepted, though it is not applicable for the woody species of arid thin forests and *Zelkova carpinifolia*, among them, as in the complicated edaphic and climatic conditions of East Georgia propagation of Zelkova by seed is not satisfactory. It mostly occurs in forest clearings and windows in the form of single individuals of different age and height. On the study area natural regeneration of a species mostly can be viewed as root offsprings around the different age *Zelkova carpinifolia* trees and, depending on the level of density of hornbeam cover, their number fluctuates within the rage of 6-15 individuals of different age, height and diameter. Number of root suckers is less around the big diameter (>25 cm) and tall (>10 m) trees ad it does not exceed 5-8 individuals.

On the study area *Zelkova carpinifolia* stand is mostly mixed with oak and hornbeam 7zlk+2hrnb+1oak+1ash. Small part of the population is represented as a pure stand. The first storey is represented by *Zelkova carpinifolia* and the second – by hornbeam (*Carpinus orientalis*). The studied stand is of mixed age – 3-5 years old years growing individuals as well as more ripe and completely ripe 85-92 years old trees of Caucasian elm grow here.

I. Abashidze [2] notes that stands of *Zelkova carpinifolia* in Georgia are mostly spread in humid areas, where mean annual precipitation is high. According to literary sources, mean annual precipitation on our study area does not exceed 400-560 mm, though in the main area of its distribution mean annual precipitation is the following: 1157 mm - in Ajameti Reserve and 900-1100 mm - in Babaneuri State Reserve.

Thus studying of taxation indexes of *Zelkova carpinifolia* through time is of interest both from theoretical and practical standpoints, as this study allows for the analysis of the entire cycle of growth and development of *Zelkova carpinifolia* from germination to withering in conditions of dry environment.

With this aim for the complex analysis a model tree of 5.44 m height and 18.55cm diameter has been chosen. Dendrometric data of the stem of *Zelkova carpinifolia* tree (Table) have shown that its height at 5 years age was 52 cm, and at 10 years age it attained 104 cm, indicating that by 10 yeas age longitudinally it grew annually by 10.4 cm.

Table *Dendrometric data of Zelkova carpinifolia stem*

Age (year)	Height (m)	Diameter (cm)	Height increment (m)		Diameter increment (cm)	
			average	current	average	current
5	0,52	-	0.25	-	-	-
10	1.04	2.41	0.31	0.30	0.24	-
15	1.55	5.15	0.38	0.33	0.34	0.55
20	2.21	7.90	0.39	0.34	0.41	0.63
25	2.94	10.65	0.41	0.35	0.43	0.48
30	3.64	13.32	0.41	0.35	0.43	0.44
35	4.30	15.54	0.40	0.32	0.40	0.32
40	4.95	17.29	0.31	0.30	0.40	0.31
44	5.44	18.55	0.29	0.16	0.36	0.26

At 15 years of age the rate of longitudinal growth of *Zelkova carpinifolia* slightly, but improves and reaches the maximum by 25 years of age, when its annual increment reaches 14.6 cm. From 25-30 years of age growth in height is slightly slowed down, indicating that despite the unfavorable edaphic and climatic conditions, existing on the site, *Zelkova carpinifolia* is characterized by stable growth and development, that is well expressed on the graph (Fig. 2).

Diameter of shoot offspring of *Zelkova carpinifolia* at 10 years of age was 2.41 (Table). At 15 years of age it was 5.15 cm and at 20 years of age - 7.9 cm, indicating that annual increment of the tree diameter was 2.74-2.75 cm. Similar increment of diameter is registered by 30 years of age. After 30 years increment of diameter gradually decreases and at 44 years of age it is only 1.26 cm, that is well reflected in the Fig. 3.

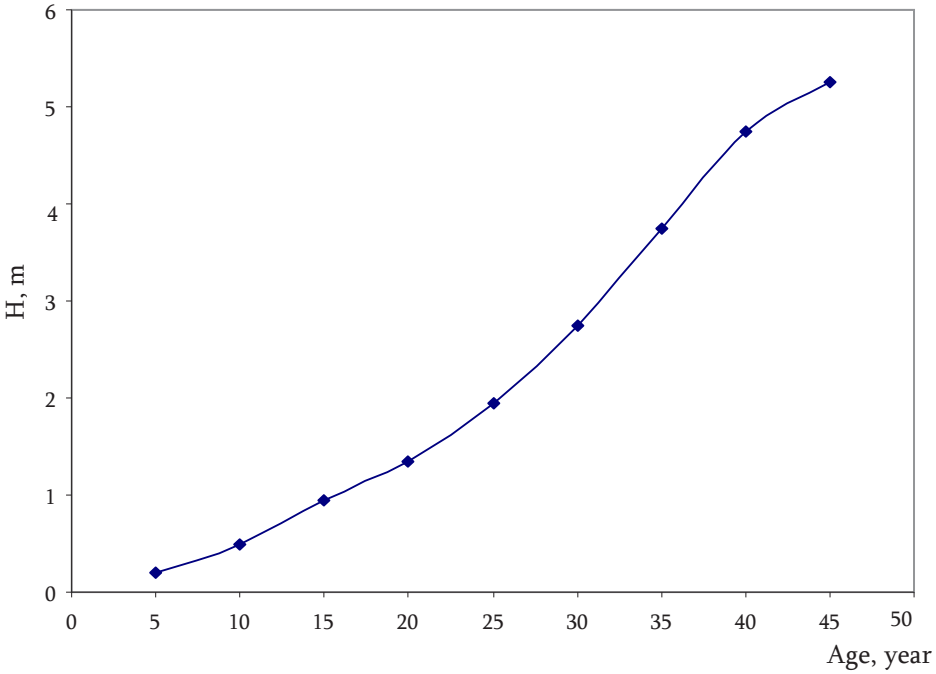

Fig. 2. *Longitudinal growth of Zelkova carpinifolia*

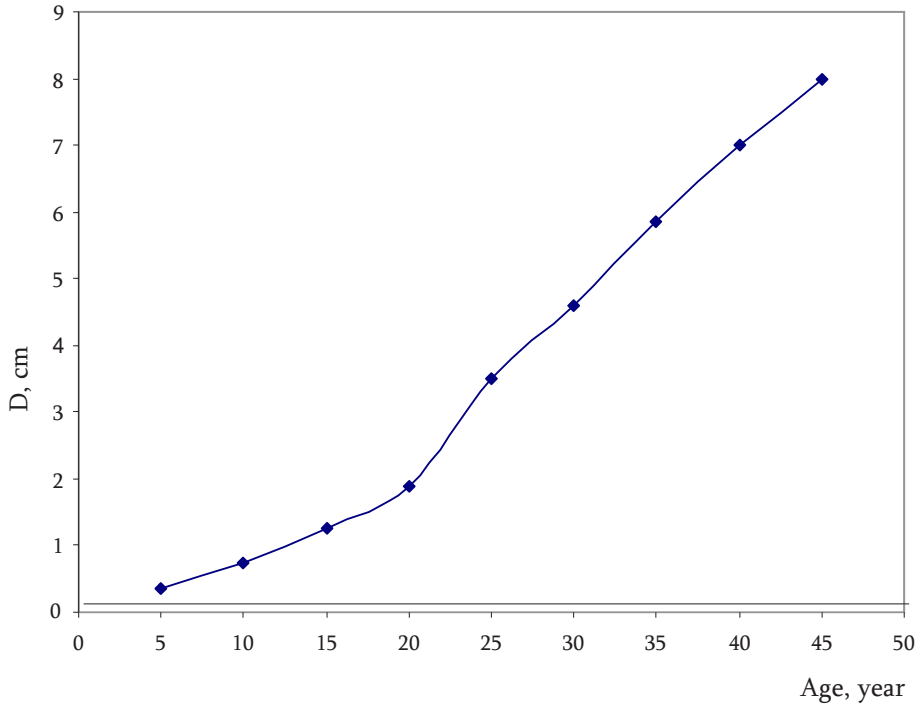

Fig. 3. *Growth of Zelkova carpinifolia in diameter*

A quite high increment of root offsprings of *Zelkova carpinifolia* in diameter and height until 25-30 years of age is conditioned by the influence of the root system of a mother tree. Upon establishing of the own root system this influence ceases.

Indexes of average and current increment in height and diameter (Fig. 4, 5) reach their maximum at 25-30 years of age and from 30 years of age they gradually decrease.

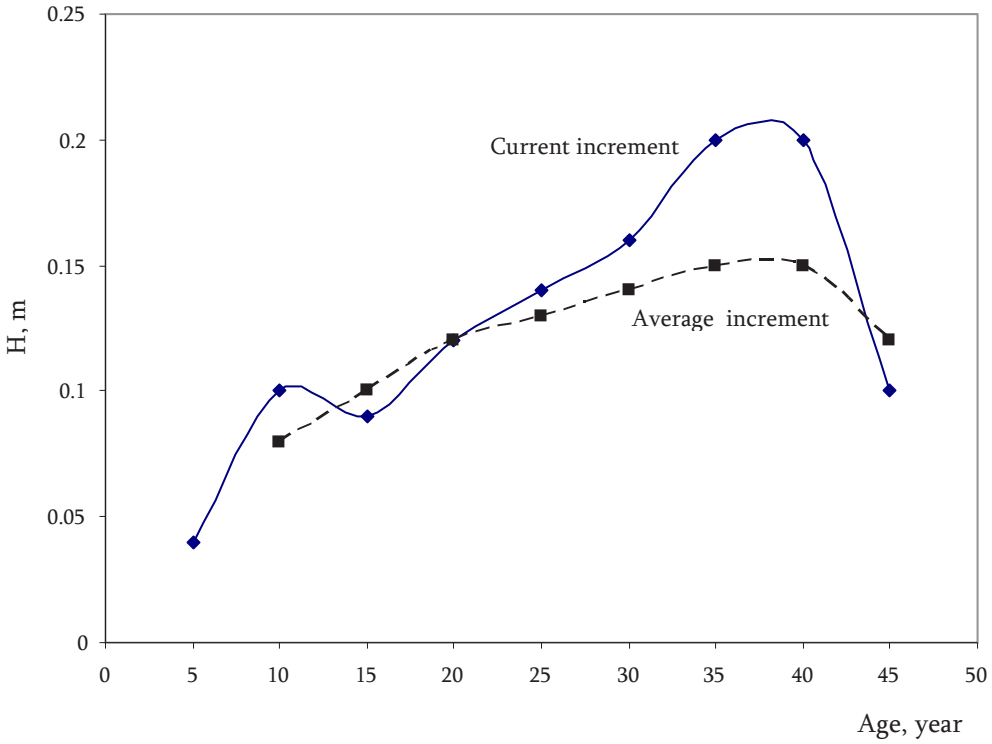

Fig. 4. *Current and average height increment of Zelkova carpinifolia*

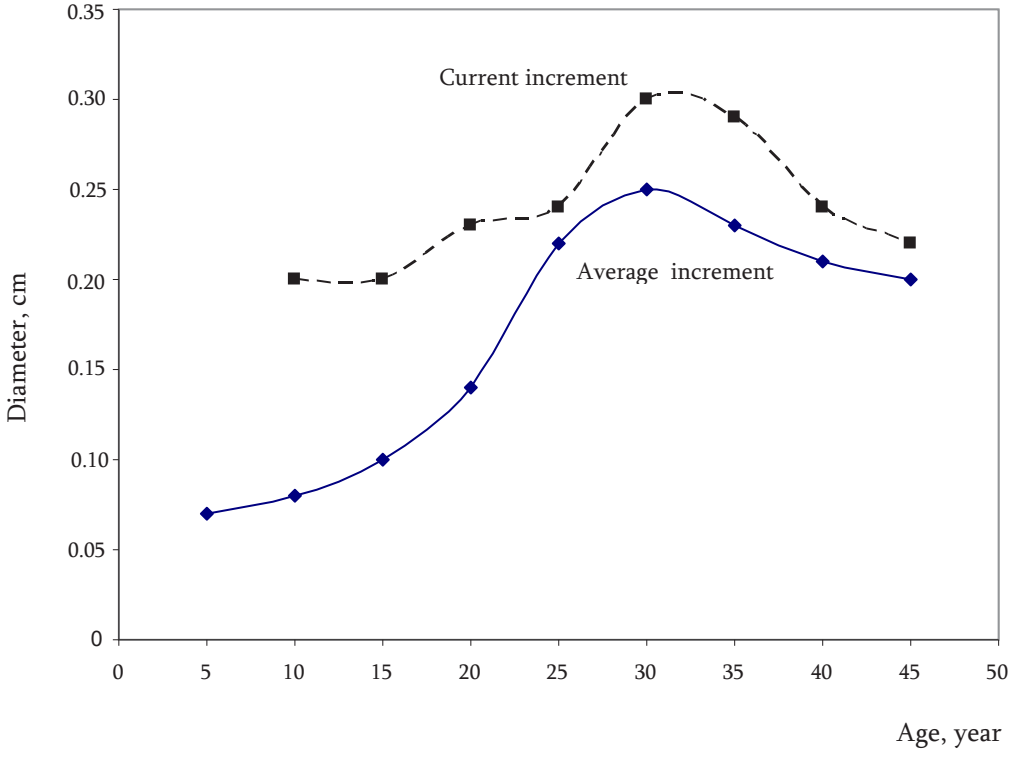

Fig. 5. *Current and average increment in diameter of Zelkova carpinifolia*

Dendrometric data of growth of *Zelkova carpinifolia* show that despite the complicated edaphic and climatic conditions, offspring of Zelkova is characterized by good growth, compact and well developed crown. Due to these features, the species can be recommended for the wide use in city greening in East Georgia.

Conclusion

1. A new site, supporting the population of *Zelkova carpinifolia*, situated north of the Jvari Monastery, which formerly was part of Tbilisi National Park, is characterized by hot summer and warm winter. Total atmospheric precipitation here is twice as lower, than in other known populations of this species. Discovery of a new population of *Zelkova carpnifolia* in environs of Tbilisi (eastern Georgia), in a habitat with different climate is of great interest for the study of biogeography and history of this ancient species.
2. Soils underlying *Zelkova carpinifolia* are cinnamonic, which are characterized by the dark coloration of the humic horizon, clayey texture and substitution of the profile with clay.
3. General regeneration of Zelkova in a newly discovered population near the Jvari Monastery proceeds by root suckers and slightly by seed. Natural propagation by seed is very low and mostly takes place on clearings and forest windows. Regeneration of Zelkova on the studied site mostly proceeds by root suckers.
4. Intense growth of *Zelkova carpinifolia* in length and diameter is marked until the 25-30 years of age, after which it slows down growth in height and in diameter, but it is characterized by stable growth until 80-90 years of age.
5. Growth and development of *Zelkova carpinifolia* plants, propagated both by seed and root suckers is positive, the trees are distinguished by compact and nicely branched crown. Due to these features *Zelkova carponifolia* deserves more attention and it can be recommended by the use in city greening.

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Evaluation of multi-model hindcasts for land surface air temperature over Georgia

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A B S T R A C T

Present study evaluates hindcast over the Caucasus Region of the multi-model system, comprising from 4 ERA-Interim-driven regional climate models (RCM) and the high resolution GCM-MRI-AGCM3 of Meteorological Research Institute (MRI). In total, five climate models simulations were assessed against the CRU observational database. Present work focuses on the mean surface air temperature. The study shows the performance of the members of ensembles in representing the basic spatiotemporal patterns of the climate over the territory of Georgia for the period of 1991–2003. Different metrics covering from monthly and seasonal to annual time scales are analyzed over the region of interest: spatial patterns of seasonal mean, annual cycle of temperature, as well monthly mean temperature bias and inter annual variation. The results confirm the distinct capabilities of climate models in capturing the local features of the climatic conditions of the Caucasus Region. This work is in favor to select models with reasonable performance over the study region, based on which a high-resolution bias-adjusted climatic database can be established for future risk assessment and impact studies.

Keywords: Regional climate models (RCMs), Reanalysis, Domain, Hindcast, Gridded data, Boundary conditions.

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Introduction

Assessing the impacts of the anticipated climate variations and change on regionally important sectors is growing in their importance. The primary tool for projecting climate are global climate models (GCMs) that are typically run at course horizontal resolutions, because of their massive computational and data storage requirements. GCMs' output cannot be directly used for impact study and requires downscaling on a finer scale, for which regional climate models (RCM) are developed. RCM data are essential for assessing the impact of climate change on different socio-economic sectors.

Climate model evaluation is a fundamental step in estimating the uncertainty in future climate projections. Because of the crucial role of climate models in this process, it is essential to characterize their strengths, weaknesses and uncertainties. Impact assessments typically start with climate data as inputs, and involve a series of calculations using multiple models through which information propagates hierarchically. Climate model evaluation is used not only for model development and improvements but also for assessing and correcting model biases. Model evaluations are also used to weight individual models in multi-model ensembles, alleviate the

effect of model error on assessment models, and estimate the range of uncertainty in projected impacts.

Uncertainties in climate projections come from multiple sources including incomplete model formulations, future emissions scenarios, and how these are folded together with elements of human behavior, technological advancements and social/government structures. Among these, model errors are probably the most viable to characterize, and potentially remedy to reduce the uncertainty. Model evaluations are typically performed by comparing model outputs against reference data from observations or reanalysis using suitable metrics [1-3], and can be further used to guide model improvement and/or for bias correction [4]. Measuring model performance objectively is of a particular importance in the practice of applying climate model outputs to climate change impact assessments which employs bias correction and/or multi-model ensemble [5-9]. Previous studies attempt to identify a single parameter representative of overall model performance [10-12], that can be applicable to objective multi-model ensemble and/or bias correction. Caution must be exercised in such attempts and application of their results, however, because spatial and temporal variations in model performance can introduce a substantial amount of uncertainties in calculating such indices. GCM evaluations have been well-established [13-17], but collective and systematic evaluation of RCMs is much less mature [18-21]. Considering the importance of RCMs in studying climate change and assessing its impacts, it is critical to apply as much observational scrutiny as possible to RCMs.

Systematic multi-model RCM experimentations and observation-based evaluations are much less mature than those for GCM studies. The long history of GCM analyses for assessments and other climate variability issues has resulted in a mature process of model experimentation and evaluation [22-25]. Evaluation of the fidelity in simulating the present-day climate of multiple GCMs that have contributed to the archives of phase 3 of the Coupled Model Intercomparison Project (CMIP3) used for the Intergovernmental Panel on Climate Change (IPCC) Fourth Assessment Report (AR4). The CORDEX program was established as the first activity of the Task Force on Regional Climate Downscaling established by World Climate Research Program (WCRP). Common experimental designs in CORDEX are advantageous for many practical purposes including model evaluations, uncertainty assessments, and constructing multi-model ensemble (ENS).

This study evaluates the 4 RCM and a high resolution global model simulations over the Georgia using Regional Climate Model Evaluation System (RCMES). We include in this research 2 RCMs' (RegCM v 4.7.0 and WRF-ARW v3.9.1.1) simulations over the domain centered to Georgia performed by us and 2 simulations over different domains from the CORDEX program. Namely RCA4 over MENA (Middle East and North Africa) and HadRM3P over CAS (central Asian domain). Such a choice has resulted in the fact that only these two domains overlaps our target area and the evolutionary simulations are available only for these 2 models on ESGF (Earth System Grid Federation)-CORDEX archive. High resolution GCM - MRI-AGCM3.2 output was provided from the Meteorological Research Institute of Japan Meteorology agency.

Section 1 provides details of the experimental design including the evaluation domain, RCMs, reference datasets, section 2 – climate description of Country, section 3 provides details of the Regional Climate Model Evaluation System (RCMES) used in the model evaluation. Section 4 presents the evaluation of RCM skill in simulating the targeted variables and examines the uncertainties in model evaluation related with reference data. Results are summarized in Sect. 5.

Main part

1. Data. In this paper, we used several data archives, most of them are available from the federative ESGF infrastructure, including Coordinated Regional climate Downscaling Experiment (CORDEX). We downloaded CORDEX simulations over central Asia (CAS) and the Middle East and North Africa (MENA) domains, covering South Caucasus territory.

Hindcast from the high-resolution atmospheric general circulation model of the Meteorological Research Institute (MRI) and two our simulations from two regional models RegCM4 and WRF with different configuration over the same domain (centered to South Caucasus territory) and with the same resolution have also been evaluated.

As for observations, we used global gridded observations and reanalysis of 2-meter air temperature data.

1.1. Observational data. For validation of individual models, also for ensemble the gridded global data set of the Climate Research Unit (CRU) was used. The monthly CRU data represent one of the

most comprehensive observational data sets available and have been widely used for former studies. These observational data sets are based on statistical interpolation methods, which are a gridded time-series dataset. We used latest version TS 4.03, released 15 May 2019, covering the period 1901-2018 Coverage: All land areas (excluding Antarctica) at 0.5° resolution for six Variables [26].

Despite the existing studies reveals that there are clear biases between the observed surface air temperature and the (GHCN + CAMS) data sets, they vary in space and seasons. We decide to compare this two gridded temperature dataset over Georgia. This data set at 0.5 X 0.5 latitude-longitude resolution is different from some existing surface air temperature data sets in: (1) using a combination of two large individual data sets of station observations collected from the Global Historical Climatology Network version 2 and the Climate Anomaly Monitoring System (GHCN + CAMS) [27].

1.2. Reanalysis data. As all Regional climate models are forced with ERA-Interim - reanalysis of the global atmosphere dataset, compatibility between reanalysis and observations (CRU) was also established.

The main objectives of the ERA-Interim project were to improve on certain key aspects of ERA-40 (previous version of ERA-Interim), such as the representation of the hydrological cycle, the quality of the stratospheric circulation, and the handling of biases and changes in the observing system. These objectives have been largely achieved as a result of combination of factors, including many model improvements, the use of 4-dimensional variation analysis, a revised humidity analysis, the use of variation bias correction for satellite data and other improvements in data handling.

The ERA-Interim atmospheric model and reanalysis system uses cycle 31r2 of ECMWF's Integrated Forecast System (IFS), configured for the spatial resolution - T255 spherical-harmonic representation for the basic dynamical fields and a reduced Gaussian grid with approximately uniform 79 km spacing for surface and other grid-point fields [28-29].

2. Climate models used in the study. Regional climate models (RCMs) are useful tools for the projection of climate change on regional scales. Unlike GCMs, the model domain of an RCM does not cover the entire globe. It is restricted to a certain area of regional scale.

This restriction allows for long-term simulations with higher resolutions. On the other hand, this implies that information about the lateral and lower boundary conditions (LBCs) has to be provided. These LBCs can be derived from GCM simulations or from observational data sets (usually reanalysis products).

RCA4. Since 1997 the Rossby Centre has developed an international standing in the field of regional climate modelling with the development of the atmospheric model RCA, at SMHI.

RCA is based upon the numerical weather prediction (NWP) model HIRLAM. The RCA4 dynamical core is a two time-level, semi-Lagrangian, semi-implicit scheme with six-order horizontal diffusion applied to the prognostic variables. Grid boxes in RCA4 can include fractions of sea (with fractional ice cover) or lake (with ice or not) and land. The land fraction can be further subdivided into forest and open land, where both can be partly snow covered. Each sub-grid scale tile has a separate energy balance equation and individual prognostic surface temperatures.

The model was driven by European Centre for Medium-Range Weather Forecasts (ECMWF) ERA-Interim reanalysis data to run the CORDEX Evaluation experiment, representative of the period from 1981 to 2010, over the Middle East and North Africa (MENA) domain called CORDEX-MENA [30-31].

HadRM3P is Hadley's Regional limited-area regional climate model widely used worldwide as part of the PRECIS (Providing Regional Climates for Impacts Studies) system, which was developed at the Hadley Centre of the United Kingdom Met Office. HadAM3P is a grid-point model which solves equations of motion, radiative transfer and dynamics explicitly on the same scale as the grid. The atmospheric equations are a quasi-hydrostatic version of the primitive equations with full representation of the Coriolis force. Other, mostly thermodynamic, processes that occur at the sub-grid-scale are represented by physical parametrizations. Model has 0.44 x 0.44 degrees' resolution with a rotated pole to achieve approx. 50 km x 50 km resolution on 19 levels (used for the EU region, South Asia, planned for East Asia). Also used is a double resolution variant at 0.22 x 0.22 degrees (Western US, planned for EU and Africa).

The model was driven by European Centre for Medium-Range Weather Forecasts (ECMWF) ERA-Interim reanalysis data to run the CORDEX Evaluation experiment, representative of the period from 1990 to 2011, over Central Asia domain with 0.44-degree resolution (CAS-44 domain) [32, 33].

RegCM v 4.7.0. Regional Climate Model version 4 - RegCM model is limited-area regional climate model. It uses the radiation scheme of the NCAR CCM3, the cloud scattering and absorption parameterization follow that of *Slingo* (1989), whereby the optical properties of the cloud droplets (extinction optical depth, single scattering albedo, and asymmetry parameter) are expressed in terms of the cloud liquid water content and an effective droplet radius. The soil hydrology calculations include predictive equations for the water content of the soil layers.

Compared to previous versions, RegCM4 includes new land surface, planetary boundary layer, and air-sea flux schemes, a mixed convection and tropical band configuration, modifications to the pre-existing radiative transfer and boundary layer schemes, and a full upgrade of the model code towards improved flexibility, portability and user friendliness. The model can be interactively coupled to a 1D lake model, a simplified aerosol scheme (including organic carbon, black carbon, SO4, dust and sea spray) and a gas phase chemistry module (CBM-Z) [34-36].

WRF-ARW v3.9.1.1 - Weather Research and forecasting model (Grell scheme). The WRF model is a state-of-the-art, next-generation mesoscale numerical weather prediction system designed to serve both operational forecasting and atmospheric research needs (http://www.wrf-model.org). It is a non-hydrostatic model, with several available dynamic cores as well as many different choices for physical parameterizations suitable for a broad spectrum of applications across scales ranging from meters to thousands of kilometers. The physics package includes microphysics, cumulus parameterization, planetary boundary layer (PBL), land surface models (LSM), longwave and shortwave radiation.

The WRF model is a state-of-the-art, next-generation mesoscale numerical weather prediction system designed to serve both operational forecasting and atmospheric research needs (http://www.wrf-model.org). It is a non-hydrostatic model, with several available dynamic cores as well as many different choices for physical parameterizations suitable for a broad spectrum of applications across scales ranging from meters to thousands of kilometers. The dynamic cores in WRF include a fully mass- and scalar-conserving flux form mass coordinate version. The soil scheme solves the thermal diffusivity equation using five soil layers and the energy budget includes radiation, sensible, and latent heat fluxes. It treats the snow-cover, soil moisture as fixed quantities with a land use and season-dependent constant value. The terrain, land use and soil data are interpolated to the model grids from USGS global elevation, 24 category USGS vegetation data and 17 category FAO soil data with suitable spatial resolution (arc 5 minutes) to define the lower boundary conditions [37-38].

MRI-AGCM3. A new version of the atmospheric general circulation model of the Meteorological Research Institute (MRI), with a horizontal grid size of about 20 km, has been developed. The previous version of the 20-km model, MRIAGCM3, which was developed from an operational numerical weather-prediction model, provided information on possible climate change induced by global warming, including future changes in tropical cyclones, the East Asian monsoon, extreme events, and blockings. For the new version, MRI-AGCM3.2, various new parameterization schemes have been introduced that improve the model climate. Using the new model, a present-day climate experiment has been performed using observed sea surface temperature. The model shows improvements in simulating heavy monthly-mean precipitation around the tropical Western Pacific, the global distribution of tropical cyclones, the seasonal march of East Asian summer monsoon, and blockings in the Pacific [39, 40].

2. Country climatology. Georgia's location on the northern edge of the subtropical zone between the Black and Caspian seas, on the one hand and on the other hand, complexity of its special topography determines the variety of climate conditions. Local climate creates Black Sea and the Caucasus. The last protects Georgia from direct invasion of cold air masses from the North and the Black Sea makes moderate temperature fluctuations and contributes large amount of precipitation, especially in western Country. Very important is the Likhi Range, running from the North to the South and dividing the Country into its eastern and western parts, with quite different climatic pictures.

Temperature regime of the territory, as a climate in whole, is characterized by a range of peculiarities stipulated mainly by the geographic location of Georgia, complex relief of the occupied territory, radiation pattern and prevailing atmospheric circulation processes. As orography on the territory varies from 0÷5068 m correspondently mean annual temperature fluctuate in the range of -5, $+15^0$C approximately.

Table 1. *Summary of data used in this study*

Data set/version	Time range	Resolution	References
Observation & reanalysis			
CRUvTS 4.03	1901-2018	0.5^0 X 0.5^0	Climate Research Unit (CRU)
GHCN + CAMS	1948- to near present	0.5^0 X 0.5^0	NOAA/OAR/ESRL PSD
ERA-Interim	1979- to near present	79 km	ECMWF
Climate models			
SMHI-RCA4	1981-2010	0.22^0 X 0.22^0	SMHI/Rossby Centre
HadRM3P	1990-2011	0.44^0 X 0.44^0	Met Office
RegCM v 4.7.0	1985-2015	15 km	ICTP
WRF-ARWv3.9.1.1	1985-2015	15 km	NCAR/NCEP
MRI-AGCM3	1979 -2003	20 km	MRI -JMA

Due to the Country climate regime, territory was divided in 8 sub-regions to examine the simulation performance across the experiments on different sub-regions. These regions mostly cover Georgia's territory but also include some other parts, according to the factors of local climate formation. On Fig.1. location and names of sub-regions are presented, where R01, R02 and R03 are, respectively, Western, Central and Eastern parts of Greater Caucasus mountains, R04 - Kolkheti Lowlands, R05 - Central part of Georgia including Likhi range, R06 – Adjara Black Sea coastal zone with adjacent mountains, R07 - Lesser Caucasus mountains, R07 - Eastern Country plane territory.

3. The Regional Climate Model Evaluation System (RCMES). RCMES is an open, publicly accessible process enabled by leveraging the Apache Software Foundation's OSS library, Apache Open Climate Workbench (OCW). RCMES provides datasets and tools to assess the quantitative strengths and weakness of climate models, typically under present climate conditions for which we have observations for comparison, which then forms a basis to quantify our understanding of model uncertainties in future projections.

RCMES is composed of two main components, the Regional Climate Model Evaluation Database (RCMED) and the Regional Climate Model Evaluation Toolkit (RCMET). RCMED can reside on a single server or be distributed on multiple servers to allow efficient data management and sharing while reducing the hardware and software burdens for handling the data storage and traffic. Observed data are fundamental to model evaluation. The demands from higher resolution and multivariate evaluation make the scientific and logistical process of model evaluation ever more challenging. RCMED bringing together massive amounts of observational and model data, but also dealing with the wide variety of sources and formats of data, necessitating significant investments in computer and personnel resources to transfer, decode, (re)format, (re)archive, and analyze the data. Such steps can make the process of performing robust model evaluations extremely difficult and time consuming even for highly trained scientists.

RCMET includes a software suite for calculating statistical metrics popularly used in model evaluations and visualizations. Model-evaluation metrics and visualization generally vary widely according to users and targets; RCMET includes the capability to incorporate user-defined metrics as well as pathways to extract partially processed data (e.g., both model and reference data regridded onto a common grid) so that users can do their own specific data processing and visualizations.

4. Results. The spatial distribution and annual cycle of mean monthly temperature along with the bias averaged over the entire analysis region and eight sub-regions (Fig.1), selected to examine climate models skills across varied geographical landscape are presented in this work. The analysis focuses on how the model simulates surface climate (temperature) in response to the large-scale forcing

imposed by the ERA-Interim reanalysis and by local topographical features. All the analysis presented here is carried out over the interior domain to eliminate the buffer zone where the direct effect of the lateral boundary conditions is maximum.

4.1. Evaluation metrics. Different metrics have been used in order to represent the performance of climate models in simulating climatic conditions. Besides computing the mean bias and root mean square error (RMSE), the degree of statistical similarity between two climatic fields was quantified in the form of normalized Taylor diagrams that can be considered as the combination of different measures such as the centered (or bias removed) RMSE, spatial standard deviation (STD), and spatial correlation. The Taylor diagrams reported in the present study are based on 13-yr annual and seasonal means in grid points.

4.2. Uncertainties assessment. The accuracy of reference data is among the most important concerns in model evaluation. All observations and/or analyses include errors of unknown/estimated magnitudes; e.g., analyses based on surface station data are directly affected by local station density. This especially true for the Caucasus region in which station density varies substantially according to regions. Uncertainties in model evaluation originating from reference data are examined using three different reference datasets. In addition to CRU, ERA-Interim (ECMWF Re-Analysis) and the GHCN_CAMS (Global Historical Climatology Network v2 and the Climate Anomaly Monitoring System) are selected for the same period as models evaluation was performed. All CMs yield higher spatial correlations with the CRU than GHCN_CAMS and ERA-Interim. The standardized deviations and RMSE are smaller against CRU and GHCN_CAMS, i.e. the spatial variability of the ERA-Interim data is larger than other two datasets (Fig.2).

Fig.3 shows the spatial distribution of mean annual temperature biases averaged over the entire 13-yr period compared to the CRU, GHCN and ERAINT datasets. In all seasons (not shown) the temperature bias against CRU data ranges between -4° and 4°C over the most of domain, except in winter and for annual means, when the bias mostly ranges between -3° and 3°C. Differences between models and ERAINT and GHCN_CAMS data range between -4° and 4°C in summer and for annual biases, but in the rest of seasons it is increased up to -6° and 6° C. As for GHCN_CAMS dataset, all models deviate from observation in the range -4° and 4°C, increasing to -6° and 6°C in winter season.

Best fit was revealed with CRU, biases of models are the smallest, but there are some systematically occurred features in the spatial distribution of these differences, i.e. relative to CRU and GHCN_CAMS it is noticeable cold bias over lowlands and plain territory and warm bias is the most evident in summer and winter over Caucasus. As for ERAINT, in contrary, for all simulations cold bias is occurred over mountainous areas, especially over Greater Caucasus range, warm bias – over lowlands and plain territory, that is the most evident in summer. Although evaluation of models against all three observation datasets demonstrates the spatial features of temperature biases and bias pattern is comparable with the terrain profile. It must be noticed, that moving towards the originally higher resolution information, the finer the details are in the spatial distribution of the seasonal temperature fields and the spatial features of deviations of mean seasonal temperature fields of ensemble simulations even reveal the ranges of Likhi Mountains (dividing west and east Country), which is in fact is especially clearly seen in winter season for all simulations.

The differences between the temperature evaluations based on the three observation datasets, may have resulted from the difference in the observational platform and methodologies. This examination shows that, quality control and cross-examination of reference datasets are important for model evaluations.

4.3. Evaluation results. In this study the baseline evaluation of the mean surface air temperature is presented against CRU dataset. As it was already mentioned, the most noticeable feature is the general warm bias over the Greater and Lesser Caucasus mountains and cold bias in the lowlands and plain territory (Fig.3). The spatial patterns of cold biases for all simulations except AGCM3 are similar, with the largest magnitudes being located in west Georgia lowlands. However, the cold biases in the WRFC and RegCM4 simulations are generally larger and extended over east Georgia plain territory. The warm bias is found in all simulations except RCA4. RCA4 is an outlier among these five CMs in the sense that it generates general cold biases over almost the entire study area. Another difference between the five simulations is that in the HaDRM3P simulation warm biases of more than 2°C are largely confined to Greater and Lesser Caucasus highlands, reaching its maximum magnitude up to 3°C in DJF season, when this warm bias

clearly depicted in other three simulations (except RCA4) as well. On the contrary, the only GCM (AGCM3) (Fig.3) generates overall warm biases. Unlike in the AGCM3 simulation, annual bias is the smallest (±1°C) but without clear dependence on topography, only in winter months there is evident positive (up to 3°C) deviation area covering mountainous part of the Country.

As it seems all RCMs except RCA4, overestimate the surface temperatures over the high elevation regions and underestimate low elevations resulting the least deviated ENS results regarding to observation in the range ±1.5°C.

Overall, all models simulate the spatial variations in the annual mean temperatures over Georgia with the spatial pattern correlation coefficients between 0.95 and 0.99 and standardized deviations (the spatial standard deviation of the simulated surface air temperature normalized by that of the observed data) of 0.8–1.15 with respect to the CRU data, except WRFC with much lower STD of 0.65 (Fig.2). Fig.2 also shows that the multi-model ensemble mean (ENS), along with AGCM3, yield the smallest RMSE.

Comparison of the simulated annual cycle against the CRU analysis for the sub-regions shows that the multi-model ensemble is generally in well agreement with observed climatology in these regions and all five simulations have almost identical annual cycle and a similar range in monthly mean temperatures averaged over sub-regions, with differences up to 5°C between separate models. However, despite the reasonable performances, model biases vary noticeably according to regions and seasons (Fig.4).

Fig.4 shows some time dependency of model deviations, as temperature biases are not constant in time. They have a more or less clear annual cycle: there is one of five CMs (RCA4) with a constant negative temperature bias through the entire year, for other four models temperature is generally overestimated in winter (exceeding 4°C in January and February), whilst underestimated to a varying extent in the rest of the year resulting ensemble simulations negative bias. Therefore, the seasonal variation in the magnitude of the bias in area-average temperature means that the ENS simulation has a less extreme annual cycle than the annual cycle of the observations. In the transient seasons (spring, autumn), all regions of the study territory have a cold bias. This appears to be largest over the west Georgia lowlands. Warm bias in area-mean temperature is greatest during winter. In this season, warm biases extend over entire mountainous regions including the Greater and Lesser Caucasus. This may be related to the simulation of cold-season snowpack in the high-elevation regions and/or the lack of resolutions both in model simulations and the CRU data, suitable for representing the large orographic variations and associated variations in surface temperature in the mountainous region.

Fig.5 presents the normalized biases and interannual variability in terms of the percentage of the temporal standard deviations of the CRU data over the 13-yr period, of the simulated surface air temperatures in the eight sub-regions during each season. The temporal standard variations are adopted as the measure of the interannual variability. The scaled model bias shows that the warm bias over the Caucasus mountains is common for nearly all models (except RCA4) only in winter; RCA4, which generates quite strong (by 50%–150% of the observed interannual variability) cold biases over the region in winter, is the only exception. As for cold bias on the intermountain low elevation area negative bias is systematic regardless of models and seasons for west Georgia and more evident in transient seasons for east Country plains.

Models skill in simulating the interannual variability of the seasonal temperature is further examined using RMSE and the temporal correlation coefficients between the simulated and CRU data over the 13-yr period. The resulting RMSE (Fig.5) generally exceeds the interannual variability of the CRU data (i.e., normalized RMSE>100%), especially during autumn. In spring and summer clear overestimation is also revealed. For winter, the RMSE varies according to models in most regions; the normalized RMSE for RegCM4, as well as the multi-model ensembles, is less than 100% while that for AGCM3 is well above 150% for all sub-regions. As for ENS, it yields the smallest RMSE in summer and winter due to opposite signs of deviations for different models, but for annual means and in transient seasons because of mostly underestimation is evident, ENS RMSE is greater than for separate models, that are AGCM3 or HaDRM3P having positive bias.

The spread of bias fields mostly ranges between –3°C and +3°C, only AGCM3, HaDRM3P (overestimation), and WRFC (underestimation) models are slightly exceeding these limits in summer. WRFC and RCA4 typically show a strong

cold bias when compared to the CRU observational dataset. In general, RegCM4 and AGCM3, having higher initial (before regridding) resolution, performs among the best climate models: i.e., producing close to zero mean annual bias due to the least biased performance during the period of March-August. Again, higher resolution simulations (RegCM4, AGCM3) are not expected to decrease the mean bias fields, and actually the standard deviation of bias averaged over the region of interest in each season is larger in case of RegCM4 and AGCM3 compared to the ensemble (Fig.5). The wide range of the spread in seasonal biases can be directly attributed to the different topography and parameterizations implemented in the evaluated climate models simulations.

Overall, models show consistently better skill in simulating the monthly-mean surface air temperature in the cold period (September-February) than for warm period (March-August) of the year (Fig.6).

The model biases also vary systematically according to regions. For spring, the most noticeable systematic biases are the cold bias in the entire western part including and no systematic warm bias revealed in this season. For autumn, the most systematic biases are the cold bias in the central mountainous regions including Likhi Range and South Caucasus highlands. In winter warm bias is evident relative to other seasons and this bias varies closely with orography as shown in Fig.5. This feature of orography dependence bias is noticeable during whole year but most evident in winter. The evaluation of the temporal standard deviation, a surrogate for the interannual variability, shows that all models perform reasonably well in simulating the interannual variability of winter and summer temperatures for all sub-regions. Most of CMs overestimate the interannual variability of the transient season's temperatures; overestimation is greatest for RCA4 in spring and AGCM3 in autumn. For all seasons, ensemble simulations have the least STD.

The correlation coefficients between the simulated and CRU time series (Fig.5) also shows that climate models examined in this study generally perform better in simulating the phase of the interannual variation in the surface air temperatures during winter than in other seasons, the poorest correlation was found in spring. In contrary with annual correlations, overview of seasonal means revealed that AGCM3, the only GCM in this study, almost not correlated with observation, whilst all RCMs has a quite high score as for annual, as well for seasonal means.

5. Discussion and Conclusion. In the present study, five climate models have been evaluated over a 13-yr reference period (1991−2003) against the CRU observational dataset. Overarching aim of the present study is to provide useful information on general capabilities of given models in reproducing climatic conditions over the Caucasus Region. By and large, the annual temperature cycle averaged over the study region is well represented by ensembles simulation. According to the spatial distribution of seasonal temperature, models performing well for annual temperature do not necessarily perform well in separate seasons and model performance varies widely and, often systematically, according to regions and seasons. These characteristics in model errors make it difficult to design a set of model weightings that can be universally applied to the construction of multi-model ensemble.

According to the findings reported in the present work, the following considerations can be made: (1) there is not a single model outperforming the other ones in all aspects, but it is also important to note that all models have their strength and weaknesses; (2) higher resolution simulations may adequately resolve the temperature variations in the region; (3) due to the amplification of biases or the increased internal variability on small scales induced by strong local surface heterogeneities within the regional domain, higher resolution simulations not necessarily reduce the uncertainties; (4) model performances are also influenced by observational uncertainties.

We assess that the model can provide useful information on variables that are important for the assessment of climate change impacts. We therefore plan to use this model configuration in simulations of other essential climate variables and construction future climate scenarios for Caucasus region.

Appendix:

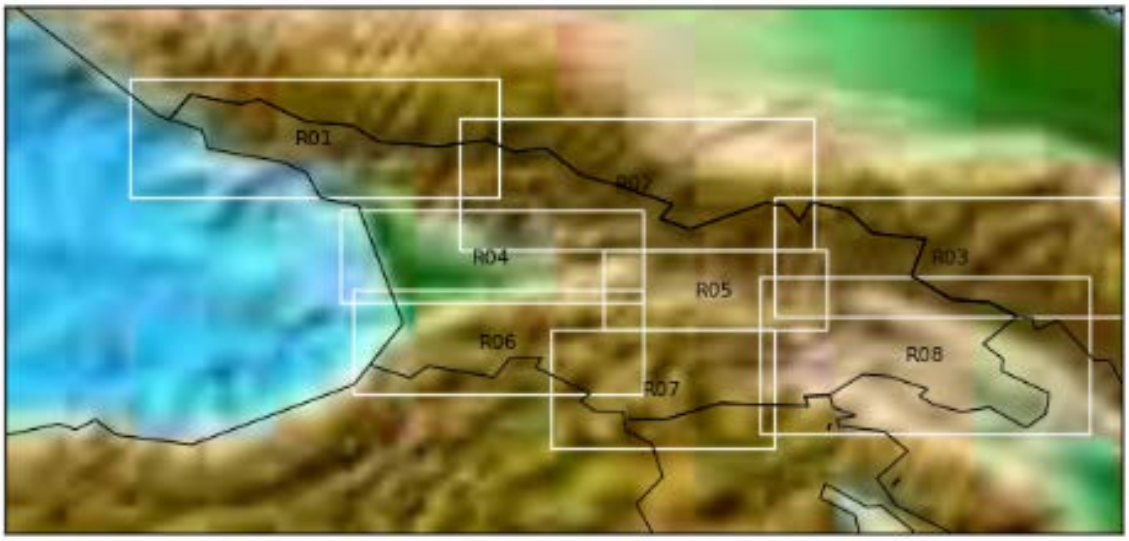

Fig. 1. *Study (Caucasus) domain. The color contours represent the terrain elevation. The numbered boxes with white boundaries indicate the eight sub-regions in which the area-mean time series are evaluated.*

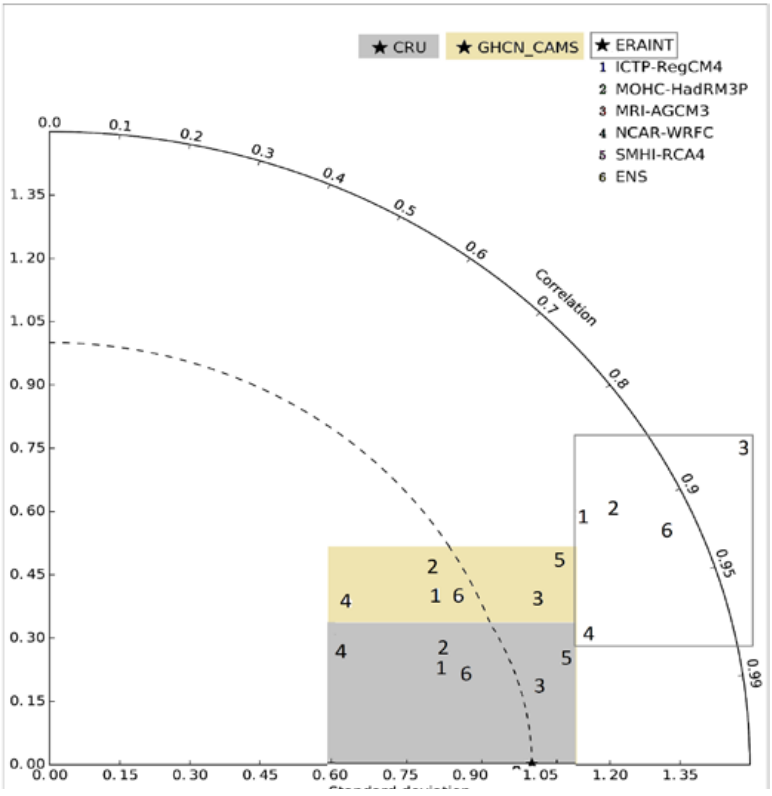

Fig. 2. *Evaluation of the simulated temperature climatology over the land using three different reference datasets. The dots on grey (CRU), yellow (GHCN_CAMS) and squared (ERAINT) backgrounds, respectively, indicate the model ensemble evaluated against different reference data.*

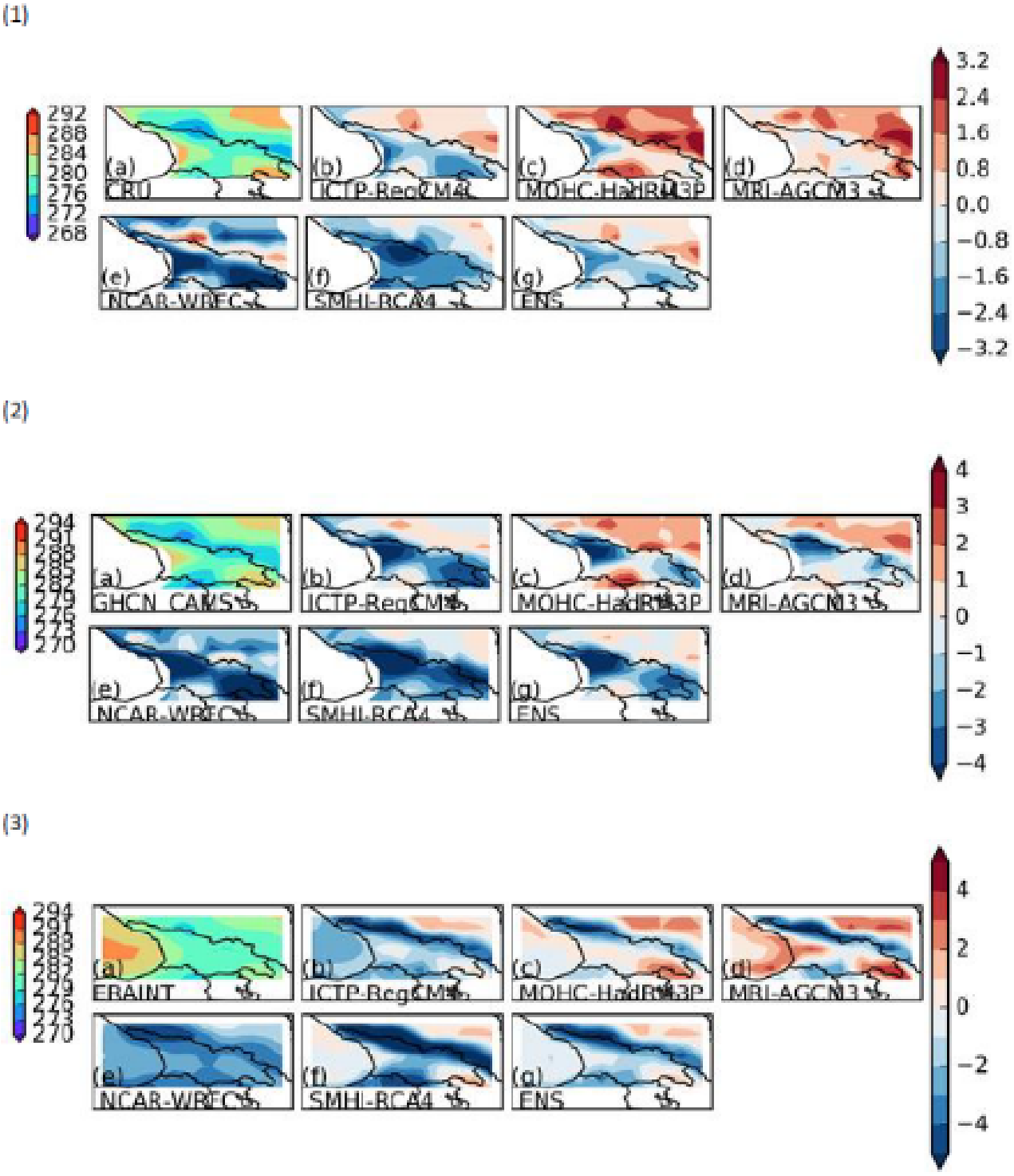

Fig. 3. *Annual-mean surface air temperature (°C) from the CRU (1), GHCN_CAMS (2) and ERAINT (3) analysis. The biases (°C) from the reference data for (b)–(f) the individual models and (g) the multi-model ensemble (ENS).*

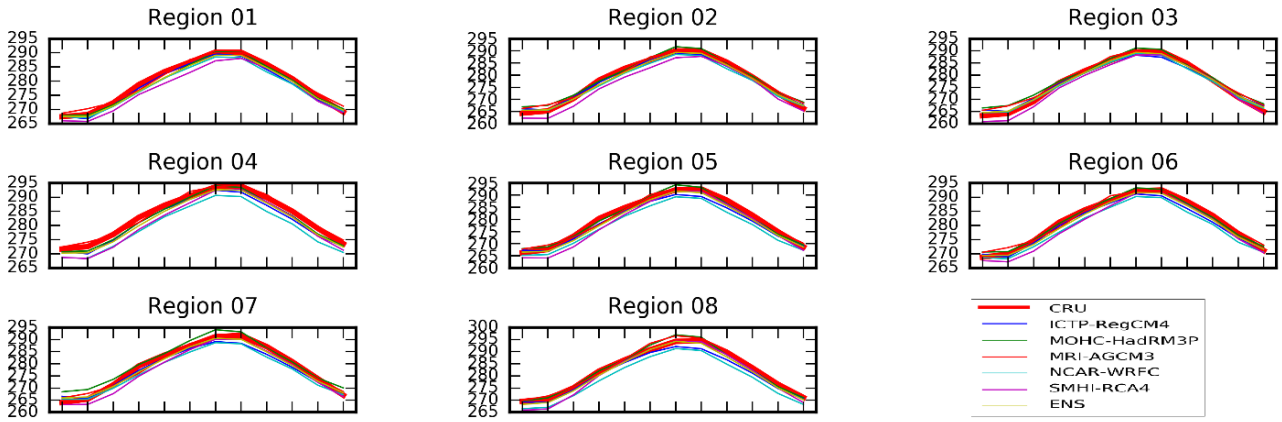

Fig. 4. *Simulated and observed (CRU, thick red) temperature annual cycle (°C) for the eight sub-regions. The thin yellow line indicates the multi-model ensemble temperature.*

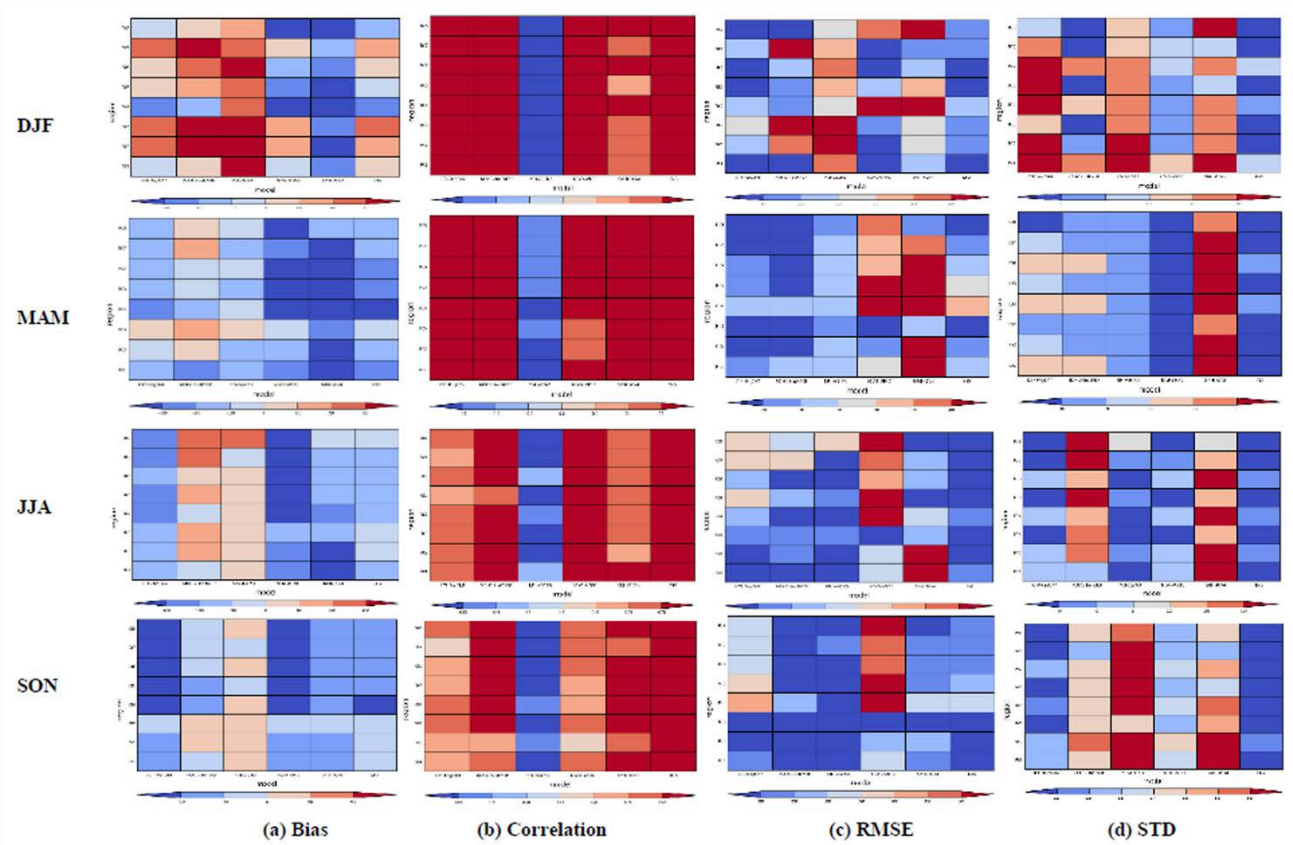

Fig. 5. *Regional (a) bias, (b) temporal correlation coefficients, (c) root mean square error and (d) temporal standard deviation of simulated average seasonal air temperatures relative to CRU observations. Seasons are defined as follows: winter-DJF (December–February), spring-MAM (March–May), summer-JJA (June–August) and autumn-SON (September–November). The bias, standard deviation, and RMSE are normalized by the standard deviation of the CRU data.*

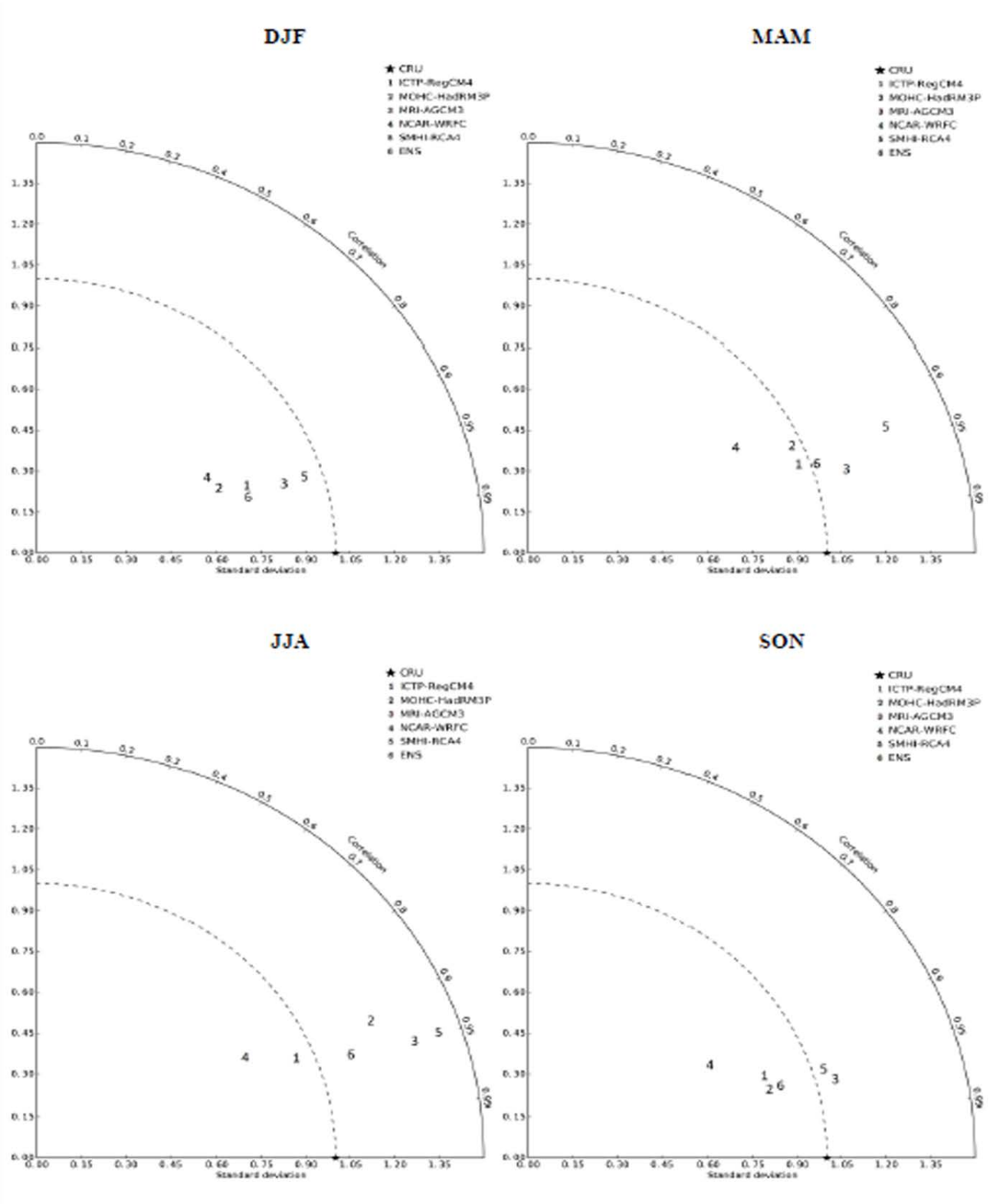

Fig. 6. *Standardized deviations and spatial pattern correlations of temperature between the CRU data and the individual model results for separate seasons over the land surface. Seasons are defined as follows: winter-DJF (December–February), spring-MAM (March–May), summer-JJA (June–August) and autumn-SON (September–November).*

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The vegetation of Mtatsminda-Kustba area (Mamadaviti Ridge, East Georgia, South Caucasus)

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A B S T R A C T

The vegetation of the eastern part of Mamadaviti Ridge – Mtatsminda-Kustba area is studied for the first time. The vegetation is highly modified due to human-induced impacts. It is a mixture of natural and near natural vegetation and the artificially planted woody plantations. Vegetation units of both mesophilous and hemixerophilous ecosystems are present. Wetland vegetation communities are formed on the shores of the lake Kustba. Natural and near natural vegetation includes broad-leaved forests, shibliak-type hemixerophilous shrubberies, meadow-steppes and steppes, as well as vegetation of rock and scree ecotypes. Common reed plant communities (Phragmitetum australis) occur locally. The area is marked by extensive zones covered with highly diverse artificial plantations. There are both coniferous and deciduous, as well as mixed plantations, which are established with sole use of introduced species. The current condition of the main units of vegetation, the degree of their modification and stress, as well as the main directions and trends of successive changes in vegetation are established. The flora of the study area is composed of ca. 750 species of vascular plants. Both boreal and Ancient Mediterranean florogenetic connections are expressed. Almost all life forms of plants of different bio-ecology are represented. Forty-eight endemic species of the Caucasus were recorded.

Keywords: Vegetation, Typological composition, Flora, Endemic species, Heterogeneity, Recreation zone.

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Introduction

Location and physiographical conditions

The study area covers the eastern part of the Mamadaviti Ridge, in particular, the Mtatsminda-Kustba (frequently referred to as Turtle Lake) section. The area is a recreational zone.

The study area is characterized by heterogeneity of physiographical conditions (terrain, soil cover, climate) as compared to the rest of Mamadaviti Ridge.

There are two macro slopes – south- and north-facing. Given the relatively small area of the territory, the altitudinal amplitude is rather high - around 485 m to 1060 m asl. The terrain is characterized by the dominance of slopes of different inclination. Slightly inclined and relatively plain sections are mainly present in the crest of Mamadaviti Ridge. The inclination of the slopes is mostly 20°-40°, although it increases to 50°-75° (80°) in some sections. The ravines and streams, that vertically intersect the slopes, are rare. The hydrological network of the study area is poorly developed. A perennial stream is observed near the landmark "Bulbulebis Chala". The Varaziskhevi river flows into the Turtle Lake through the artificial tunnel. In addition to these, there are several small springs.

The climate of the area is characterized as humid and semi-humid. The average annual precipitation is about 550 mm to 650 (700) mm. As the elevation increases, the amount of precipitation and hydration coefficient increases as well, whereas the average annual temperature decreases. Such climate change is reflected in the typological composition of the vegetation cover and the patterns of its distribution.

Below is a cluster diagram based on the data of the meteorological station on Mtatsminda (Fig.).

It should be mentioned, that due to the fact that recent data is not available, relatively old data was used [1, 2].

The study area largely supports cinnamonic soils. Brown forest soils are developed on limited areas. In most cases, the soils are thin or of medium depth, and gravelly. Bedrock is frequently visible on the soil surface. Rock and scree ecotopes occupy limited areas. They are of fragmentary distribution and are found within the areas with different soil types. Such ecotopes are of different age and mostly developed as a result of erosion processes.

Bibliographical Review

Although the study area has been under the attention of researchers for a long period of time (from the mid-19th century to the present), no comprehensive data is available on its vegetation. Fragmented data is scattered throughout various fundamental papers and scholarly articles. There is limited herbarium material collected from this area, which are preserved in different Georgian herbaria (TBI, TGM, TB, TBPH). Some information about individual plants within the study area can be found in various papers [3-14]. There are also scarce data on syntaxa distributed on the Mamadaviti Ridge, including the Mtatsminda-Kustba section [7, 10, 11, 15-19].

Despite the large number of published scientific papers, the information presented in them does not provide the overall picture of the floristic and ecosystem diversity of the study area. There are no uniform and complete data on the typological composition and distribution patterns of the vegetation.

Aim of the research

The main objectives of the present study were the following:

- identification of the typological composition of the vegetation in the study area and regularity of the distribution of recorded syntaxa;
- assessment of the degree of stress and level of modifications within the phytosociological units;
- identification of the dynamics and trends of on-going succession stages;
- study of the flora of the survey area.

Objectives and Methods

The objective of research is the study of vegetation of the eastern part of Mamadaviti Ridge - Mtatsminda-Kustba section. Phytosociological data was obtained by the route method. During the geobotanical surveys to study the structure of plant communities and identification of syntaxa, use was made of traditional geobotanical methods [20-26].

Geobotanical surveys were conducted in forests within a sample area of 400 m², whereas in shrubland and grassland communities field data was collected in plots with 25 m² area. In addition to the main vegetation characteristics for each plot, information

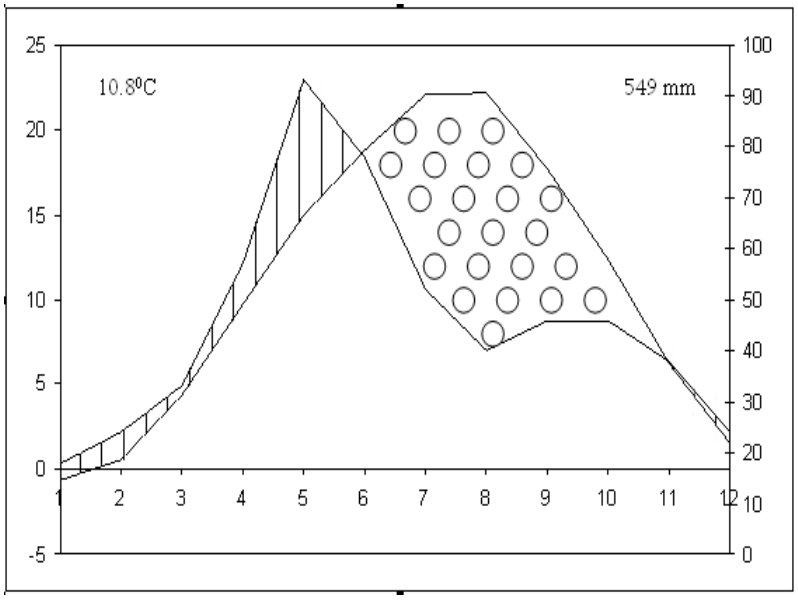

Fig. *Climograph of Mtatsminda meteorological station*

was collected on biotic and abiotic environments such as altitude, inclination, exposure, geographical coordinates, soil type, degree of disturbance of vegetation and causes of transformation. Instead of the term "association" that was widely used in Soviet literature, we use the term "plant community" accepted across the Europe.

Life forms of the plants follow Raunkiaer's system [27] and Serebriakov's classification [28].

The borders of the Ancient Mediterranean and Boreal regions are those as defined by A. Takhtajan [29]. Plant species are regarded as endemics of the Caucasus if they are found only within the borders of Caucasus Ecoregion [30].

Soil types are based on the modern classification systems [31-33].

Results and Analysis

I. Vegetation

Typology and distribution patterns

The vegetation of the study area is under heavy anthropogenic pressure and is a mixture of near natural or semi-natural vegetation units and artificially planted woody plantations. It is characterized by rich typological composition. Vegetation units of both mesophilous and hemixerophilous ecosystems are distributed. Mesophilous vegetation is mostly represented by forest stands, while hemixerophilous vegetation - by shibliak-type shrubberies and steppes. Hemixerophilous ecosystems also include vegetation of rock and scree ecotopes. Meadow-steppe plant communities are transitional between mesophilous and hemixerophilous vegetation. Wetland communities occur locally. These vegetation units are typical for the vegetation cover of Tbilisi area. With the diverse typological composition, the artificial plantations hold an important place in the vegetation of the study area.

Different types of plant communities are diffusely distributed due to the micro- and meso-mosaic variability and anthropogenic factors of local physical-geographical conditions (altitude, terrain, exposition, substrate and degree of its hydration, micro-features of climate, etc.). Despite the mosaic and, at first glance, unsystematic distribution of vegetation, some regularity is still obvious.

The main forest area covers the western and central part of the territory ("Bulbulebis Chala"), which is characterized by a humid climate. It is distributed on the slope of the northern macro exposure of the Mamadaviti Ridge. Forest stands are developed both on the slopes and in the ravines. Slope exposure and inclination varies considerably. The areas are characterized by the forest ash-grey, as well as cinnamonic soil types. The degree of soil moisture is different.

The forests are represented with the formations of European ash (Fraxineta excelsior), Georgian oak (Querceta petraea subsp. iberici) and Oriental hornbeam (Carpineta orientalis). The stands of the first two formations are primary, while the the stands formed by oriental hornbeam are of secondary origin and represent one of the initial stages of the digressive succession of oakwoods [15]. The stands formed by oak and ash are mostly characterized by brown forest soils, while those of hornbeams are also found on cinnamonic soils. These formations are characteristic units of vegetation in the environs of Tbilisi and play an important role in establishment of natural vegetal landscapes.

Shibliak-type hemixerophilous shrubberies mostly occur on the macro slope of the northern exposure of the Mamadaviti Ridge. The plant communities are confined to the North-East, North and North-West slopes, rarely recorded on Southern and South-Eastern exposure. The inclination ranges from 15°-20° to 35°-40°. Communities are developed on gravelly cinnamonic soils. In most cases, bedrock are almost on the soil surface. The main formation of shibliak-type hemixerophilous shrubberies is that of Iberian spirea (Spiraeta hypericifoliae), which covers quite large areas. It is possible, that the area covered with Iberian spirea was wider in the past and it was later replaced by artificial plantations. In addition, polidominant shrubberies are important. All these are recorded on steeper sections. Bidominant groups of Iberian spirea and Tanner's sumach (*Spiraea hypericifolia* + *Rhus coriaria*) are rare. In some sections, along with them, the Smoketree (*Cotinus coggygria*) also appears in the dominant role. The Christ's thorn communities (Paliuretum spina-christi) occupy relatively small area.

Meadow-steppes have the smallest area in the study area. They are distributed in the western part of the study area at the hypsometrically highest point (950-1050 m asl), which is characterized by a humid climate. Communities are found on the North-facing macro slopes of the Mamadaviti Ridge, on medium and thin gravelly cinnamonic soils. In a separate section the bedrock are exposed to the surface. Despite the small size of the area, the meadow-steppes are represented by grass-forb communities of different modifications.

The present area of steppe vegetation is fragmented, which, along with the natural physiographical conditions, is largely due to anthropogenic factors - most of the steppe area is replaced by artificial plantations.

Steppe communities are common on both South- and North-facing macro slopes of the Mamadaviti Ridge. It is clear that steppe vegetation would have been a leading vegetation type in the past in the zones of present-day artificial plantations planted on the slopes of the South-facing macro slopes. Communities are developed on medium and thin gravelly cinnamonic soils. In some sections the bedrock is exposed. Slope exposure and inclination are different. Although the steppes do not cover large areas in the study area, they are presented in different modifications. The main ones are plant communities belonging to Fescue formations (Festuceta valesiaci). In addition, there are plant communities dominated by Feather grasses (Stipetum spp.).

The vegetation of rock and scree ecotopes occupies a prominent place in the study area. They are mostly of secondary origin and are associated with different types of erosion processes. However, the existence of primary ecotopes is not excluded. They are fragmented in different parts of the study area and are associated with different types of vegetation. The typological composition of the vegetation of the rock and scree ecotopes is not formed. They are represented by floristic grouping of different species composition.

Wetland plant communities occur along the shores of Lake Kustba. They are represented by Common reed beds (Phragmitetum australis).

Artificial plantations are widely established almost throughout the study area. They are presented on the slopes of different exposure and inclination and on different soils. In some parts of the area, their development is fragmented and intruded into the area of natural vegetation. The artificial plantations are largely cultivated in a significant part of the area and are the key components of the phyto-landscape. Their main area is in the Northern and Eastern part of the territory, while it covers relatively small areas in the Western ones. There is a lack of systemic cultivation - artificial plantations and natural vegetation occasionally alternate diffusely and unsystematically. The typological composition of artificial plants is rich. There are both coniferous and deciduous as well as mixed plantations. Coniferous plantations are mainly composed of pine trees (*Pinus nigra* subsp. *nigra, P. nigra* subsp. *pallasiana, P. sylvestris* subsp. *hamata*). Small areas include Deodar cedar (*Cedrus deodara*) and Oriental arborvitae (*Platycladus orientalis*) groves. Deciduous plants are cultivated in small areas and are represented by groupings of Common lilac (*Syringa vulgaris*) and Golden rain (*Laburnum anagyroides*). An extensive area is occupied by mixed plantations with both deciduous and coniferous trees and shrubs. A different variant of artificial plantations is the complex habitat composed of mixed plantations combined with natural vegetation.

A brief description of the natural and near to natural vegetation

The coverage of canopy layer ranges from 0.5 to 0.7 (0.8). The undergrowth is developed distinctively in different groves. Its coverage is between 3-5% and 30-40%. Characteristic shrubs are *Ligustrum vulgare*, *Lonicera caprifolium*, *Euonymus verrucosus*, *Swida australis*, *Cornus mas*, *Crataegus kyrtostila*, *Lonicera caucasica*. The floristic composition of the grass cover is dissimilar in different stands. The main species are as follows: *Melica uniflora*, *Melica picta*, *Poa nemoralis*, *Brachypodium sylvaticum*, *Brachypodium pinnatum*, *Carex humilis*, *Viola alba*, *Viola odorata*, *Silene italica*, *Primula macrocalyx*, *Primula woronowii*, *Aegonychon purpurea-coeruleum*, *Campanula rapunculoides*, *Vincetoxicum amplifolium*, *Klasea quinquefolia*, *Alliaria petiolata*, *Albovia tripartia*, *Physospermum cornubiense*, *Vicia truncatula*, *Laser trilobum*, *Anthriscus nemorosa*, *Lapsana grandiflora*, *Polypodium vulgare*, *Tamus communis*, *Corydalis angustifolia*, etc.

The floodplain forest derivative occurs locally on the southern macro slope of the Mamadaviti Ridge in the vicinity of Okrokana village. Despite the small size of the area and the natural structure disturbed by the influence of anthropogenic factors, it occupies a drastically different place in the surrounding vegetation. It is characterized by the diversity of woody plant species (up to 20 species). Their composition is complex - typical representatives of the floodplain ecosystems, trees and shrubs characteristic to foothill, lower mountain and middle mountain belt forests are present. The typical layer structure of the forest is disrupted. The canopy layer is not uniform and is composed of trees of different height. The height of canopy varies from 8 to 16m. Its coverage also ranges from 0.6 to 0.7. It is composed of *Salix* sp., *Quercus petraea* subsp. *iberica*, *Fraxinus excelsior*, *Acer laetum*, *Prunus*

avium, *Prunus malaheb*, *Ulmus minor*, *Crataegus kyrtostila*. The undergrowth is characterized by a similar heterogeneous structure and uneven height - it is composed of shrubs of different height and bio-ecology (*Carpinus orientalis*, *Berberis vulgaris*, *Rubus* sp., *Salix caprea*). The average coverage of the undergrowth is up to 70%, although it is unevenly distributed. Important components of this ecosystem are lianas (*Hedera helix*, *Smilax excelsa*, *Vitis* sp.), which are characteristic to the floodplain forest.

Woodland-like plant communities are developed in relatively moist ravines. Under such ecological conditions, along with trees, mesophilous and xerosophilous shrubs are widely represented. Consequently, the floristic composition of this vegetation unit is complex. Due to the increased humidity, the cover of both trees and shrubs is high. The structure of these types of plant communities is not stable and well-established. In some sections, shrubs play a dominant role. The floristic composition of different ravines is distinct. The main species are *Fraxinus excelsior*, *Acer campestre*, *Acer laetum*, *Swida australis*, *Celtis caucasica*, *Prunus avium*, *Ulmus minor*, *Rosa* spp., *Rubus* spp., *Cotoneaster meyeri*, *Clematis vitalba*, *Hedera helix*, *Ligustrum vulgare*, *Crataegus* spp., *Berberis vulgaris*, etc.

In addition to the dominant species, shibliak-type hemixerophilous shrubberies are composed of the following species: *Prunus incana*, *Jasminum fruticans*, *Cotoneaster* spp., *Cytisus caucasicus*, *Rhamnus pallasii*, *Ephedra major* subsp. *procera*, *Amelanchier ovalis*, *Astragalus cornutus*, *Crataegus kyrtostyla*, *Ligustrum vulgare*, *Berberis vulgaris*, *Colutea orientalis*, *Rosa canina*, *Ligustrum vulgare*. The grass cover is more or less complex - along with the characteristic species of shrubbery, steppe and meadow-steppe species are also distributed. Almost all life forms of herbaceous plants (hemicryptophytes, geophytes, annuals) are represented. Characteristic plant species are *Thalictrum collinum*, *Potentilla recta*, *Salvia nemorosa*, *Filipendula vulgaris*, *Silene italica*, *Onobrychis cyri*, *Melica transsilvanica*, *Galium verum*, *Dactylis glomerata*, *Valeriana officinalis*, *Potentilla adenophylla*, *Muscari szovitsianum*, *Veronica multifida*, *Astragalus brachycarpus*, *Thesium arvense*, *Dictamnus caucasicus*, *Fragaria* sp., *Phleum phleoides*, *Erysimum leptophyllum*, *Euphorbia boissieriana*, *Fritillaria caucasica*, *Seseli grandivittatum*, etc. The composition is enriched with characteristic species of stony and gravelly ecotopes, both semi-shrubs and undershrubs, as well as herbaceous plants (*Teucrium nuchense*, *Artemisia caucasica*, *Scutellaria orientalis*, *Psephelus carthalinicus*, *Sedum oppositifolium*, *Sempervivum transcaucasicum*, *Oxytropis pallasii*, etc.). Forest elements (*Cyclamen vernum*, *Primula macrocalyx*) are also recorded in some communities. Representatives of the family *Orchidaceae* (*Dactylorhiza romana* subsp. *georgica*, *Ophrys caucasica*, *Neotinea ustulata*, *Anacamptis morio*) are rather common in the above communities.

The floristic composition of meadow-steppes is complex; along with the typical components of steppes and meadow (*Phleum phleoides*, *Dactylis glomerata*, *Koeleria cristata*, *Bothriochloa ischaemum*, *Stipa* spp., *Poa bulbosa*, *Festuca valesiaca*, *Filipendula vulgaris*, *Coronilla orientalis*, *Echium rubrum*, *Euphorbia iberica*, *Galium verum*, *Onobrychis cyri*, *Potentilla adenophylla*, *Potentilla recta*, *Seseli grandivittatum*, *Origanum vulgare*, *Plantago media*, *Thalictrum collinum*, *Veronica multifida*, *Dianthus subulosus*, *Rumex tuberosus*, *Fragaria* sp., *Muscari szovitsianum*, etc.), plants characteristic to gravelly soils and scree ecotopes are also present (*Scutellaria orientalis*, *Alyssum tortuosum*, *Thymus* sp., *Bromus biebersteinii*, *Teucrium nuchense*, *Helianthemum nummularium*, *Psephellus carthalinicus*, *Petrorhagia saxifraga*, *Alopecurus tiflisisensis*, etc.) due to the specific soil conditions. One of the key characteristics of the vegetation is the occurrence of the species of the *Orchidaceae* family (*Anacamptis morio, Neotinia ustulata, Dactylorhiza romana* subsp. *georgica*).

Steppes are species-rich plant communities. Along with the grasses (*Festuca valesiaca*, *Bothriochloa ischaemum*, *Stipa capillata*, *Stipa lessingiana*, *Stipa arabica*, *Dactylis glomerata*, *Koeleria cristata*, etc.), the following forbs are well-represented: *Salvia nemorosa*, *Carex bordzilowski*, *Galium verum*, *Dianthus subulosus*, *Potentilla adenophylla*, *Potentilla recta*, *Seseli grandivittatum*, *Euphorbia iberica*, *Veronica multifida*, *Filipendula vulgaris*, *Plantago media*, *Rumex tuberosus*, *Onobrychis cyri*, *Medicago caerulea*, *Trifolium tumens* and others. The involvement of hemixerophilous semi-shrubs and undershrubs (*Thymus coriifolius*, *Teucrium polium*, *Teucrium nuchense*, *Scutellaria orientalis*, etc.) is directly related to gravel content in soils. Annuals also play an important role in the floristic composition, among which are weed and widespread plant species (*Alyssum alyssoides*, *Draba nemorosa*, *Erodium cicutarium*, *Medicago minima*, *Helianthemum lasiocarpum*, *Hirschfeldia incana*, *Carthamus lanatus*, *Bromus squarrosus*, *Crepis sancta*, etc.).

Steppe vegetation is mostly of secondary origin in the study area. However, the existence of primary communities is also possible. At present, it is impossible to clearly distinguish between the primary and secondary communities. The formation of secondary communities has a long history, which is primarily related to the anthropogenic factors (deforestation and burning of forests and shrubberies, grazing, etc.).

Despite the extreme conditions, the floristic composition of the vegetation of the rock and scree ecotopes is diverse. The main characteristics of the vegetation structure are not well-defined. Plants of different life forms are diffusely and unsystematically distributed. In addition to the typical petrophytes, components of different types of vegetation (mostly shibliak-type hemixerophilous shrubbers ies, arid birch forests and steppes) are distributed, which are characterized by a relatively wide ecological amplitude. Part of them belongs to the so-called coenotic relics and is remnants of the previously existed vegetation; other part penetrated into this vegetation and became well-established. The composition of life forms is also diverse - almost all life forms are represented. Shrubs include *Atraphaxis caucasica*, *Rhamnus pallasii*, *Paliurus spina-christi*, *Ephedra major* subsp. *procera*, *Cotinus coggygrea*, *Colutea orientalis*, *Pyrus salicifolia*, *Spiraea hypericifolia*, *Rhus coriaria*. Shrub layer is marked by the presence of dwarf shrub *Fumana procumbens.* The composition of herbs is rich. The main hemicryptophytes are *Centranthus longiflorus*, *Hyssopus angustifolius*, *Alyssum tortuosum*, *Asperula glomerata*, *Agropyron cristatum*, *Nepeta mussini*, *Alopecurus tiflisiensis*, *Festuca valesiaca*, *Stipa arabica*, *Oxytropis pallasii*, *Centaurea ovina*, *Psephellus carthalinicus*, *Botriochloa ischaemum*, *Isatis iberica*, *Poterium polygamum*, *Astragalus bungeanus*, *Minuartia woronowii*, *Petrorhagia saxifraga*, *Campanula alliariifolia*. The participation of succulents is noteworthy - *Sedum oppositifolium*, *Sempervivum transcaucasicum*, *Sedum pallidum*. Characteristic plants of this vegetation are the semi-shrubs and undershrubs (chamaephytes) - *Artemisia caucasica*, *Artemisia incana*, *Artemisia marschalliana*, *Teucrium polium*, *Teucrium nuchense*, *Helianthemum nummularium*, *Thymus coriariifolius*, *Scutellaria orientalis*, *Ziziphora serpyllacea*, *Dianthus orientalis*, *Cerastium argenteum*, etc. Of annuals, *Astrodaucus orientalis* is a characteristic species. The floristic composition is enriched with introduced and subsequently naturalized plant species (*Cercis siliquastrum*, *Ailanthus altissima*, *Amygdalus communis*, *Spartium junceum*, *Pinus nigra* subsp. *pallasiana*).

Current condition of vegetation cover, degree of modification and level of disturbance

Both heavily and partially modified and natural and near natural ecosystems are present in the study area. The level of ecosystem disturbance varies in different parts of the study area. The main determining factors of the levels of disturbance are anthropogenic impacts (grazing, tree and shrub felling, recreational use, pollution).

The level of disturbance in natural and near natural ecosystems is mostly observed in forests and the least - in shibliak-type hemixerophilous shrubberies and meadow-steppes.

Although the disturbance levels of forests varies in most parts of the area, their current overall condition can be assessed as satisfactory. The structure of forest canopy in most cases corresponds to the mean characteristics of forests in the vicinity of Tbilisi. In the stands and their edges there are seedlings and young individuals of key dominant species (especially *Fraxinus excelsior*). However, the condition of the undergrowth and grass cover varies in different stands, which is related to anthropogenic factors (recreational use).

The condition of shibliak-type hemixerophilous shrubberies in most parts of the area is satisfactory or good, with an expressed floristic diversity. Their level of disturbance in most parts of the area is negligible, or none. However, some degraded groupings are also present (especially in the vicinity of residential areas and trails).

The disturbance level of meadow-steppes is low. Their structure corresponds to the physiographical conditions in which they are established. Consequently, their current condition can be assessed as satisfactory.

In steppe ecosystems, the plant communities on the macro slopes of the southern exposure of the Mamadaviti Ridge, village Okrokana surroundings, are characterized by a high level of disturbance and are affected by heavy anthropogenic pressure (grazing). The structure of plant communities in this area is disrupted which is especially reflected in its floristic composition – increased number of weed species were recorded. The disturbance level in the rest of the area is insignificant and therefore their structure is satisfactory.

The condition of the rock and scree ecotope vegetation is different in various parts of the area, which is due to the different natural and anthropogenic factors.

The current condition of artificial plantations is different in various parts of the area. A significant part of the plantations is degraded and need large-scale restoration. Particular attention should be paid to the condition of pines and pine stands - their massive decline is caused by, on the one hand, climate fluctuations (prolonged droughts) and, on the other hand, mycological and entomological diseases. This process could be described as plant disease epidemics. It is irreversible today in not only in most parts of the study area, but also in Tbilisi and its immediate environs as well. The disease is rapidly transmitted to newly planted young pines. As a result, there are died back or dying pine stands. The disease progresses intensively and affects other coniferous species (cypress, thuja, cedar). In addition, the high disturbance level of artificial plantions is noteworthy, which is a result of the adverse impact of anthropogenic factors (primarily recreation and related processes). Young pine specimens have been noted adjacent to some pine stands, although they only appear as single individuals and natural regeneration of pine plantations is not expected.

Dynamics of successive processes of vegetation - direction and trends

Although the disturbance level of forests varies in most parts of the area, they are represented in the climax and near-climaz stages. Their structure is sustainable and compatible with soil, terrain and climatic conditions.

Shibliak-type hemixerophilous shrubberies, in most parts of the area, are represented in the climax and near-climax stages. Their structure is well-established and sustainable, adapted to soil, terrain and climatic conditions. These communities mostly grow on moderate and steep slopes and fully perform their anti-erosion function.

In the steppes there are plant communities in the climax and near-climax stages, as well as at the stage of digressive succession. The existence of different stages is related to the intensity of anthropogenic factors (grazing, recreational load, establishment of artificial plantations). Plant communities of the first ecological stage are found mostly in the crest part of the Mamadaviti Ridge, at the macro slopes of northern exposure. Plant communities in the stage of digressive succession are widespread on the macro slope of the southern exposure of the Mamadaviti Ridge (e.g., village Okrokana surroundings). Steppe communities are mostly established on thin soils and have an important anti-erosion function.

Despite their complex floristic composition, meadow-steppes are in the climax and near-climax stages. The structure is stable and corresponds to the transitional vegetation type. At this stage no tendencies of successive vegetation change are observed. It is distributed on gentle and moderate slopes, on thin and medium depth soils and performs anti-erosion function.

The vegetation of rock and scree ecotopes is more or less dynamic due to the substrate instability. The floristic composition varies in different parts of the area, which is due to the variability of terrain and soil conditions. Invasive plants were also recorded expanding their area of distribution. Nevertheless, the core of the floristic composition is more or less expressed. Such variable and dynamic vegetation is generally characteristic to rocky and especially sedimentary ecotopes. Due to the above mentioned, the vegetation of these ecosystems can not be attributed to the vegetation of the climax stage, especially the floristic grouping of scree and easily shredded sandy clays and sandstones. Vegetation formation processes are clearly seen on relatively newly formed bare slopes. The vegetation of long-formed rocky ecotopes is more or less stable and close to the climax stage.

The floodplain forest derivative cannot be regarded as vegetation in climax stage. As already mentioned, its structure is severely degraded and far from the typical floodplain forest structure. In addition, digression successive processes continue under the influence of anthropogenic factors.

Significant successive processes take place in artificial plantations as well, especially in some pine stands. Particularly in the western part of the study area, the abundant growth of saplings and young individuals of the key dominants (*Fraxinus excelsior*), *Quercus petraea* subsp. *iberica*, *Carpinus orientalis*) of the deciduous forest characteristic to the Tbilisi environs, is observed. Young specimens of other species (*Carpinus betulus*, *Tilia begoniifolia*, *Acer campestre*, *Lonicera caucasica,* etc.) were also observed. It is obvious that there is an on-going restoration process of deciduous forests. Seedlings and young individuals of European ash and Georgian oak are also observed in other artificial plantations, especially in the mixed deciduous stands.

II. Floristic composition

General composition and plant life forms

Despite the small teritory, the study area is distinguished by its floristic richness. About 750 species of vascular plants are distributed.

The richness of floristic composition is not expressed in just taxonomic diversity. Almost all life forms of plant [28] are observed in the study area - trees, shrubs, semi-shrubs and undershrubs, perennials, biennials and annuals. In terms of species, the most diverse is the herb composition, which forms the core of the floristic composition (about 635 species). The composition of woody plants (trees and shrubs) is also rich (about 100 species). The lowest number of species (about 15 species) are semiwoody plants (semi-shrubs and undershrubs).

Herbaceous plants are not only distinguished by their species richness, but also by their different bio-ecology and forms. According to Raunkier classification [27], hemicryptophytes, cryptophytes (geophytes and helophytes) and therophytes are recorded. It is important to note that herbaceous plants are the core of almost all types of vegetation. The hemicryptophytes recorded on the study area are mostly characteristic plants of steppes and meadow-steppes. They are also the key plants forming ground vegetation in forests (*Melica uniflora, Melica picta, Poa nemoralis, Brachypodium sylvaticum, B. pinnatum, Carex humilis, Viola* spp., *Primula macrocalyx, Primula woronowii, Campanula rapunculoides*, etc.) and various types of shrubsThe geophytes distributed in the study area are mostly characteristic to hemixerophilous vegetation (shibliak-type hemixerophilous shrubberies and steppes) (*Gagea taurica, Gagea chlorantha, Muscari szovitsianum, Iris pumila, I. reticulata, Anacamptis morio, Dactylorhiza romana* subsp. *georgica, Ophrys caucasica, Neotinea ustulata, Anacamptis morio, etc.*). The small part of them is the components of forests and forest edges (*Cyclamen vernum, Ficaria ledebouri*, etc.). *Iris caucasica* is a plant of a very gravelly soil and stony ecotopes. The participation of helophytes is small (*Phragmites australis, Typha* ssp.). Therophytes (annual plants) that are common in all types of vegetation are not characterized by high constancy. However, they enrich the floristic composition of different communities.

Trees and shrubs (phanerophytes) are among the main vegetation-forming plants in the study area. They are dominant and associate plants of forests and various types ofshrubberies. They also play an important role in forming vegetation cover of rock and scree ecotopes. Consequently, they are priority plants in forming the vegetal landscapes.

Despite the low number of species, semiwoody plants (semi-shrubs and undershrubs) hold a special place. In the study area they are mostly associated with rock and scree ecosystems and very gravelly soils (*Artemisia caucasica*, *A. incana*, *A. marschalliana*, *Scutellaria orientalis*, *Dianthus orientalis*, *Thymus coriifolius*, *Ziziphora serpillacea*, *Teucrium nuchense*, *T. polium*, *Cerastium argenteum*, *Helianthemum nummularium,* etc.). According to Raunkier classification [27], the semi-shrubs and undershrubs distributed in the target area are mostly chamaephytes.

Endemics and species of high conservation value

Forty-eight endemic species of the Caucasus were recorded in the study area. The families of *Asteraceae* (9 species), *Fabaceae* (7) and *Caryophyllaceae* (5) are represented by the largest number of endemic species (Table 1). They are characteristic plants of different types of vegetation. However, most endemic species are the components of hemixerophilous vegetation, including scree and rock ecotopes.

Some Caucasian species also irradiate mainly in eastern and northeastern Anatolia and northeastern Iran. They are as follows: *Astragalus brachycarpus*, *A. stevenianus*, *Cotoneaster meyeri*, *Onobrychis radiata*, *Erysimum leptophyllum*, *Scutellaria orientalis*, *Scorzonera biebersteinii*. These species can be considered as sub-endemics of the Caucasus.

The number of endemics and sub-endemics is quite high for such a small area. They are plants of different bio-ecology and are common in almost all natural and near natural ecosystems.

The study area supports two species included in the Red List of Georgia; they are *Ulmus minor* and *Juglans regia*. *Ulmus minor* is represented by few individuals and /or small groups and is scattered almost throughout the study area. *Juglans regia* is recorded only in the floodplain forest derivative, where it likely penetrated from adjacent private land plots of village Okrokana.

Rare species also include *Astragalus cornutus*. It is not included in the Red List of Georgia, however its distributional area in Georgia is very limited and is mainly confined to Mamadaviti Ridge [34], where it is sporadically distributed in small groups.

Table. *Caucasian endemic plants recorded on the survey area*

Family	Species	Life form	
		according to Raunkiaer, 1934	according to Serebriakov, 1964
Angiospermae			
Dicotyledoneae			
Apiaceae (*Umbelliferae*)	*Seseli grandivittatum* (Sommier & Levier) Schischk.	Hemicryptophyte	Perennial herb
Asteraceae (*Compositae*)	*Centaurea ovina* Pall. ex Willd.	Hemicryptophyte	Perennial herb
	Cousinia orientalis (Adams) K.Koch	Hemicryptophyte	Perennial herb
	Jurinea blanda (M.Bieb.) C.A.Mey.	Hemicryptophyte	Perennial herb
	Psephellus carthalinicus Sosn.	Hemicryptophyte	Perennial herb
	Scorzonera biebersteinii Lipsch.	Hemicryptophyte	Perennial herb
	Taraxacum grossheimii Schischk.	Hemicryptophyte	Perennial herb
	Taraxacum praticola Dahlst.	Hemicryptophyte	Perennial herb
	Tragopogon serotinus Sosn.	Hemicryptophyte	Perennial herb
	Tragopogon tuberosus K.Koch	Geophyte	Perennial herb
Boraginaceae	*Nonea setosa* Roem. & Schult.	Therophyte	Annual
	Symphytum caucasicum M.Bieb.	Hemicryptophyte	Perennial herb
Brassicaceae (*Cruciferae*)	*Isatis iberica* Steven.	Therophyte	Annual
Campanulaceae	*Campanula alliariifolia* Willd.	Hemicryptophyte	Perennial herb
	Campanula sibirica L. subsp. *hohenackeri* (Fisch. & C.A.Mey.) Damboldt	Hemicryptophyte	Biennial
Caprifoliaceae (*Dipsacaceae*)	*Cephalaria media* Litv.	Hemicryptophyte	Perennial herb
	Scabiosa georgica Sulak.	Hemicryptophyte	Perennial herb
Caryophyllaceae	*Cerastium argenteum* M.Bieb.	Chamaephyte	Undershrub
	Dianthus inamoenus Schischk.	Hemicryptophyte	Perennial herb
	Dianthus subulosus Conrath & Freyn	Hemicryptophyte	Perennial herb
	Gypsophila stevenii Fisch. ex Schrank	Hemicryptophyte	Perennial herb
Celastraceae	*Euonymus leiophloeus* Steven	Phanerophyte	Shrub
Cornaceae	*Swida iberica* (Woronow) Pojark. ex Grossh.	Phanerophyte	Shrub or tree
Crassulaceae	*Sedum caucasicum (Grossh.) Boriss.*	Geophyte	Perennial herb
	Sedum oppositifolium Sims.	Hemicryptophyte	Perennial herb
	Sempervivum transcaucasicum Muirhead	Hemicryptophyte	Perennial herb
Euphorbiaceae	*Euphorbia boissieriana* (Woronow) Prokh.	Hemicryptophyte	Perennial herb
Fabaceae (*Leguminosae*)	*Anthyllis lachnophora* Jus.	Hemicryptophyte	Biennial
	Astragalus bungeanus Boiss.	Hemicryptophyte	Perennial herb
	Cytisus caucasicus Grossh.	Phanerophyte	Shrub

	Lotus caucasicus Kuprian.	Hemicryptophyte	Perennial herb
	Onobrychis cyri Grossh.	Hemicryptophyte	Perennial herb
	Onobrychis iberica Grossh.	Hemicryptophyte	Perennial herb
	Onobrychis kachetica Boiss. et Buhse	Hemicryptophyte	Perennial herb
Lamiaceae (*Labiatae*)	*Hyssopus angustifolius* Bieb.	Hemicryptophyte	Perennial herb
	Teucrium nuchense K.Koch	Chamaephyte	Undershrub
	Thymus coriifolius Ronniger	Chamaephyte	Undershrub
Polygalaceae	*Polygala mariamae* Tamamsch.	Hemicryptophyte	Perennial herb
	Polygala transcaucasica Tamamsch.	Hemicryptophyte	Perennial herb
Ranunculaceae	*Delphinium ochroleucum* Steven ex DC.	Geophyte	Perennial herb
	Ficaria ledebourii Grossh. et Schischk.	Geophyte	Perennial herb
Rosaceae	*Cotoneaster saxatilis* Pojark.	Phanerophyte	Shrub
	Pyrus georgica Kuth.	Phanerophyte	Tree or shrub
	Rubus ibericus Jus.	Phanerophyte	Shrub
Scrophulariaceae	*Verbascum formosum* Fisch. exSchrank	Hemicryptophyte	Biennial
Monocotyledoneae			
Iridaceae	*Iris caucasica* Hoffm.	Geophyte	Perennial herb
Liliaceae	*Gagea commutata* K.Koch	Geophyte	Perennial herb
Orchidaceae	*Ophrys caucasica* Woronow ex Grossh.	Geophyte	Perennial herb

Invasive plants

Several invasive species were identified in the study area. They are plants of various bio-ecological characteristics and life forms. These species are *Opuntia humifusa*, *O. phaeacantha*, *Ambrosia artemisiifolia*, *Xanthium spinosum*, *X. strumarium*, *Ailanthus altissima*, *Cercis siliquastrum*, *Laburnum anagyroides*, *Spartium junceum* and *Koelreuteria paniculata*. These species are represented by micro-groups as well as single individuals.

The source, area of occupancy, abundance and invasion potential in the study area are different. The woody plants escaped from artificial plantations. The cacti species (*Opuntia* ssp.) escaped from the collections at the National Botanical Garden of Georgia and became established in adjacent areas (Tabori, Narikala, Teleti, Mamadaviti ridges, etc.); they are rapidly expanding their distributional area. The annual invasive species (*Ambrosia artemisiifolia*, *Xanthium spinosum*, *X. strumarium*) are widespread in the vicinity of Tbilisi and appear as weeds in different habitats.

Invasive species are most abundantly and intensively established in habitats with heavily modified structure. Their penetration in both natural and near natural plant communities, as well as in well-established artificial plantations, is rare. Their individuals are sometimes found in the forest edges, shrubberies and artificial plantations. Some of them (*Ailanthus altissima*, *Cercis siliquastrum*, *Spartium junceum*) are also becoming well-established in scree and rock ecotopes.

Conclusion

1. The target area is characterized by heterogeneous physiographical conditions, which contributes to the high level of biodiversity. Both ecosystem and floristic diversity was recorded.
2. Vegetation represents a mixture of, on the one hand, natural and near natural and, on the other hand, artificial plantations. No regularity in the spatial distribution of vegetation was observed. In most cases different ecosystems alternate unsystematically and diffusely with one another. However, the concentration of natural and near natural vegetation was detected in the western and central parts of the north-facing macro slope of the Mamadaviti Ridge. Artificial plantations are mostly established in the eastern and northern parts of the study area, as well as on the southern macro slope of the Mamadaviti Ridge.
3. Natural and near natural vegetation is represented by both mesophilous and semi-xerophilous and xerophilous variants. The rapid and often

mosaic interchangeability of terrain and -soil conditions causes their diffuse distribution. In such distribution of vegetation anthropogenic factors also played an important role. Wetland plant groupings occur locally along the shores of Lake Kustba.

The deciduous forests of the foothills and the lower mountain form the major part of mesophilic vegetation. They are represented by the European ash (Fraxineta excelsior), Georgian oak (Querceta petraea subsp. iberici) and Oriental hornbeam (Carpineta orientalis) forests. Shibliak-type hemixerophilous shrubberies are the main units of hemixerophilous vegetation. Steppes occupy smaller areas. Meadow-steppes are transitional communities between mesophilous and hemixerophilous vegetations. They occur at the highest altitudes. Despite the fact they occupy limited area, their role is important in terms of ecosystem biodiversity.

The vegetation of the rock and scree ecotopes is of fragmentary distribution in different parts of the survey area. These ecotopes are established as a result of erosion process, although the existence of their primary origin areas are also possible. The floristic grouping of these ecotopes significantly enrich the biodiversity of the target area.

Common reed beds (Phragmitetum australis) occur locally along the shores of Lake Kustba.

4. Artificial plantations are represented by both coniferous and deciduous stands, as well as mixed plantations. The largest area is covered by the pine stands (*Pinus nigra* subsp. *nigra, Pinus nigra* subsp. *pallasiana, Pinus sylvestris* subsp. *hamata*). The extensive area is also covered by mixed plantations, with both deciduous and coniferous trees and shrubs. A special variant of artificial plantations is the complex habitats of mixed plantations and natural (wild) vegetation. A significant part of artificial plantations are declined and need to be restored and replaced. With this regard, mass drying of conifers (especially pines) is noteworthy.
5. The main part of natural and near natural vegetation (forests, shibliak-type hemixerophilous shrubberies, steppes and meadow-steppes) is in the climax stage, their structure is sustainable and self-regenerating in the majority of the survey area. Regarding this, the vegetation of rock and scree ecotopes is different, the structure of which is more or less dynamic in most parts of the area due to the instability of the substrate. Significant succession processes are observed in artificial plantations, especially in the western part of the area. Particularly, mass regeneration of major species of native deciduous forests (Fraxinus excelsior, *Quercus petraea* subsp. *iberica, Carpinus orientalis*) is taking place in pine stands and there is a replacement tendency of pine stands with native deciduous forests.
6. The flora of the study area is rich and complex. About 750 species of vascular plants are found. All major life forms of different bio-ecology and plants of different phenologies are present. The floristic composition is enriched with introduced plants, some of which were naturalized.

   Forty-eight endemic species of the Caucasus were recorded. Besides, 7 other native species are known to occupy limited area outside the Caucasus Ecoregion and can be considered as Caucasian sub-endemic species.

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Definition of new herbal preservative optimal dose in cooked sausage meat

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ABSTRACT

The infusion made from the extracts of Oregano (Origanum vulgare L), Thyme (Thýmus), and Pennyroyal (Mentha pulegium), is a new preservative and has no impact on the sensory qualities of finished product and its safety quality. Furthermore, it allows cooked sausages to be stored at a low temperature for quite a long period. Meat processing companies operating in Georgia use *„BOMBAL® ASC Super"* preservative produced by a German company "VAN HEES GmbH" in cooked sausage production. It was determined through research that the above-mentioned preservative can be replaced by a new herbal one proposed by us. The minimum amount of the latter in terms of 100 kg sausage meat main raw material is estimated to be 1200 ml. The indicated amount of the herbal preservative when storing at low, yet positive temperatures (+3...+4^{0}C), for 30 days ensures the stability of chemical composition, physicochemical properties, and sensory qualities of cooked sausage "Lean-extra". The finished product retains high quality and safety standards.

Keywords: Cooked sausage, Food additive, *„BOMBAL® ASC Super"*, Herbal preservative, Tasting, Sensory qualities.

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Introduction

The retention of quality and biosafety of products for a particular period is considered to be one of the uppermost problems in food production [1-3]. From this perspective, the researchers employed in food industry focus their attention on avoidance of the negative effect of products, including meat and meat products on people's health through the search for new, more effective food additives [4-6].

Generally, the main purpose of using additives in food industry is to improve the physicochemical qualities, the appearance of finished products, taste, flavor, and consistency of main raw material, as well as the desire to prolong the product's shelf life and so on.

Food additives are used sparingly in the production of food products of particular brand and assortment, and this dose is considered to be safe for consumer health. On top of that, according to the existing data, a person receives on average 2,5-9,0 kg of such substances a year from various products, which is not a small figure. There is a divergence of opinions in scientific literature whether additives, including preservatives, are harmful generally. Food producers consider that otherwise the additives would not be called "food additives" [7].

Doctors, nutritionists, and a large number of consumers disagree with such interpretation of the problem. However, they point out the potential harmfulness of some additives that have already reached into our bodies, as by interacting with the metabolism, they could cause unexpected reactions, often incompatible with life. Unfortunately, the effect of specific food additives on the physiological processes happening in the body as well as the possible effects on one's genetics has not been defined. All food additives undergo chemical processing before given the final appearance. The latter should be taken into account to avoid inhomogeneous and even fatal outcomes. The risk of allergy reactions remains quite high, and their carcinogenic qualities have not been identified.

The safety quality of food additives and their permissible norm is defined by the United Nations FAO and WHO expert committee; this data is included in a specialized catalogue [8, 9] and is available for everyone interested. The relevant service agency of each particular country determines the regulation of various food additives usage [10-12].

From the safety perspective, food additives are divided into three groups in Codex Alimentarius system: I. "E" – safe, II. "E" – additives permissible for use in food production and III. "E" – forbidden. Consumer rights organizations in many countries call group number II additives "the technical components of food", and the use of which is essential for development of food industry. They claim these additives are safe for consumers. Meanwhile, it has been determined that not everything is so uniform and attractive. Their negative impact on human body has also been revealed [13].

The additives offered nowadays contain substances of various composition and effect mechanisms. They include emulsifying agents, flavouring agents, antioxidants, dye, preservatives as well as a wide range of so called technical additives. „E**", i.e. E200-E299 substances and preservatives, represent the main concern of production engineers and nutritionists. It is the topic of heated discussions and serious doubt. The disastrous effect of these additives on microorganisms has been identified, which allows to avoid food contamination by microorganisms for a long period, that is to grow its shelf life. Thus, the effect of food additives is similar to antibiotics, but the mechanism which dictates the suppression of microorganisms is certainly different. When consuming preservatives that are presently approved, the following is expected to occur: digestive system and some internal organs dysfunction, the development of allergic reactions, migraine, skin diseases, breathing problems, carcinogenic formation and so on [14].

Of various cooked sausage storage facilitation substances, Georgian meat processing factories use *„BOMBAL® ASC Super"* preservative (produced in Germany by „VAN HEES") [15]; it constitutes a mixture of three additives: Sodium Pyrosulphite (E223), Sodium Acetate (E262), and Sodium Citrate (E331). It was also determined that when these substances get into the digestive tract, it leads to a high level of risk [16]. The same is true for all non-organic preservatives [17, 18].

In the scientific literature, there had been a presumption, which was later proved in practice, that herbal preservatives possessing antimicrobial qualities prolong the shelf life of sausages. Some of them even hinder the lipid oxidation process and/or the change in sausage meat color [19]. Moreover, the research showed that some herbs with preservative qualities, for example Rosemary extract, are useful for human health and protect it from various malignant tumour formations.

Based on the above-mentioned fact, a research dedicated to the discovery of new substances and materials of herbal origin that represent a lower risk for the human body (or even the absence of any risk), providing similar effect, is becoming quite significant and, due to its urgency, expands to a larger scale.

It has been determined by us that adding 1,5 l of Oregano (Origanum vulgare L), Thyme (Thýmus), and Pennyroyal (Mentha pulegium) extracts (these species grow naturally in Georgia and are cultivated as well) in 100 kg of sausage meat guarantees storing sausages for up to one month, retaining their quality. In particular, organoleptic and microbiological research showed that sensory qualities of finished products are practically unaffected by the 30th day after production. Microbial contamination level is lower than the minimal requirement identified for products [20-22].

Materials and methodology

The experiment was held on the base of Georgian meat producing company "Iveria" (ISO 22 000). In order to determine the minimum amount of Oregano (Origanum vulgare L), Thyme (Thýmus), and Pennyroyal (Mentha pulegium) extract in "Lean-Extra" sausage meat, we prepared and studied 2 control and 3 trial variants of sausages, including the following: sausage meat in the I control group was prepared without preservatives; we added 0.80, 1.20 and 1.50 l of herbal preservative to sausage meat in control groups number II, III, and IV respectively, in terms of 100 kg of main raw material. As to group number V, 0,3 g of *"BOMBAL® ASC SUPER"* was added according to the approved formulation applied by the company.

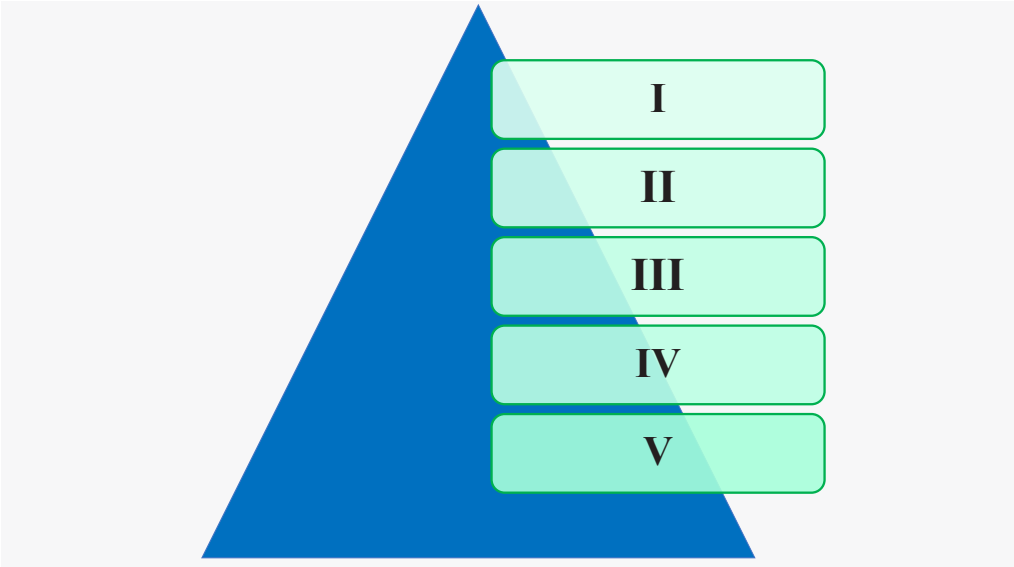

Fig. 1. *Trial and control variants of cooked sausage "Lean-Extra" produced by adding various types and amounts of preservatives.*

Conditional observations:

I control group: without preservatives; II trial group: 800 ml herbal preservative per 100 kg ground meat; III trial group: 1200 ml of herbal preservative per 100 kg ground meat; IV trial group: 1500 ml of herbal preservative per 100 kg ground meat; V control group: 0.3 g "BOMBAL® ASC SUPER" per 100 kg ground meat.

The amount of other auxiliary ingredients of the formulation in trial and control variants in terms of 100 kg ground meat was the same and amounted to 7.25 kg. 17.5 kg of water (flake ice) was added to control variants I and V ground meat. The amount of water in II, III and IV variants was regulated in terms of the herbal preservative water solution in physical form and constituted 16.70, 16.30, and 16.00 kg respectively.

Main raw materials, as well as flavoring and functional additives, were produced according to the regulation applied by the company. The ground meat was prepared in a vacuum cutter-mixer in the following order:

According to the formulation, first we placed the main raw material consisting of 20 kg mechanically softened ground mixture of meat and chicken in the machine bowl, added pork fat emulsifier, pork skin protein-fat emulsifier. After rotating the bowl twice, we added specifically defined amount of herbal extract and flake ice in II, III, and IV variants of ground meat; while "BOMBAL® ASC SUPER" and flake ice were added in V ground meat variant. On top of that, we added table salt, spices, Sodium Nitrite and other ingredients. After 2.5-3 minutes of mixing, starch and milk serum protein were added in the bowl, after that the bowl was covered with the lid and vacuum pump of the machine was turned on. The chopping and mixing process took 8-10 minutes, at +9 ^{0}C temperature. Number I sausage ground meat variant was also made under the described regulations, but without adding preservatives.

After finishing the mixing process, the finished ground meat remained in the bowl for 30 minutes, was weighed and placed into a vacuum pump stuffer receiver. In order to pump the ground meat, "Agro-Pac" 60 mm diametre multilayer polyamid casing was employed, which along with having antimicrobial resistance, mechanical resistance and flexibility, hardly allows any moisture or air through. These features are particularly essential in cooked sausage storing process.

The ground meat stuffing pressure was 4-6 kg/ cm^2 (3,92-5,88 $\cdot 10^5$ Pascal); placed in casings, semi-finished ground meat was divided by hand stationary clips machine into 150-160 mm long, ~400 g loaves that were then hung on a wheeled rack to drain for 1 hour at +20...+25 ^{0}C.

Sausage loaves were boiled in water at +83 ^{0}C, (reaching +74 ^{0}C in the center of a loaf), which took 70-75 minutes. Boiled loaves were placed under a cold water shower, where the cooling procedure lasted for 1 hour and 40 minutes until the temperature in the upper layers of the loaves reached +25 ^{0}C. Next the sausage loaves were transferred to the

storing facility for 12 hours under the conditions of +4 ^{0}C and 95% relative moisture until they reached the selling temperature (+12 ^{0}C and less).

In order to study storing stability, the sausage loaves were stored in a domestic fridge at a low, but positive temperature (+3...+4 ^{0}C) and 85-90% moisture.

Results and analysis

The trial and control sausage meat contained 20 kg of main raw materials; the amount of other components, materials and additives complied with the factory approved formulation, whereas preservatives and flake ice were added in accordance with the scheme designed by us, based on the amount of main raw material. Finally, the mixed masses of all variants of prepared ground meat made up 25,95 kg (Table 1).

We studied the chemical composition of finished products in the laboratory of Agricultural University of Georgia under the requirements of GOST R 52196-2017 [23]. It was determined that the chemical composition indices of the trial and control samples complied with the developed technical regulation of the factory and were hardly different from one another. For instance, according to the data in Table 2, sausages of control variant I and trial variants II, III, and IV differ in moisture and dry substances by 1.10-1.23, in fats – by 0.81-1.10, in proteins – by 0.12-0.13, and in carbohydrates – by 0.15-0.38 g per 100 g. Products of control variant V and trial variants II, III, and IV differ in the same substances by 0.42-0.55, 0.06-0.22, 0.09-0.16 and 0.27-0.50 g per 100 g respectively.

The minimal difference in the chemical composition of the trial and control sausages resulted in an almost similar energy value, which fluctuated within 181-192 Cal, i.e. 760-806 kJ per 100 g.

Table 1. *"Lean-Extra" trial and control sausage meat formulation*

Names of Ingredients	Unit of Measurement	Variant				
		I Control	II Trial	III Trial	IV Trial	V Control
Main raw material	kg	20	20	20	20	20
"BOMBAL® ASC SUPER"	Kg	0,00	0,00	0,00	0,00	0,08
Herbal preservative	ml	0,00	0,160	0,240	0,300	0,00
Other auxiliary ingredients	kg	1,45	1,45	1,45	1,45	1,45
Flake ice	kg	3,5	3,34	3,26	3, 20	3,50
Total	kg	24,95	24,95	24,95	24,95	25.03

Table 2. *Chemical composition of trial and control sausage samples (g/100 g) and energy value (Cal/kJ) per 1000 g.*

Index	Variant				
	I, Control	II, Trial	III, Trial	IV, Trial	V, Control
General Moisture	68,98	67,75	67,85	67,88	68,30
Dry Substance	31,02	32,25	32,15	32,12	31,70
Fat	13,59	14,69	14,56	14,40	14,62
Protein	10,07	10,19	9,94	10,19	10,05
Carbohydrate	4,36	4,51	4,74	4,67	4,22
Table Salt	2,26	2,20	2,21	2,24	2,23
Mineral Substances	0,74	0,66	0,70	0,62	0,58
Energy Value	181 / 760	192 / 806	191 / 801	191 / 801	190 / 796

Prior to distribution, the chemical laboratory of the producing factory studies chemical composition of finished products and their physicochemical properties as well as conducts tasting. In our case, by studying the chemical composition it was determined that sausages of all variants contained 2.2 % of table salt, their moisture was 70%, and active reaction (pH) was within the range of 6.7. Every product also gained a positive appraisal from the members of engineering staff who participated in the tasting process.

According to the methodology, the final outcome of the commercial value determination of sausage as a food product depends largely on consumer opinions, i.e. the outcomes of tasting.

Complying with the research technique, in order to evaluate gustatory and sensory properties of the trial and control sausage variants, tasting was held at the Faculty of Agricultural Sciences and Biosystems Engineering of Georgian Technical University GOST 9959-2015 [24] and GOST R ISO 8588-2008 [25]. Complying with the given regulations, the professor-lecturers, technical staff and PhD students of the university participated in the tasting. The tasting involved two steps: at the initial stage of the production, on the 3rd day, and on the last possible point past expiry date, i.e. on the 30th day. The participants evaluated the gustatory (taste, flavor) and physicochemical (color, consistency, succulence) properties by a 9-grade scale. First they evaluated the product appearance: the correlation between the condition of sausage loaf surface and its shape, the color of sausage meat, porosity and solidity, then the flavor, taste, consistency and succulence.

The results of the tasting cards empirical data collation are shown in Table 3 (drawings 2 and 3).

It was determined that the sample taken on the 3rd day after production had not caused significant differences in judgements. Variant IV (trial), sausage cooked with herbal preservative in terms of 1.5 l per 100 kg of ground meat, also gained the highest average evaluation according to the five indices. Variant I (control), cooked by the traditional formulation applied in the factory, but without preservatives, received the lowest evaluation.

When tasting sausages on the 30th day after production, and observing the appearance of the slices, the participants' attention was drawn to the sausages cooked without preservatives. The surface of ground meat was clearly porous and had occasional moist patches, in comparison with the other samples.

Sausage meat slices of other samples were solid and dry. As to the sausage meat cooked with herbal preservative, its slice had a more attractive pinkish, milky pink color, than when using „BOMBAL® ASC Super ".

The processing of empirical data of tasting conducted on the 30th day after production showed that according to color, taste and consistency, variant I sausage, which had been cooked without preservatives, received the lowest average mark - 6.53.

Table 3. *Results of tasting cooked sausage "Lean-Extra" control and trial samples*

Variant	Color	Flavor	Taste	Consistency	Succulence	Average mark
On the 3rd day after production						
I - control	7,4±0,460	7,2±0,412	7,1±0,227	7,3±0,375	7,4±0,324	7,30±0,254
II - trial	7,6±0,375	7,1±0,350	7,6±0,324	7,5±0,463	7,6±0,420	7,50±0,288
III - trial	7,8±0,313	7,5±0,379	7,8±0,366	7,0±0,534	7,5±0,378	7,50±0,302
IV - trial	8,0±0,189	7,9±0,350	8,3±0,313	8,1±0,295	8,4±0,263	8,12±0,230
V-control	–	–	–	–	–	–
On the 30th day after production						
I - control	6,8±0,563	6, 5±0,365	6,4±0,516	6,3±0,803	6,3±0,847	6,53±0,489
II - trial	7,3±0,843	6,7±0,342	7,7±0,558	6,8±0,792	6,3±0,381	6,93±0,191
III - trial	7,8±,0792	7,5±0,342	8,2±0,543	7,7±0,61	7,5±0,563	7,73±0,399
IV - trial	7,8±0,619	7,5±0,843	7,5±0,428	7,0±0,730	7,7±0,957	7,50±0,422
V-control	7,5±0,477	7,0±0,342	7,0±0,428	6,6±0,730	6,8±0,494	6,98±0,397

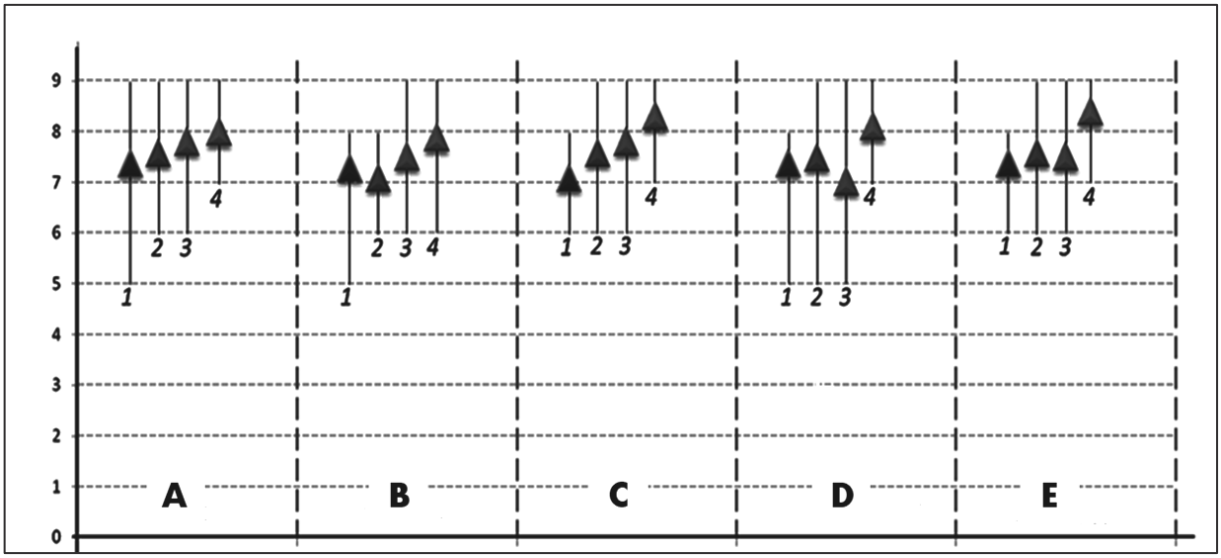

Fig. 2. *Results of sausage tasting on the 3rd day after production*
Notational Conventions - ▲ - average mark; ⊥ - minimal mark; ┬ - maximal mark; A - colour; B - flavour; C - taste; D -consistency; E -succulence; 1, 2, 3, 4 variants.

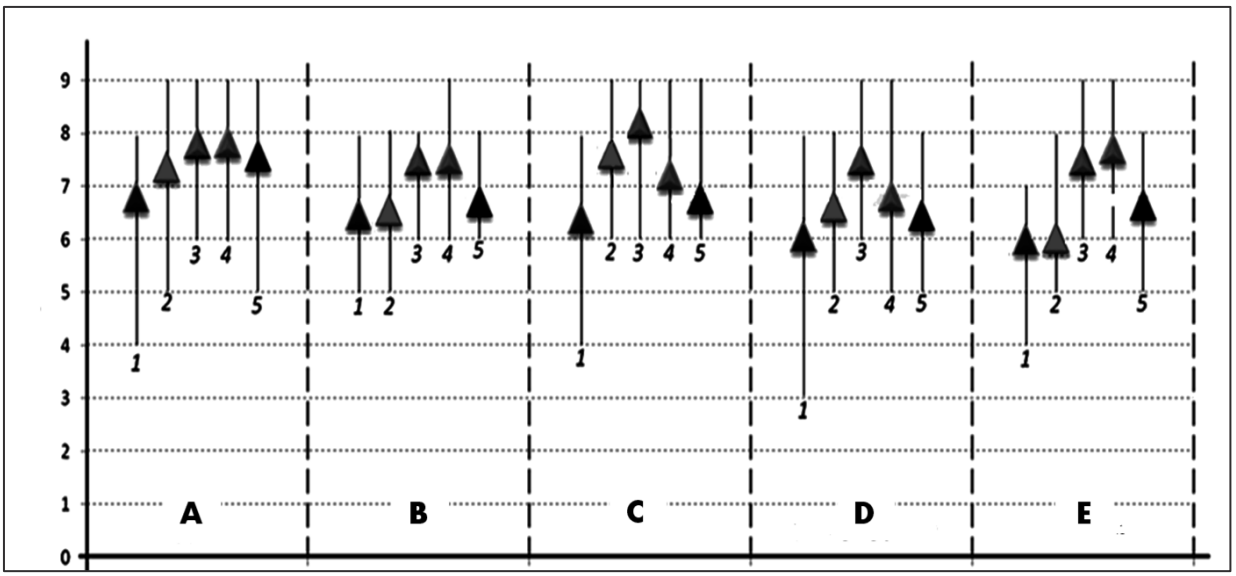

Fig. 3. *Results of sausage tasting on the 30th day after production*
Notational Conventions-▲ - average mark; ⊥ - minimal mark; ┬ - maximal mark; A - colour; B - flavour; C - taste; D -consistency; E -succulence; 1, 2, 3, 4, 5 variants.

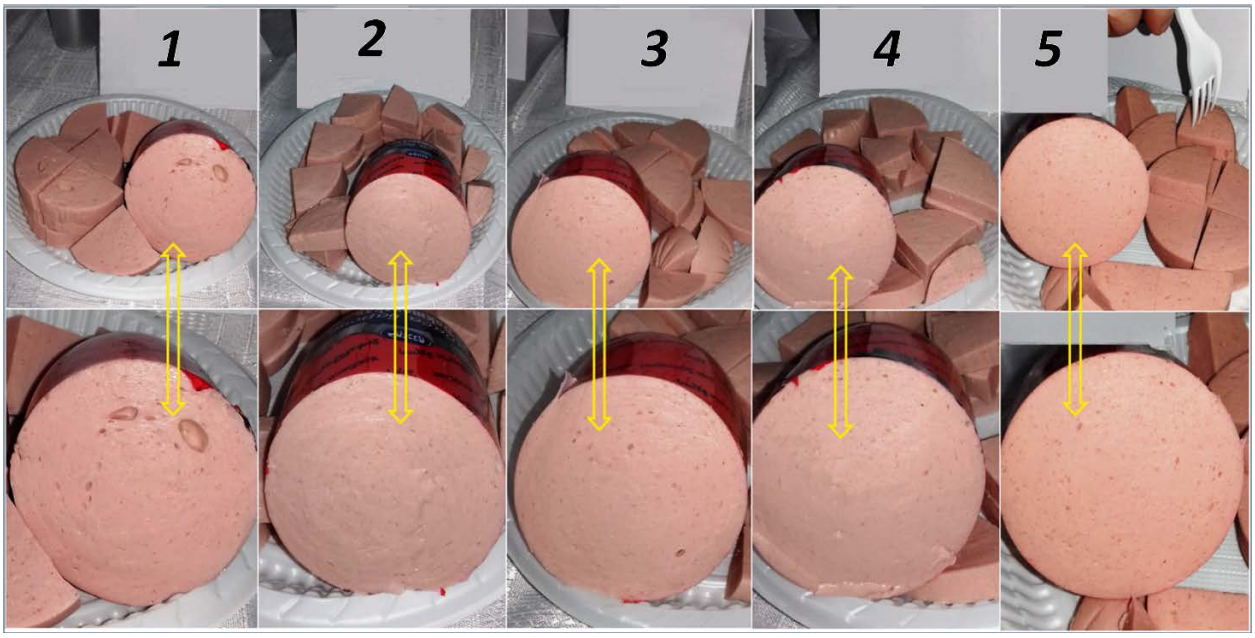

Fig. 4. *Sausage trial and control samples on the 30th day after production*
Notational Conventions - 1, 2, 3, 4, 5 – presented at the tasting the samples of cooked sausage "Lean-Extra" I, II, III, IV and V variants.

According to the five parameters, III and IV variants products received the highest average mark. These samples had been made by adding 1.2 and 1.5 of herbal preservative per 100 kg of main raw material. As to the average evaluation of sausage cooked by the traditional formulation applied at the factory, i.e. using "BOMBAL® ASC SUPER", it was insignificantly, by 0.52-0.75 marks, lower than III and IV variants produce (Table 3).

It must be mentioned that variant I and II products received significantly higher evaluation when tasting was conducted on the 3rd day after production than on the 30th day. As to variants III and IV sausage, their evaluation results received on 30th day hardly differed from those received on the 3rd day.

Conclusion

Introducing Oregano (Origanum vulgare L), Thyme (Thýmus), and Pennyroyal (Mentha pulegium) extract 0.8; 1.2 and 1.5 l per 100 kg ground meat, instead of "BOMBAL® ASC SUPER" included in cooked sausage "Lean-Extra" formulation, doesn't practically change the chemical composition and physicochemical properties of finished product.

Sausage meat made with the herbal preservative is homogeneous, more attractive, of pinkish (milky pink) shade and hardly differs from the products made by following regulations applied in the factory.

All control and trial samples received quite high evaluation in every parameter in tasting that was held on the 3rd day after production.

After tasting samples that had been stored at low positive temperatures on the 30th day it was determined:

- sausage samples that had 1.2 -1.5 l of Oregano (Origanum vulgare L), Thyme (Thýmus), and Pennyroyal (Mentha pulegium) extract added per 100 kg of main raw material received a higher mark. As to sausage made by adding 0.8 l of herbal preservative per 100 kg of main raw material, it received a lower mark than variants III and IV as well as variant V.
- according to the standard regulation applied in the factory, the average evaluation of cooked sausage made with "BOMBAL® ASC SUPER" is a little lower than variants III and IV that had been made with herbal preservative.
- sausage meat made without adding a preservative was porous, had slightly changed color and occasional moist patches on the slice.

In terms of 100 kg of main raw material, 1.2 l of Oregano (Origanum vulgare L), Thyme (Thýmus), and Pennyroyal (Mentha pulegium) extract helps to maintain physiochemical and sensory properties of finished product stored at +3, +4 ^{0}C for 30 days.

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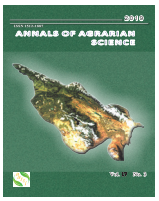

Effectiveness of pheromone for the control Box Tree Moth – *Cydalima perspectalis* in Georgia

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A B S T R A C T

The Box Tree Moth (BTM) - *Cydalima perspectalis* (Walker, 1859) (Lepidoptera; Crambidae) was introduced in 2012 in Georgia and in the next year it began to defoliate *Buxus* spp in large quantities. Today the situation is quite alarming in Western Georgia, with BTM damaging *Buxus colchica*, which is an endemic species of the Caucasian flora and is threatened now by habitat loss. The larvae feed on leaves and shoots, causing serious damages, defoliating box trees, leading to economic, social and environment problems. During 2017-2018, a WitaTrap® Funnel trap system, with pheromone CYDAWIT® (Witasek, Pflanzenschutz, GmbH, Austria), was installed for the monitoring and control of BTM. A long term trapping was conducted at two location of boxwood forest in Tsageri - Ambrolauri (South slop of Grate Caucasian mountain range) in West Georgia. In total 450 pheromone traps were set out on the 150 ha at least ten days before the pest was expected to emerge and at the proper height above the ground or in the plant canopy. Three traps per ha were placed, where prevailing winds were carrying the pheromone into the forest area. BTM moths attracted by the pheromone fall into a capture container and cannot fly out anymore. The pheromone traps were emptied and new dispensers were added at two times during the flying period of *C. perspectalis*. The number of captured adults variated from 11 to 176 moths per trap. In total approximately 93000 (2017) and 74000 (2018) *C. perspectalis* were captured in Tsageri – Ambrolauri region during this monitoring period.

Keywords: *Cydalima perspectalis*, Pheromone trap, Monitoring, Number of captured, Box Tree Moth, Caucasian flora.

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Introduction

The Box Tree Moth (BTM) - *Cydalima perspectalis* (Walker, 1859) (Lepidoptera; Crambidae) is an insect of Asian origin [1-3] that recently invaded most of Europe, Turkey [4-6] and the Caucasus, causing serious damage to ornamental box (*Buxus* sp.) shrubs and trees [7, 3].

In Georgia BTM was introduced in 2012. During the preparation to the 2014 Winter Olympics in 2012 it was introduced from Italy to Sochi with the planting stock of *Buxus sempervirens*. It then arrived in Georgia and in the next year it began to defoliate *Buxus colchica* in large quantities [8]. *Buxus colchica* is an endemic species of the Caucasian flora. BTM larvae cause damage not only in native habitats of box trees, also in public and private gardens and parks [9]. The larvae feed on leaves and shoots, causing serious damages, defoliating box trees, leading to economic, social and environment problems (www.tzona.org.ge, 23 April, 2016).

Today the situation is quite alarming in Western Georgia, where *Buxus colchica*, is threatened by habitat loss.

The aim of this research is to test sex pheromone trapping, in order to determine the efficacy of the baits for detecting the male flights of BTM.

Material and methods

Site of Investigation

The study of distribution of BTM - *C. perspectalis* individuals was conducted in the summer of

2016, in *Buxus colchica* occurring in nature in different regions and locations in Georgia: Imereti, Racha, Tsageri, Samegrelo and Adjara

In every location visual assessments are made in at least 50 individuals of box trees and hedges. Doing this, evaluated plants were distributed in 5 different levels of damage, based on the assessment of the degree of defoliation of box trees by pest larvae, using a 5 point scale (Table 1).

Table 1. *Injury scale of defoliation of box trees by Cydalima perspectalis Walker in percent*

#	Defoliation (%)	Damage level	Damage significance
1	0	0	undamaged
2	1-25	1	weak
3	26-50	2	middle
4	51-75	3	strong
5	> 75	4	very strong

Pheromone traps

During 2017-2018, a WitaTrap® Funnel trap system, with pheromone CYDAWIT® (Witasek, Pflanzenschutz, GmbH, Austria), was installed for the monitoring and control of BTM.

A long term trapping was conducted at two locations of boxwood forest in Tsageri - Ambrolauri (South slop of Grate Caucasian mountain range) in West Georgia.In total 450 pheromone traps were set up on the 150 ha, at least ten days before the pest was expected to emerge and at the proper height 2 m above the ground or in the plant canopy. Three traps per ha were placed, where prevailing winds were carrying the pheromone into the forest area.

Results and discussion

This study to establish the degree of damages caused by *C. perspectalis* to *Buxus colchica* was conducted in locations with confirmed presence of this pest in 2016. The results of the research are presented in Table 2.

The results show that *C. perspectalis* is a serious pest on the box trees in the majority of studied localities. The percentages of defoliation ranged from less than 25% to more than 75%. In general it has been observed that the damage level of box tree varied between weak to very strong (15%-100%).

During 2017-2018, a funnel trap system, with pheromone traps, was installed for the monitoring and control of BTM in different regions of Georgia. In Figure 1, the areas (in hectares) with native *Buxus* sp. monitored by pheromone traps in different regions of Georgia is represented.

Table 2. *C. perspectalis damage level in different locations of Georgia (2016)*

#	Region /Area	Defoliation (%)	Damage level	Damage in %	Damage Significance
1.	Imereti	1-25	1	15,7-24.8	weak
2.	Racha	26-50	2	30,5-47,6	middle
3.	Tsageri	51-75	3	65,5-72,3	strong
4.	Samegrelo	51-75	3	52,5-69,5	strong
5.	Adjara	≤ 75	4	75-100	very strong

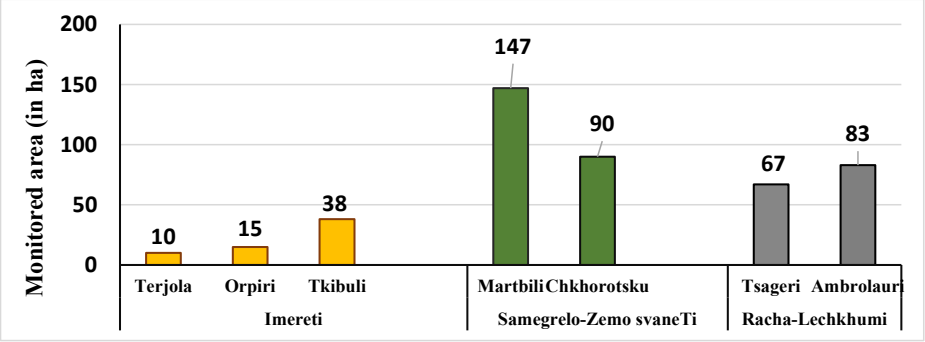

Fig.1. *The areas (in ha) in different regions of Georgia with pheromone traps for the monitoring of C. perspectalis, 2017-2018.*

As described in methods, 450 pheromone traps were installed on 150 ha in Racha-Lechkhumi (Tsageri-Ambrolauri) Region, where we calculated number of baited moth Moth attracted by the pheromone fall into the capture container and cannot fly out anymore (Fig.2).

The pheromone traps were emptied and new dispensers were added at two times during the flying period of *C. perspectalis*. The number of captured adults varied from 11 to 176 moths in one trap. In total approximately 93000 (2017) and 74000 (2018) of moths were captured in the Tsageri – Ambrolauri (Racha-Lechkhumi region) (Fig.3).

Analyzing the results of study relating to the aim of this research, to use sex pheromone trapping, in order to determine the efficacy of the baits the male flights of BTM, is effective means for monitoring. Moreover, use pheromone traps for control this insects in the area of South slop of Grate Caucasian mountain range is very important tool, that for its landscape it is difficult to use techniques for insecticides application.

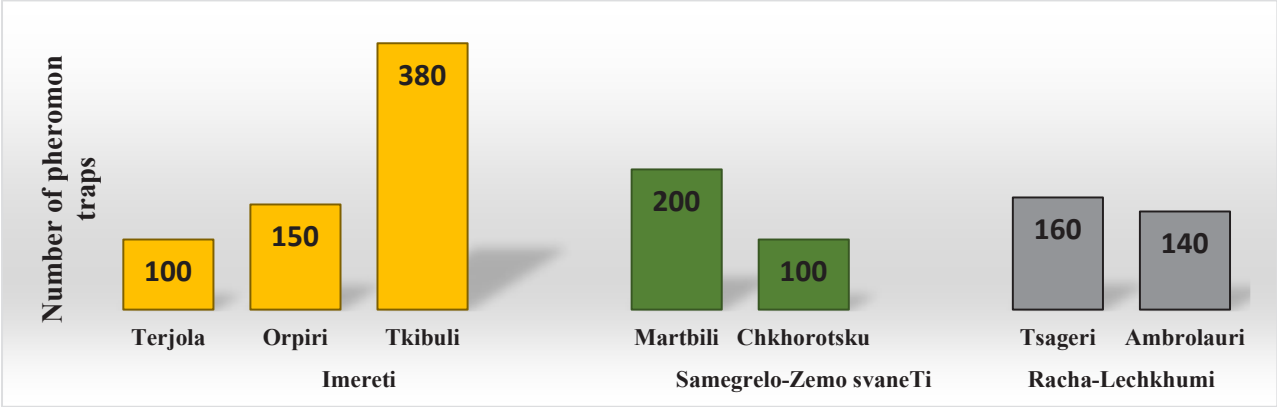

Fig.2. *Pheromone traps set up different region of Georgia for monitoring C.perspectalis*

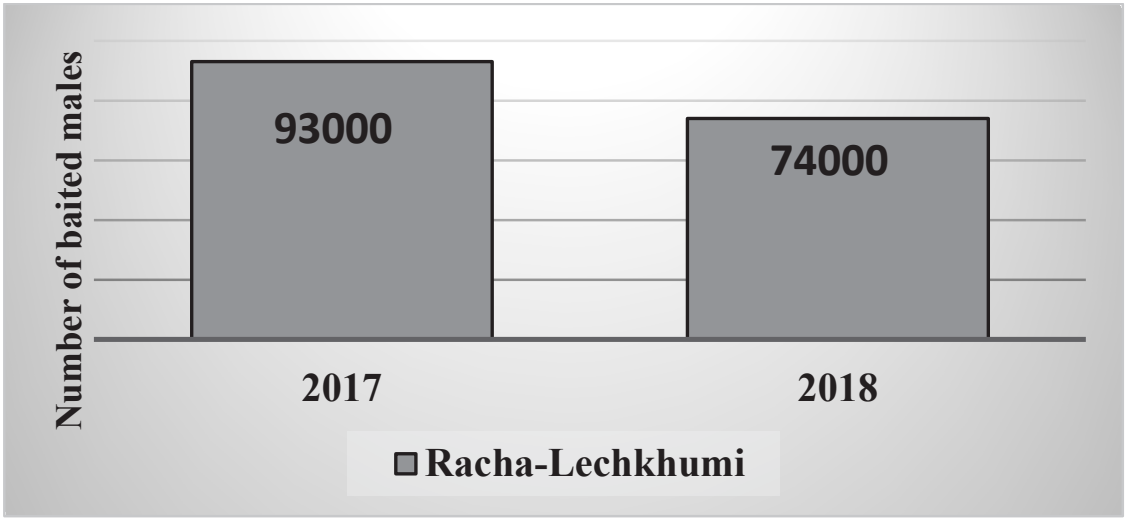

Fig. 3. *Total number of C. perspectalis captured in the Tsageri – Ambrolauri region 2017-2018*

Acknowledgements

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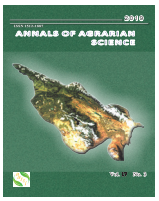

Proteomic responses of oxidative stress-resistant *Arthrobacter globiformis* 151B to Cr and Mg and the influence of Mg on the uptake process of Cr(III) and Cr(VI)

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ABSTRACT

In nature, there are some heavy-metal and oxidative stress resistant bacterial species, which can be used effectively for the environmental biotechnological purposes. They can participate in bioremediation processes: degrade aromatic compounds, uptake radioactive or heavy metals and reduce their carcinogenic activity. Different metals reveal synergist or antagonist effects on each other during their bioaccumulation processes. Knowing of the effects of a metal on another metal uptake processes by bacteria, is one of the actual issues for environmental protection biotechnologies. *Arthrobacter globiformis* 151B is an aerobic, basalt dwelling bacteria, isolated from heavy metal polluted Kazreti region (Georgia). Here we report the influence of magnesium (Mg) ions on the uptake capability of Cr(III) and Cr(VI) by *A. globiformis* 151B. The Influence of Mg(II) and Cr(VI) ions on the proteome of *A. globiformis* 151B have been studied. For identification the significantly differentially expressed proteins, two-dimensional (2-D) gel electrophoresis and Liquid chromatography mass spectrometry (LC-MS/MS) methods were used. We demonstrated that Mg(II) increased the accumulation of Cr(III) by *A. globiformis* 151B and decreased the uptake process of Cr(VI). Significant changes in protein expression involve different groups of proteins. Most remarkable changes were associated with proteins participating in oxidation-reduction processes. According to our earlier and present data, *A. globiformis* 151B is reactive oxygen species (ROS) resistant bacteria with great potential for bioremediation purposes.

Keywords: *Arthrobacter globiformis* 151B, Cr(VI), Mg(II), Bioremediation, Heavy metals, Metal-resistance.

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Introduction

Heavy metals, most important industrial contaminants and systemic toxic agents, exert harmful effects on living organisms. Cr(VI) is a highly water soluble, toxic and carcinogenic metal, which is released into the environment due to different industrial and antrophogenic factors [1- 3]. Cr(VI) occupies the prior place between the most acute environmental pollutants [4, 5]. It can easily penetrate the biological membranes of any living cell. Different cellular components are capable to reduce Cr(VI) using the specific or nonspecific reductants such as glutathione, cysteine, NADH and others [6, 7]. In the cell, Cr(VI) can be reduced to its subsequent Cr(V)→Cr(IV) →Cr(III) forms [7]. During the reduction process, the reduction by products: free radicals, reactive oxygen species (ROS), H_2O_2, reactive nitrogen species (RNS) are formed, which attack the DNA, RNA, protein or lipid molecules, damage them and cause mutations [7]. Reduced Cr(III) form is less toxic and less soluble and it is even essential for different living systems [8, 9]. Different types of living cells are experiencing difficulties to counteract oxidative stress processes caused by heavy metals such as Cr(VI). By this point of view,

there is a high interest in the Cr(VI) resistant endogenous bacterial species of *Arthrobacter* genera. *Arthrobacter globiformis* 151B is an aerobic, basalt-dwelling bacteria, isolated from the metal contaminated (Cr, Pb, Zn, V, Ni, Mo) Kazreti region (Georgia) [10], which reduces toxic Cr(VI) into Cr(III) form even of its high concentrations (Cr(VI) >1000 mg/l) and survives [11]. Since the soil microorganisms are influenced by many different metals concomitant action, it is important to study the simultaneous action of diverse metals on resistant bacterial species, during metal accumulation processes. Some metals cause synergic or negative effects upon the accumulation/uptake capabilities by different bacteria. It is shown, that zinc can increase Cr(VI) reduction and accumulation intensity by *A. globiformis* 151B [10]. Magnesium (Mg) is among the most abundant element in the earth's crust and plays an important role for all living systems. Natural habitats are often characterized by the coexistence of Cr and Mg. As a cofactor, Mg participates in different energy-producing enzymatic reactions, in oxidative phosphorylation or glycolysis [12, 13]. Does the presence of Mg(II) ions influence capacity of *A. globiformis* 151B to accumulate and reduce Cr(VI)? This issue is very important for polluted environments. The purpose of the present study was to determine the effect of Mg(II) ions on *A. globiformis* 151B Cr(VI) and Cr(III) accumulation properties and to study the proteomic responses of bacteria under the influence of Mg(II) and Cr(VI) joint action.

Objects and Methods

Reagents and Chemicals

K_2CrO_4, $MgCl_2$, $CrCl_3$, TSB (Tryptic Soy Broth), TSA (Tryptic Soy Agar), reagents for 2-D gel-electrophoresis and mass spectrometry analyses were purchased from Sigma and Thermo Fisher (all ACS grade).

Sample Preparation for Cr(VI), Cr(III) and Mg(II) Uptake Processes, for Proteomic and Spectral Analyses

Bacterial cells of *A. globiformis* 151B were grown aerobically in 250-ml Erlenmeyer flasks as a 100-ml suspension in TS broth at 26˚C. The cells were grown with constant shaking conditions during 40 hours (at a speed of 100 rpm).

To examine the uptake capability of research elements by *A. globiformis* 151B, we conducted two sets of experiments:

First type of experiments were carried out on the following groups of bacterial cells of *A. globiformis* 151B which were grown during 40 hours in the culture medium containing: (**A)** 7.1 μg/ml Cr(III) and 4.1 μg/ml Mg (controls); (**B)** 40 μg/ml Cr(VI) and 4.1 μg/ml of Mg; (**C)** 40 μg/ml Cr(VI) and 50 μg/ml of Mg; (**D**) 7.1 μg/ml Cr(III) and 50 μg/ml of Mg. After 40 hours of metals exposure, bacterial cells were harvested from the nutrient medium by the centrifugation (3,000g, 15 min, 4˚C), rinsed three times in bi-distilled water for atomic absorption spectroscopy (AAS), 2D-gel electrophoresis and Mass spectrometry (MS) analysis. Bacterial culture growth proceeded without medium renewal. Culture growth was monitored by measuring optical density at 490 and 590nm and by weighing of dried bacterial biomass after their centrifugation and lyophilization.

For the second type of experiments we decided to study the effect of high concentration of Mg(II) on Cr(III) accumulation process on different time points by AAS method. The concentration of Mg(II) was 1600 μg/ml and the concentration of Cr(III) was 7.1 μg/ml in the TS broth for *A. globiformis* 151B. Bacterial cells were incubated at 26°C, during the different time points: 16 hours, 24 hours, 48 hours, 96 hours, and 144 hours. After, they were harvested and prepared for spectral analysis. K_2CrO_4, $CrCl_3$, $MgCl_2$ salts were used respectively for both types of experiment.

AAS measurements

For the determination of the concentrations of Cr and Mg(II) by bacteria itself, wet biomass of bacterial pellet (after centrifugation and washing procedures) was placed in an adsorption-condensation lyophilizer and dried following the procedure reported in [14, 15]. Dried cells were ashed in nitric acid, diluted with bi-distilled water and analyzed by AAS method in an acetylene air flame. Analyst 800 (Perkin Elmer) was used. The detection was carried out at 357.9 nm for Cr and at 285.2 nm for Mg(II).

Statistical analysis

The changes in the concentration of Cr and Mg was analyzed separately with one-way analysis of variances (ANOVA) with factor- cultivation media. Two-way ANOVA was applied used for the analysis of data of Cr(III) uptake in time. The factors were: Time and cultivation media. Planned comparisons

were carried out with student t-test. All comparisons were two-tailed. For the statistical analysis GraphPad Prism 5 software was used.

Bacterial cell wall lysis and protein extraction for 2-D Gel Electrophoresis and MS Analyses

The sample preparations for 2-D electrophoresis and MS analysis were carried out essentially as described in [15, 16]. Bacterial pellets were resuspended in buffer (20 mM Tris-acetate, pH 7.8, 20 mM NaCl, 2 mM EDTA, 100μg/mL lysozyme). Samples were incubated for 30 min at 37°C with intermittent vortexing. 9M Urea, 4% Tween 40, 2% Pharmalyte, 2% Mercaptoethanol, 2% protease inhibitor (bacterial) were added and lysates were centrifuged at 15,000 × g for 30 min at 4°C. Protein concentration in supernatants was quantified by a micro-BCA kit (Pierce, Thermo Scientific) in quadruplicate. Appropriate buffer controls were used.

Isoelectric focusing, Equilibration, SDS Electrophoresis, Staining, Scanning, In-gel Digestion and MS analysis were carried out according the [15, 16]. MS/MS spectra data were analyzed using SEQUEST (Proteome Discoverer 1.4), searching against UniProt UniRef 100 *Arthrobacter* species protein databases.

Results and Discussion

It is known, that Mg(II) ion is the important co-factor-component of different macromolecules such as glutathione, enzymes, polycarbohydrates, ATP, RNA, etc. These molecules actively participate in almost all redox reactions occuring in the cell. We examined the changes in bacterial proteome under the influence of Mg(II) and Cr(VI)+Mg(II) as compared to control cells, using 2-D gel electrophoresis and MS analyses. MS analysis were used to determine the identity of the excised proteins. The 2-D gel electrophoresis of *A.globiformis* 151B protein extracts were carried out initially with two pH gradients: 3.0–11.0 and 4.0–7.0. The majority of the proteins on the 3.0 – 11.0 pH gradient gels were concentrated between pH 4.0 and 7.0. Thus, for the better resolution and identification of differentially expressed bands 2-D gel electrophoresis were continued using strips with a pH gradient from 4.0 to 7.0 (Fig. 1 A, B, C, D). During comparative studies of the influence of Cr(VI) and Mg(II) ions action using 2D electrophoresis on the bacterial proteome, reveal that Mg(II) and Cr(VI) joint action can increase protein content on the 2D electrophoregrams, when 40 μg/ml Cr(VI) and 50 μg/ml Mg(II) were added in a growth medium.

Differently expressed bands were identified by MS. The list of differentially expressed proteins are given in the Table (1). Different microorganisms have their diverse protection capabilities from heavy-metal toxicity by various mechanisms such as uptake, methylation, oxidation or reduction [17]. The disturbed cellular metal ion homeostasis, caused by the increased Cr(VI) and Mg(II) level in the growth medium and further reduction process of Cr(VI) inside the cell, produced the formation of ROS and RNS in high quantity, which may cause oxidative damage to biological macromolecules. The 40 hours exposure of *A. globiformis* 151B cells with the concomitant action of Cr(VI) and Mg(II) or single Mg(II) action, resulted in statistically significant differential expression of 30 proteins out of 556 (Table 1). Functions of statistically significantly differentially expressed proteins between Mg(II) and Cr(VI) treated bacteria (**C**) versus control groups (**A**), or between Mg(II) treated bacteria (**D**) versus control groups (**A**) are associated with different activities. The function of proteins, which appeared or increased in Mg(II) or Mg+Cr(VI) treated cells characterized by oxido-reduction activity, metal ion binding, metal ions transport activities, cell SOS-response, nucleotide -excision repair activities, electron-transport activity, riboflavin synthase complex synthesis activity (which is one of the important reductant in the cell) and the other cellular activities related to the oxidative stress response (Table). During Cr(VI) reduction to Cr(III) form a wide spectra of ROS (superoxide anions, hydrogen peroxide, hydroxyl radicals) are generated and as a result the activities of the enzymes involved in the detoxification of reactive oxygen species are changed [18].

The biomass of *A. globiformis* 151B cells were significantly decreased after the treatment of single Cr(VI) or joint Mg(II)+Cr(VI) ions action due to the elevated oxidative stress (Fig. 2). Between the four combinations of metal mixtures (A, B, C or D), 40 μg/ml Cr(VI) in growth medium (**B**) revealed detrimental effect upon the bacterial biomass and decreased it by 1.8 fold. Mg(II) and Cr(VI) joint (**C**) combination decreased bacterial growth by 1.5 fold. The combination (**D**) decreased bacterial biomass only by 1.3 fold.

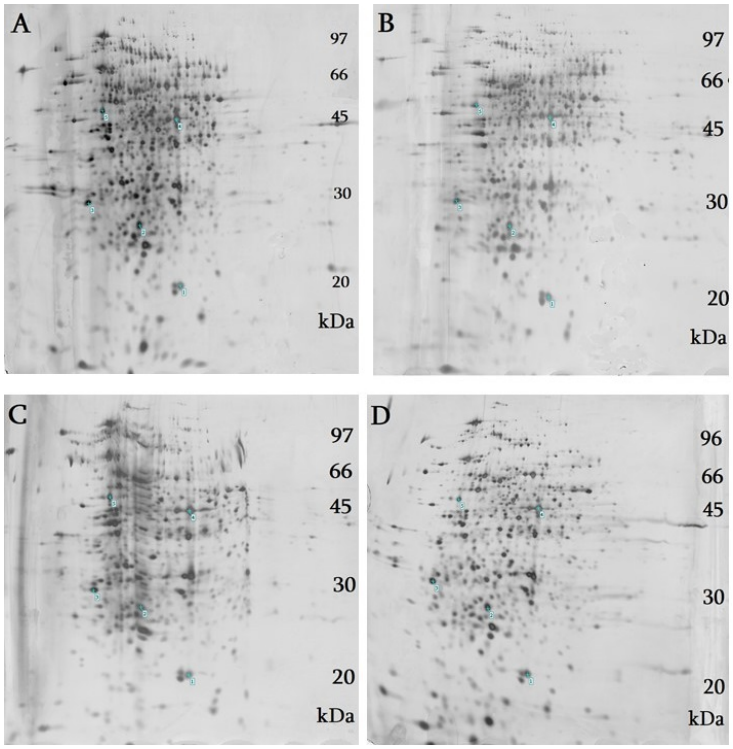

Fig. 1. *Representative images of silver-stained 2-D gel electrophoresis gels on strips with pH linear gradient 4.0 - 7.0 of A. globiformis 151B protein extracts of 40 hours of growth. (A) protein extract from low concentrations of Cr(III) –(7.1 µg/ml) and Mg – (4.1 µg/ml) treated cells (controls); (B) protein extract from 40 µg/ml Cr(VI) and 4.1 µg/ml of Mg treated cells; (C) protein extract from 40 µg/ml Cr(VI) and 50 µg/ml of Mg treated cells; (D) protein extract from 7.1 µg/ml Cr(III) and 50 µg/ml of Mg treated cells. light green color points represent the programme marks done by ImageMaster 2-D platinum 7.0 software. They indicate the matched starting identical points (ID similarities) for matching the rest of the protein ID-s, to find the significantly diferentially appeared protein bands (dots) between A, B, C or D gel-figures. Different ones are cut and analysed by MS for the identification.*

Table. *The list of statistically significantly differentially expressed proteins extracted from Mg(II)+Cr(VI) treated cells versus control cells and Mg(II) treated cells versus control cells of A. globiformis 151B.*

Proteins appearing and their expression increasing in Mg(II)+Cr(VI) treated cells ↑	Molecular/biological functions	**Proteins appearing in Mg(II) treated cells ↑ their expression increases in Mg treated cells**	Molecular/biological functions
Carboxymuconolactone decarboxylase	carboxymuconolactone decarboxylase activity; peroxiredoxin activity, thiol-containing-reductant: hydroperoxide oxidoreductase activity	30S ribosomal protein S1	Nucleic acid binding
DNA-directed RNA polymerase subunit beta	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substratesMolecular functions: DNA binding; DNA-directed 5'-3' RNA polymerase activity ribonucleoside binding	Dimethyl sulfone monooxygenase	Monooxygenase activity, oxidorductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
Capsular glucan synthase	Transferase activity, glucan biosynthetic process	SAM-dependent methyltransferase	Metal ion binding, methyltransferase activity

Aldehyde dehydrogenase	aldehyde dehydrogenase (NAD) activity; aldehyde: NADP+ oxidoreductase, glyceraldehyde-3-phosphate dehydrogenase (NAD+) (non-phosphorylating) activity; NAD binding; NADP binding; Ligand for NAD and NADP, Oxidoreductase activity	NUDIX hydrolase	Hydrolase activity
HNH endonuclease	endonuclease activity; nucleic acid binding; is an integral component of membrane	Two-component sensor histidine kinase	ATP binding, phosphorelay sensor kinase activity
Phenylacetate-CoA oxygenase	2 iron, 2 sulfur cluster binding; electron transfer activity; metal ion binding	UDP-glucose-6-dehydrogenase	NAD binding, oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor polysaccharide biosynthetic process.
ABC transporter	ATPase activity; ATP binding; DNA binding; exonuclease ABC activity; zinc ion binding; nucleotide-excision repair; SOS response	PHB domain-containing protein	integral component of membrane
Glycosyltransferase	guanosine phosphorylase activity; purine-nucleoside phosphorylase activity; pyrimidine-nucleoside phosphorylase activity; thymidine phosphorylase activity; uridine phosphorylase activity	Thiamine-monophosphate kinase	Magnesium ion binding, ATP binding, thiamine-biosynthetic process
ABC transporter substrate-binding protein	metal ion transport	DNA modification methylase	DNA binding, N-methyltransferase activiy, DNA-mediated transposition activity
FAD-linked oxidoreductase	FAD binding; oxidoreductase activity	Transposase	Transposase activity, DNA binding
Undecaprenyl-diphosphate phosphatase	diacylglycerol diphosphate phosphatase activity; phosphatidate phosphatase activity; phosphatidyl glycerophosphatase activity; undecaprenyl-diphosphatase activity	Glutamate synthase	Glutamate synthase activity
1-acyl-glycerol-3-phosphate acyltransferase	1-acylglycerol-3-phosphate O-acyltransferase activity; phospholipid biosynthetic process; an integral component of membrane;	4-aminobutyrate--2-oxoglutarate transaminase	4-aminobutyrate transaminase activity, pyridoxal phosphate binding
6,7-dimethyl-8-ribityllumazine synthase	riboflavin biosynthetic process; 6,7-dimethyl-8-ribityllumazine synthase activity; riboflavin synthase complex	Putative glutamate--cysteine ligase	Glutamate cysteine ligase activity, ATP binding
		FAD-binding protein	D-arabino-1,4-lactone oxidase activity, FAD binding
		L-asparaginase	Asparaginase activity
		Putative oxygenase	Oxidoreductase activity, iron ion binding, 2 iron, 2 sulfur cluster binding
		DNA helicase	ATP binding, DNA binding, DNA helicase activity

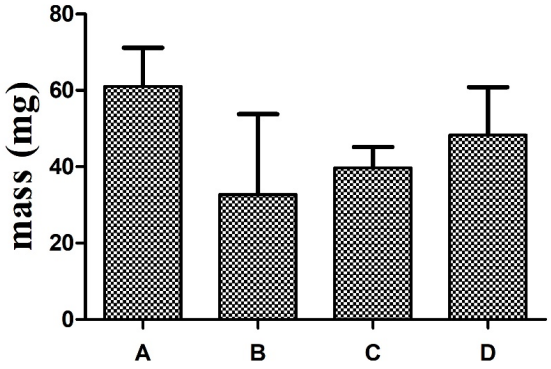

Fig. 2. *The effects of metals on the biomass of A. globiformis 151B. Cultivation time 40 hours. Biomass of the cells which were grown in the culture medium: (A) 7.1 µg/ml - Cr(III) and 4.1 µg/ml Mg (controls); (B) 40 µg/ml Cr(VI) and 4.1 µg/ml of Mg; (C) 40 µg/ml Cr(VI) and 50 µg/ml of Mg; (D) 7.1 µg/ml Cr(III) and 50 µg/ml of Mg .*

By our observations with AAS method, the effect of cultivation medium was significant on the Cr accumulation [$F_{3,11}$=124.09, P=0.0001], whereas Mg(II) accumulation was not changed significantly [$F_{3,11}$=1.87, P=0.214]. *A. globiformis* 151B effectively increased accumulation of Cr(VI) from the growth medium, where the Cr(VI) concentration was 40 µg/ml and Mg(II) concentration was low (Fig. 3, condition **B**) [T = 24.98 P= 0.0001 DF = 4 as compared to condition (**A**)]. Increase in the concentration of Mg(II) ions up to 50 µg /ml (see Fig.3, condition **C**) significantly decreased the uptake of Cr(VI): [condition (**C**) vs condition (**B**) T= 3.93 P = 0.017 DF = 4], whereas the uptake of Cr(III) was strongly enhanced by the same Mg(II) concentration: condition (**D**) vs condition (**A**) [T = 25.18 P= 0.0001 DF = 4]. It seems, that at this time point divalent Mg decreases Cr(VI) assimilation capability by bacteria, but by the other hand Mg(II) ions increased Cr(III) uptake significantly.

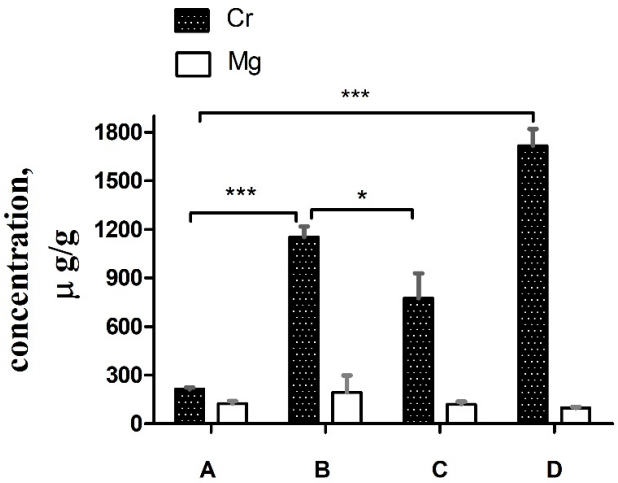

Fig. 3. *Concentration of Cr (dark bars) and Mg(II) (white bars) in A. globiformis 151B from different metal containing medium.*

*The mean values ± sem of Cr and Mg(II) concentrations are indicated on y axis. Culture mediums are denoted on x axis. Culture medium contains: (A) 7.1 µg/ml - Cr(III) and 4.1 µg/ml Mg (controls); (B) 40 µg/ml Cr(VI) and 4.1 µg/ml of Mg; (C) 40 µg/ml Cr(VI) and 50 µg/ml of Mg; (D) 7.1 µg/ml Cr(III) and 50 µg/ml of Mg. For statistical analysis one-way ANOVA with factor- culture medium were made. Planned comparisons were carried out with student t-test. All comparisons were two-tailed. For Cr accumulation, factor- culture medium was significant $F_{3,11}$ = 124.09, P=0.0001, For Mg(II) accumulation, factor- culture medium was not significant: $F_{3,11}$ = 1.87, P=0.214. All the significant differences were marked as follows: *p = 0.017 and ***p = 0.0001. Cultivation time 40 hours.*

The effect of time as well as cultivation medium on Cr(III) uptake was significant [$F_{4,29}$ = 71,73, P=0.0001 and $F_{1,29}$ = 85.92, P=0.0001 correspondingly (Fig.4)]. The interaction of factors was also significant [$F_{4,29}$=45.98, P=0.0001]. The uptake of Cr(III) without Mg(II) displayed significant decrease (e.g. 48h). The addition of Mg(II) shifts uptake uniformly to strong increase with the highest point at 144h (Fig.4). We suggested that, Cr(III) concentration increased in cells, when 1600 μg/ml Mg(II) was added in the growth medium (Fig. 4). Cr concentration significantly (p = 0.0005) increased at the cultivation time of 48 hours (increased by 2.3 fold). Significant changes were observed at the 96 hours of cultivation, when Cr concentration increased by 1.3 fold (p = 0.0099).

Based on our present data, we can suggest, that Mg(II) increases Cr(III) assimilation capability for *A. globiformis* 151B and decreases Cr(VI) accumulation. During Cr(VI) and Mg(II) concomitant action expression of certain group of proteins are increased. Differentially expressed protein bands functions are mainly associated with oxido-reductase and metal ions or different functional groups transport activities. According to the data, *A. globiformis* 151B belongs ROS resistant bacterial species and is promising candidate for bioremediation purposes.

Conclusion

We conclude, that Mg(II) and Cr(VI) joint action caused oxidative stress in *A. globiformis* 151B. Bacteria effectively scavenged ROS and other free radicals produced during Cr(VI) reduction process. Mg(II) ions increased the bioaccumulation of Cr(III) ions in time but at the same time they decreased the uptake process of Cr(VI) by bacteria.

Acknowledgement

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Conflict of Interest

The authors declare no conflict of interest.

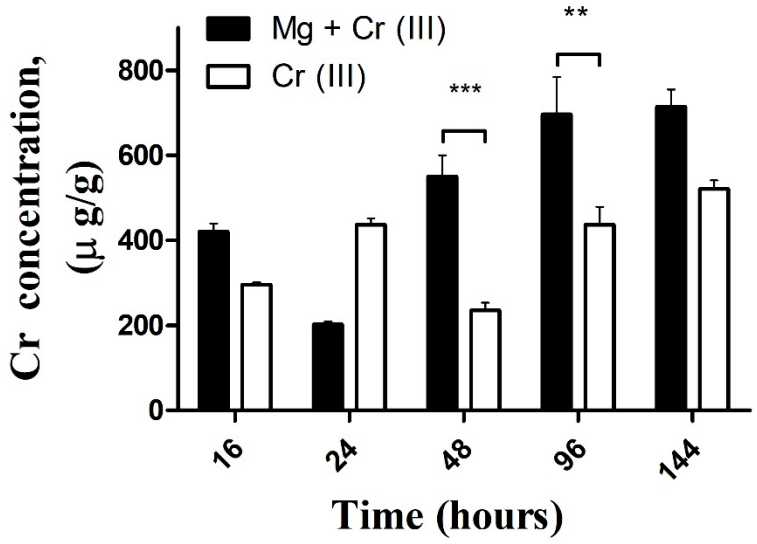

Fig. 4. *The Effect of Mg (1600 μg/ml) on Cr(III) uptake capability by A. globiformis 151B in time. The mean values ± sem of Cr(III) concentrations are indicated on y axis. The time of cultivation is denoted on x axis. Black bars indicate Cr concentration in cells, which were triated by 1600 μg/ml of Mg(II) and 7.1 μg/ml Cr(III); White bars indicate Cr concentration in cells, which were triated by 7.1 μg/ml Cr(III); Two-way ANOVA was applied for the analysis of data of Cr(III) uptake. The factors were: Time and cultivation medium and their effects on Cr(III) uptake were significant. Planned comparisons were carried out with student t-test. All comparisons were two-tailed. The effect of factor- time: $F_{4,29}$ = 71,73, P=0.0001; The effect of factor-cultivation medium: $F_{1,29}$ = 85.92, P=0.0001. The interaction of factors was also significant $F_{4,29}$=45.98, P=0.0001. All the significant differences were marked as follows: **p = 0.0099, ***p = 0.0005.*

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The influence of partial oscillations on the vibratory displacement of grainy material under different frequency vibration mode

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A B S T R A C T

Vibrational transportation-technologic (VTT) process is a dynamically sensitive process, in which many physically different components are involved: vibrodrive, elastic system, working member (absolutely or finitely rigid), various grainy loads. The interactions of those components define the behavior of grainy material on the surface of working member. As a main defining factor of the accuracy of the process, precise transmission of vibration to the working member is of particular importance. In the manuscript, the special dynamical model of the VTT system and corresponding mathematical model of the VTT process are presented, where the movement of grainy material is described under the conditions of spatial vibration of the working member, which is caused by possible errors in the manufacture and assembly of the vibration machine. Mathematical modeling has been carried out under continuous operating conditions (amplitude, frequency) of electromagnetic resonance vibrofeeder, when simultaneously alternating amplification of separate non-working spatial vibration and revelation of its impact on the process takes place. The results of modeling in the form of graphs and the influence of non-working vibrations on the law of the material displacement are presented in the manuscript.

Keywords: Vibratory displacement, Partial oscillations, Grainy material, Resonant vibration, Mathematical modeling, Vibratory Feeder.

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1. Introduction

Vibrational transportation-technologic (VTT) machines are widely used in many fields of manufacturing in the world for vibrational processing and transportation of different kinds of materials [1-11].

VTT machines (drawing.1c) are used in construction, mining, agriculture, chemical and confectionery manufactures and others, therefore, the dimensions of machines vary, as well as their power, shapes of working member and types of vibro-drive and elastic systems.

VTT process is a dynamically sensitive one, in which many physically different components are involved: vibro-drive, elastic system, working member (absolutely or finitely rigid), various grainy loads [10, 12-17]. The interactions of those components define the behaviour of grainy material on the surface of working member.

Due to various construction, installation or manufacturing errors in spring vibrating machines [12, 17], Also, due to the specificity of the springs, there is a deviation of the excitation force from the calculating direction. As a result, spatial oscillations occur along with the operating oscillations of the working member [17-19], which, in the case of amplification (e.g., under resonance conditions), can have a significant impact on the vibrational displacement pattern of loose material.

Based on the above, it is advisable to develop a generalized (spatial) mathematical model of a loaded vibrational technological machine, where the possible cinematic and dynamic connections and interactions between the constituent components will be reflected. Such an approach will allow us to investigate through modeling

the influence of a wide range of parameters on the regularity of movement of material (technological load).

2. Spatial dynamical model of vibrational displacement of grainy material

A spatial dynamical model of vibrational transportation of grainy material has been developed to investigate this problem (Fig. 1). A three-mass (vibro exciter – working member – grainy load) dynamical model of the vibro-feeder is shown in the figure, where the following assumptions are adopted:

- Working member, as an asymmetrical rigid body, performs spatial (rotational-linear) vibrational motion;
- Grainy material is considered as a solid body, which will be equipped with conditional elastic-damping elements, which characterize (describe) the rheological properties of grainy material;
- The movement of grainy load is considered in three linear directions;
- The third mass - vibroexciter is considered immobile , from which the vibration is transmitted to the working member in a single direction;
- The excitation force is not transmitted precisely to the center of gravity of the working member, but is missed due to various permissible structural and physical inaccuracies [5, 19], which generates force projections and moments towards the center of gravity.

The working member $(O_1x_1y_1z_1)$ with elastic system 1 (fig. 1*a*) from one side is connected to vibroexciter $(O_2x_2y_2z_2)$ and from other side – to the material to be displaced $(O_2x_3y_3z_3)$ with one-sided connection 2. Free point A_i of the working member is vectorially connected to the vibratory exciter and its own center of gravity (O_1), just as the point of the material is connected to the O_1 and O_3 points (such connection of the points A_i and B_i are used to obtain the mathematical model (9) of spatial motion of the system [11, 17].

Fig. 1*c* shows an analog of dynamical model (fig.1 a) – vibratory feeder with vibratory exciter and grainy material.

The grainy material (M_3) model (Fig.2) is a cube-shaped body, in which the whole mass is concentrated and equipped with conditional elastic-damping elements (k_x, k_y, k_z, $k_{\square}$, $k_{\square}$, $k_{\square}$) unbound from one side (from the side of the working member surface).

They describe the properties of a particular material and vary depending on the working mode of the vibratory machine (moving along the surface of the working body or separately).

Presentation of grainy material in such a way on the one hand allows us to include it in the overall oscillation system (Fig. 1 a), as a solid body (describe spatial motion), and on the other hand describe its deformation (rheological) properties with elastic elements.

The rotational motions of each mass of the system are described in Euler angles (Fig. 1 b), for both towards its own center and from one mass towards another.

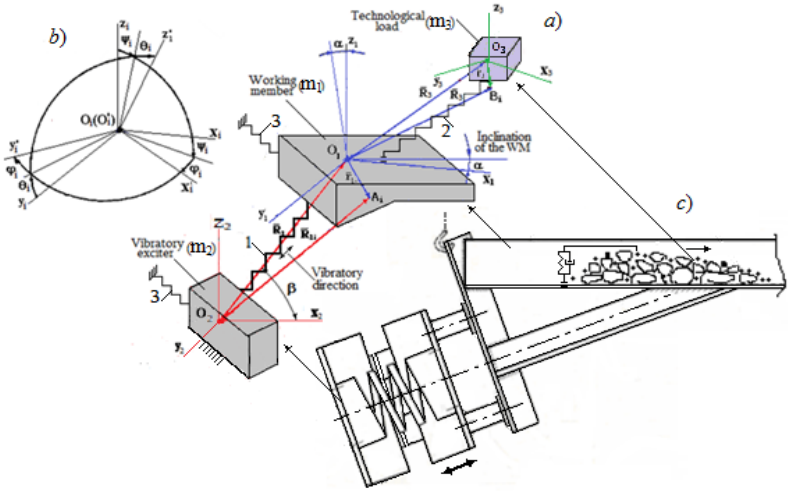

Fig. 1. *The dynamical model of vibratory technological machine: a) three mass spatial model, b) rotational motion, c) physical analogue of the model*

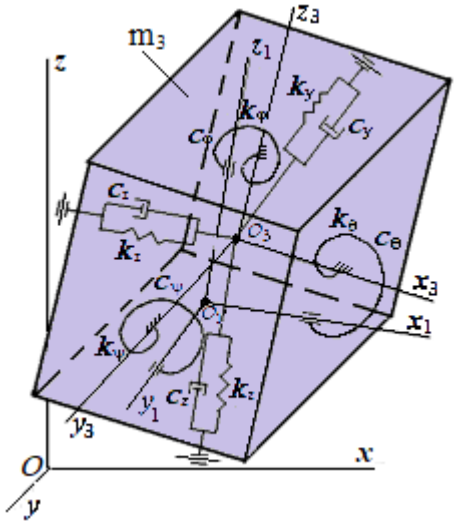

Fig. 2. *The spatial model of grainy material*

3. Friction force between the working member (m1) and the surfaces of the material (m3)

The problems of the friction force between the working member and the material to be displaced are studied for piece details in detail and substantially [1-3, 20]. They examine the typical forms of frequently used parts (rectangular, spherical, cylindrical, etc.), as well as polyhedral parts with rolling properties for which the friction force is only present at the points of contact of the working member and the load.

Unlike singled out "hard" materials, for grainy materials, there is no definite boundary of point of contact. This view stems from the fact that for dispersive (grainy) materials the detachment from the surface or attachment to it does not happen instantaneously, but along with transitional elastic-damping process. To describe the friction force between the working member and grainy material, different approaches are used [21-25], the essence of which lies in that the reaction of the load on the working member represents the function of load movement and velocity; At the same time, the internal resistance of the material, as well as the resistance of the environment in which the movement takes place (air, liquid, etc.) is taken into account.

$$N_q = f\ (\dot{q},\ q), \qquad (1)$$

where q takes the values: $x_{1,3}$, $y_{1,3}$, $z_{1,3,}$ and the friction force will be

$$(F_{fr})_q = f_q N_q;$$

When considering the spatial (rectilinear or rotational) motion of a technological load, in addition to the normal reaction, as a result, moments of those forces arise on this or that surface of the working member (Fig. 3):

$$(M_{fr})_q = (F_{fr})_q \cdot r_q, \qquad (2)$$

Where f_q is the friction coefficient between the load and the working body (f_q is normally obtained in each cycle of the variable motion depending on the dynamical condition of the load – sliding on the surface, stoppage, etc.); r_q is the distance from friction surface to the center of gravity of the load along the coordinates.

The components of the friction force can be expressed as follows:

$$F_{x_3} = f_x N_z sign(\dot{x}_3);$$

$$F_{y_3} = f_y N_z sign(\dot{y}_3); \qquad (3)$$

$$F_{z_3} = f_z N_y sign(\dot{z}_3),$$

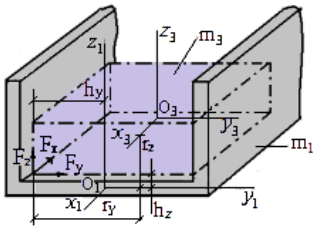

Fig. 3. *To determine the friction forces and moments affecting the grainy load*

where f_x, f_y, f_z are the friction coefficients between the load and the working member in the directions of x, y, z (subsequently will be obtained: $f_x = f_y = f_z = f$); N_y – normal reaction of the load on the lateral surface (Fig. 3); N_z – normal reaction of the load on the bottom; sign represents a nonlinear function and is determined depending on the sign of velocity V: sign = 1, when $V < 0$ and sign = -1, when $V > 0$.

The moments of the friction forces towards the axes are expressed as follows:

$$(M_{fr})_{x_3} = (F_{z_3} r_y - F_{y_3} r_z) sign(\dot{\theta}_3);$$

$$(M_{fr})_{y_3} = F_{x_3} r_z sign(\dot{\psi}_3); \quad (4)$$

$$(M_{fr})_{z_3} = F_{x_3} r_y sign(\dot{\varphi}_3);$$

where r_y, r_z are the distances from friction surface $O_3 x_3 y_3 z_3$ to the system axes. The friction force moments towards the system (working member) $O_1 x_1 y_1 z_1$ are expressed as follows:

$$(M_{fr})_{x_1} = (F_{z_3} h_y - F_{y_3} h_z)$$

$$(M_{fr})_{y_1} = F_{x_3} h h_z); \quad (5)$$

$$(M_{fr})_{z_1} = F_{x_3} hhhh_y);$$

where h_y, h_z are the distances from the friction surface $O_1 x_1 y_1$ to the system axes.

In the given example, working member is bound from two sides with the planes: $O_1 x_1 y_1$, $O_1 z_1 x_1$, whereas in the direction of $O_1 x_1$ it is open; in this case, on $O_1 y_1 z_1$ surface the friction force is not present, and therefore, r_x, h_x multiplier members in (4), (5) expressions equal to zero.

4. The excitation force

In a real machine, as a result of initial errors, the excitation force may not be exactly in the center of gravity, but be moved with eccentricities e_x, e_y, e_z (which was mention above as well); Besides, when the mass M_1 is dynamically affected, the mass deviates with respect to the external (excitation) force by x_1, y_1, z_1 coordinates at the expense of deformation of the elastic system.

As an illustration, on Fig.4, the condition of M_1 mass is shown before and after the engagement of excitation $Q(t)$ force; M_1 mass is shown in two different conditions – I, II; I corresponds to the initial condition, when $Q(t)$ direction coincides the direction of non-elastic spring axis and passes on the center of gravity OO_1 of M_1; II corresponds to the real condition of M_1 mass, i.e. considering the deviations caused by tolerances on the machine manufacturing and installation [3, 5, 23, 26, 27]. The deviations, which are characterized with corners and eccentricities represent the reason of generation of friction forces, which, along with bending deformations of the spring 1, cause the vibration of mass M_1 in space.

The projections of force Q on the axes of coordinate system $O_1 x_1 y_1 z_1$ will be expressed as follows:

$$Q_{x_1} = Q[(\psi_1 - \psi_2)\sin\alpha_1 + \cos\alpha_1];$$
$$Q_{y_1} = Q[\varphi_2 - \varphi_1 \cos_1 - \theta_1 \sin\alpha_1]; \quad (6)$$
$$Q_{z_1} = Q[(\psi_1 + \psi_2)\cos\alpha_1 + \sin\alpha_1)];$$

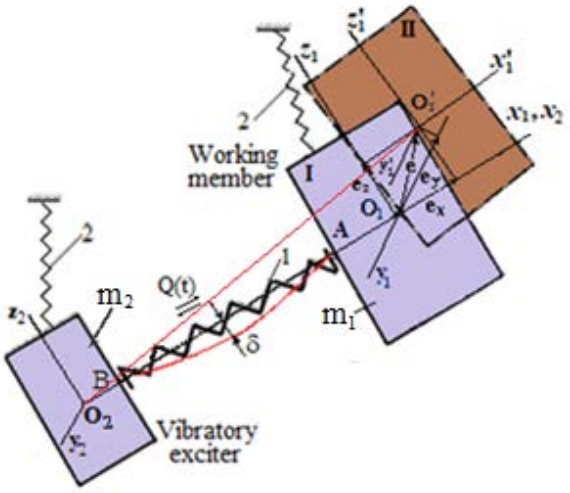

Fig. 4. *The position of the working member before and after the engagement of the force*

The moments of Q force are defined by the formulas of vector algebra theory [6]; If we define point N, on which Q force vector passes, then the moment of this vector towards $O_1 x_1 y_1 z_1$ coordinate system axes will be as follows:

$$M_{X_1} = e_{Y_1} Q_{Z1} - e_{Z_1} Q_{Y_1};$$
$$M_{Y_1} = e_{Z_1} Q_{X_1} - e_{X_1} Q_{Z_1}; \quad (7)$$
$$M_{Z_1} = e_{X_1} Q_{Y_1} - e_{Y_1} Q_{X_1}.$$

5. Mathematical model of "Grainy material – working member" system spatial vibratory motion

To obtain the differential equation system of load's spatial vibratory movement, the dynamical model presented on Fig.1 was considered. The systemic approach [17] was used, which allows to fully describe they dynamic interaction of masses.

To obtain the full mathematical picture of the material and working member interaction, we used the classical theory [17, 19] of relative (technological load), translational (working member) and absolute (technological load) motion of bodies, which corresponds to the principles of vibratory displacement of one body in relation to the other.

This approach involves obtaining vector equations of velocities of A_i and B_i free points of the masses (relative, translational, absolute) and their expansion on the coordinate axes, then their projection on the coordinate axes of the working member using Euler's angles [17]. Subsequently, by obtaining the functions of the total kinetic and potential energies and resistance, and using the 2nd order Lagrange equation, we obtain the differential equation of spatial motion (9) of each mass.

If we assume that the reaction of the grainy load in the equations of the working member (1) is equal to 0 and also in the left part of the equations we consider only linear constituents, then the system of differential equations of the spatial motion of the working member can vectorically be expressed in this way:

$$M_i\ddot{q}_i + c_i\dot{q}_i + b_iq_i - d_iq_j = A_qQ_q(t), \quad (8)$$

where M_i is the mass of the working member and the moments of inertia during the rectilinear and rotational motion respectively; q_i is the coordinates of spatial motion: x_1, y_1, z_1, θ_1, ψ_1, ϕ_1; b_i is the coefficient of resistance towards the axes of spatial coordinates; b_i, d_i coefficients express the interdependence of linear-spatial motions; q_j - the corresponding rotational motion of a linear motion on different axes; F_q - the projection of excitation force and moment on the corresponding axis; A_q - force coefficient on the corresponding axis.

As mentioned, certain structural, physical, and other permissible errors occur during the manufacturing and installation of the vibratory machine [12, 19], which is why the excitation force is not transmitted to the center of gravity of the working body precisely; as a result, the force constituents on the coordinate axes are obtained, as well as the corresponding moments towards the center of gravity. Normally, such deviations are not taken into account due to their smallness, but in resonance machines, they can have a significant influence on the technological process.

After determining the total kinetic and potential energies of the masses M_1 and M_3 and obtaining the analytical expressions (the expansion on the coordinate axes considering Euler's angles [17], working member inclination and angles of vibrations), using the 2nd order Lagrange equation, the system of differential equations of material's spatial motion is obtained:

$$\begin{aligned}
&m_3\ddot{x}_3 + m_3[(\ddot{x}_1 - \ddot{z}_1\psi_1)\cos\alpha_1 - (\ddot{z}_1 + \ddot{x}_1\psi_1)\sin\alpha_1 + \ddot{\psi}_1 z_3 - \ddot{y}_1\varphi_1 + 2\dot{\psi}_1\dot{z}_3 - \\
&-\ddot{\varphi}_1 y - 2\dot{\varphi}_1\dot{y}_3] + h_x(\dot{x}_1\cos\alpha_1 - \dot{z}_1\sin\alpha_1 + \dot{x}_3) + h_{x_3}\dot{x}_3 - m_3g(\sin\alpha - \psi_1\cos\alpha) = \\
&= -(f_{x_3}N_z + f_{y_3}N_y)sign(\dot{x}_3), \\
&m_3\ddot{y}_3 + m_3[\ddot{y}_1 + (\ddot{z}_1\theta_1 - \ddot{x}_1\varphi_1)\cos\alpha_1 + (\ddot{x}_1\theta_1 - \ddot{z}_1\varphi_1)\sin\alpha_1 - \ddot{\theta}_1 z_3 + 2\dot{\theta}_1\dot{z}_3 + \\
&+2\dot{\varphi}_1\dot{x}_3] + h_y(\dot{y}_1 + \dot{y}_3) + h_{y_3}\dot{y}_3 + k_{y_3}y_3 + m_3g(\varphi_1\sin\alpha + \theta_1\cos\alpha) = -f_{y_3}N_z sign(\dot{y}_3), \\
&m_3\ddot{z}_3 + m_3[(\ddot{z}_1 + \ddot{x}_1\psi_1)\cos\alpha_1 + (\ddot{x}_1 - \ddot{z}_1\psi_1)\sin\alpha_1 - \ddot{y}_1\theta_1 + \ddot{\theta}_1 y_3 + 2\dot{\theta}_1\dot{y}_3 - \\
&-2\dot{\psi}_1\dot{x}_3] + h_z(\dot{z}_1\cos\alpha_1 + \dot{x}_1\sin\alpha_1 + \dot{z}_3) + h_{x_3}\dot{z}_3 + k_z z_3 + m_3g(\cos\alpha - \psi_1\sin\alpha) = \\
&= -f_{z_3}N_y sign(\dot{x}_3),
\end{aligned} \quad (9)$$

where $\alpha_1 = \alpha + \beta$, α is the inclination of working member towards the horizon and β – the angle of vibration (Fig.1).

In the presented work, with the help of equations (9), this time we consider movement of the material only in the linear spatial directions and study of the influence of various non-working vibrations (y_1, z_1, θ_1, ψ_1, ϕ_1) of the working member.

In the process of mathematical modelling, the change of the spatial vibrations (strengthening, weakening) occurs not by variation of the vibratory exciter force, but by its own vibration entering resonance in different directions with frequency (vibration) of the constant excitation force and therefore its amplitude changes (increases). Such an approach allows us to investigate and establish the influence of each non-working vibration of the working member on the VTT process, when the working member operates in normal resonance vibratory regime and acts in combination with the aforementioned vibration.

As an example, let's examine the lateral displacement equation (10) of the working member from the vector expression (8). Let's assume that the excitation force changes accordimg to sinusoidal law, with frequency - w

$$M_1\ddot{y}_1 + c_y\dot{y}_1 + b_y y_1 - d_\psi\psi_1 == Q_y sin\omega(t); \quad (10)$$

To amplify it, b_{y_1} should change so that its own frequency ω_{y_1} approaches 50 Hz (should enter in resonance with the excitation force); Such approach allows to observe the tendency of its influence on the parameters of material displacement (Fig.5).

6. Some of the modeling results

In the figures are given the graphs, where the influence of some partial (of non-working

direction) vibrations on the process of material's displacement is shown.

Fig.5 shows the impact of transverse vibration (y_1) on vertical displacement (z_3) and velocity (V_x); As in other instances (Figs. 6, 7, 8), x_1 is the amplitude of working vibration and as a consequence of the modeling condition, it is constant for each experiment, when ω_{exc} takes the values: 25, 50, 100 Hz (as indicated on the figures).

Fig. 6 shows the change (increase) of vertical partial amplitude (z_1) of the working body and the corresponding changes of dynamical parameters of motion (z_3, V_x, N_z – reaction of the material on the bottom and N_y – reaction of the material on the lateral surface).

It can also be noticed that different partial vibrations' (y_1, z_1, etc.) entry into resonance causes changes in the inertial members associated with it in equations (8) (for example, $\ddot{y}_1\varphi_1$, $\ddot{z}_1\theta_1$, etc.), which is reflected in the change in material velocity and other dynamical characteristics. (working vibration frequency ω_{exc} = 100 Hz).

Fig. 7 presents the influence of transverse partial vibration (y_1) on the dynamical characteristics of the material, under the conditions of working vibration (ω_{exc} = 25 Hz); z_3, y_3 are vertical and transverse displacements, V_x – the velocity of displacement in transverse (x_3) direction.

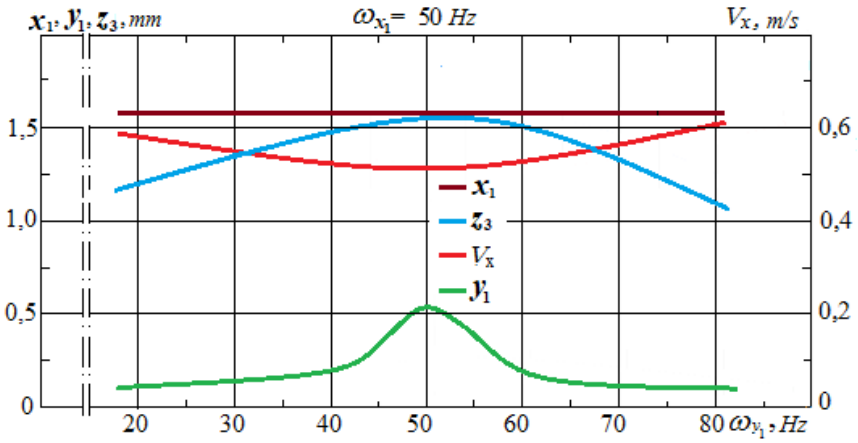

Fig. 5. *The dependence of material movement velocity (V_x) and vertical trajectory (z_3) on the transverse vibrations of working member (y_1), when working vibration (x_1) value is constant*

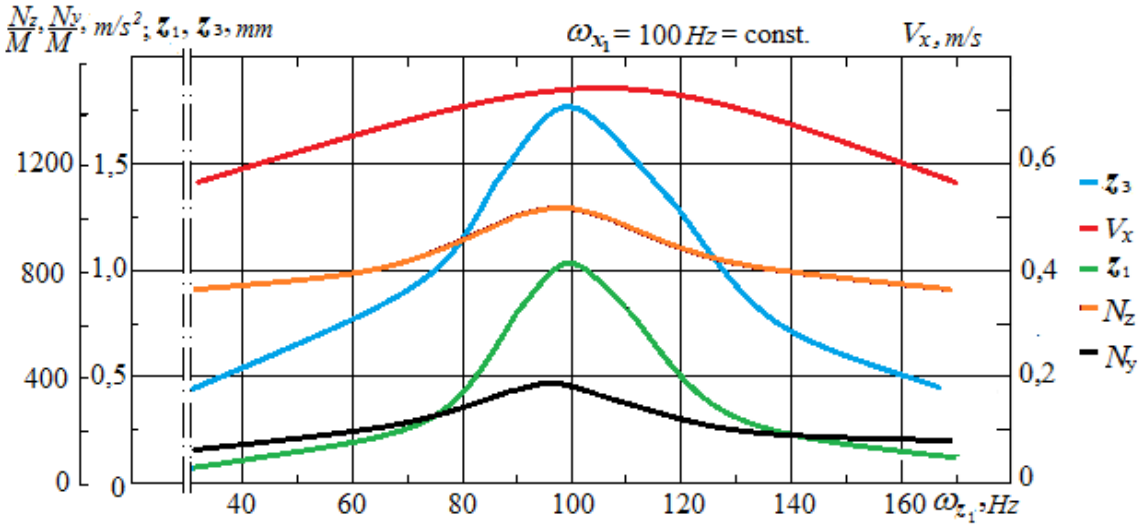

Fig. 6. *The dependence of material movement velocity (V_x), vertical trajectory (z_3) and reaction forces (N_z , N_y) on the vertical vibrations of working member (z_1)*

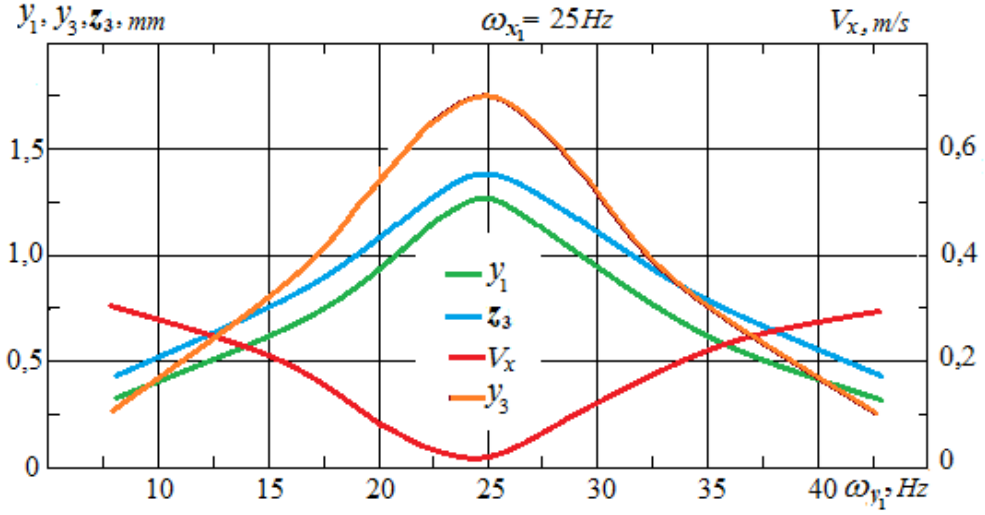

Fig. 7. *The dependence of material movement velocity (V_x), vertical trajectory (z_3) and transverse trajectory (y_3) on the transverse vibrations of the working member (y_1)*

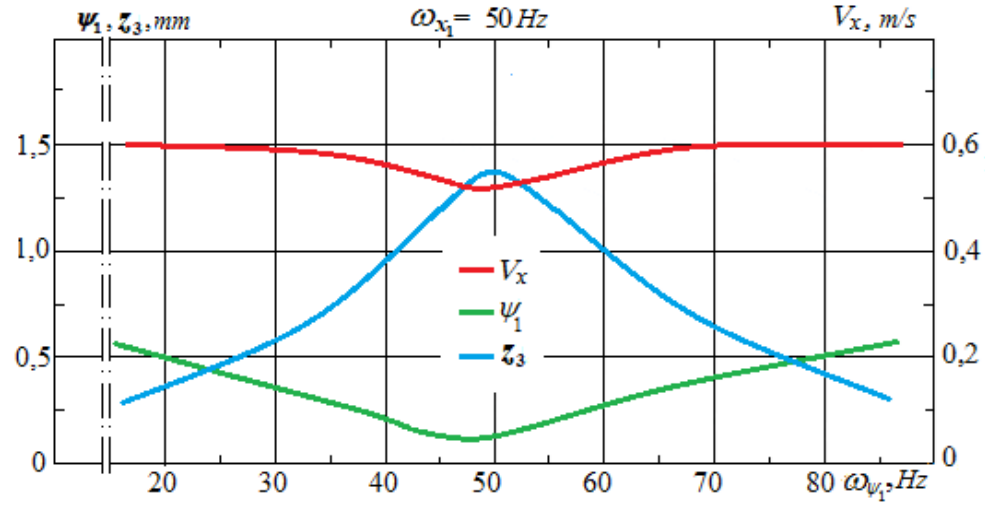

Fig. 8. *The dependence of material movement velocity (V_x) and vertical trajectory (z_3) on the rotational vibrations of the working member (ψ_1)*

7. Conclusion

1) The modeling has shown that the partial oscillations of resonance vibrofeeder working member, in combination with main (working) vibration, significantly influence the regularity of material displacement; 2) Some partial vibrations (for example, in vertical direction) increase the velocity of material displacement, which indicates the advisability of constructional modernization of the machine; 3) Most partial oscillations have a negative effect on the performance of the machine (displacement velocity reduces), which indicates the need for manufacturers to reduce the tolerances on the accuracy of machine manufacturing and installation.

Acknowledgement

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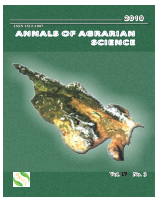

Developing the methods and principles to ensure safety of the population living in the high-risk zone flooded as a result of possible accident of Zhinvali earth dam

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ABSTRACT

The work describes the state of Zhinvali Earth Dam and development of the Dam control risk frame by using the critical state and risk portfolio analysis (GAPRA). By using the theoretical and experimental global positioning device (GPS) and geographical information system (GIS) software, a digital map of the flooded area was compiled. The article describes the modern methods and principles to ensure safety of the population living in the high-risk zone flooded as a result of possible accident of Zhinvali Earth Dam.

Keywords: Reservoir, Flooded area, Population safety, Risk, Digital map, Portfolio analysis.

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1. Introduction

Examples from recent history of operation of hydraulic structures of reservoirs evidence that all over the world, including Georgia, the protection of the population, significant infrastructure, surrounding areas and agricultural lands against floods and mudflows in case of possible accidents resulting in unfavorable environmental problems and consequently, social and economic problems for humanity is an urgent problem. By considering that it is almost impossible to accurately predict the expected accidents of hydraulic structures (dams, coast protection walls, reservoirs, etc.) due to a great variety of their causes and their formation at different times, there is currently no universal method to assess the said process. Modern methods are necessary to develop based on the scientific studies by using novel tools and technologies.

One of the most urgent issues, which is of a state importance in our country, is the development and introduction of the national safety strategy and risk management action plan, and the assessment of the risks for vulnerable infrastructure by considering the threats caused by natural and anthropogenic (including terrorist acts) catastrophes.

The project envisages active cooperation between the governmental and non-governmental organizations in contemporary management and realization of risks what will allow developing an efficient, integrated and consistent national platform for risk management to prevent and neutralize natural and anthropogenic catastrophes.

Besides, in line with the memorandum concluded between Tsotne Mirtskhulava Water Management Institute of Georgia of Georgian Technical University and the University of Maryland (USA) (2011), a Critical Asset and Portfolio Risk Analysis (CAPRA) model is planned to use. The model envisages the quantitative assessment, and testing and introduction of all expected risks.

The author of CAPRA theory is Bilal M. Ayyub, the editor in chief of journal “Risk and Uncertainty in Engineering Systems” with ASCE (American Society of Civil Engineering) classification and

the consultant scientist of the present project. The journal is published in almost all leading countries of the world. Based on the above-mentioned, it is planned to publish the scientific results gained within the scope of the grant study in the above-said journal [1,2].

The object of the study is to develop the methods and principles to ensure the safety of people living in the risk zone flooded as a result of a possible accident of Zhinvali Earth Dam. The article accents the development and assessment of methods and principles to evaluate the impact of the expected catastrophe on the people living in the risk zone; increasing the awareness of the local people and designing, developing and introducing preliminary preventive measures.

2. Thorough assessment of Zhinvali Earth Dam

Zhinvali Reservoir is a reservoir with a complex designation in East Georgia, Dusheti Municipality, north of Zhinvali settlement, 70 km from Tbilisi. It is located in the middle course of Aragvi, between Alevi, Gudamakari and Kartli Ridges. Its area is 11,5 km^2; the water volume is 520 mln. m^3; the useful capacity is 370 mln. m^3, and the maximum depth is 75 m. Zhinvali Reservoir was built in 1985 within the scope of Zhinvali HPP construction project in the Aragvi River gorge [3]. Its generated electricity capacity is 130,000 kW. The section of the Aragvi River where Zhinvali Reservoir was built is abolished and no river ecosystem exists along this former section of the river [3]. Zhinvali Reservoir plays an important role in the water supply of Tbilisi. It is noteworthy that Zhinvali Reservoir supplies about half of the population of Tbilisi with drinking water. Due to the construction of Zhinvali hydraulic complex, the XII century Jvarpatiosani Church was flooded. The Church is under the water for 6 months a year and can be seen above the water for the rest 6 months [4,5].

The complex of Zhinvali structures contains an earth dam and clay core, with deep water intake and deep idle open outlets.

Normal flooding level of the Reservoir is 810,0 m, maximum flooding level is 812 m and the minimum flooding level is 770 m. Generally, the Reservoir is prohibited to operate below 766 m level.

Particularly important is the increased frequency of recurrence of natural anomalies, landslides and mudflows in particular, denivellation events in the Reservoir coastal line and surrounding area affecting the environmental balance, the possibility of transporting the drift with the rivers forming the coastal line, dynamics and intensity of the underground water levels and potential impacts of various nature and strength on the artificial structures.

Figure 2 shows Zhinvali Reservoir plan in isogyphs by using GIS technologies.

Following the above-mentioned, the intensity of silting the Reservoir, as compared to the design data, is increased by 2,5 times. Mean annual silting value is 4,5 mln. cub.m. At present, the total volume of the deposited mass is 126,0 mln. cub.m.

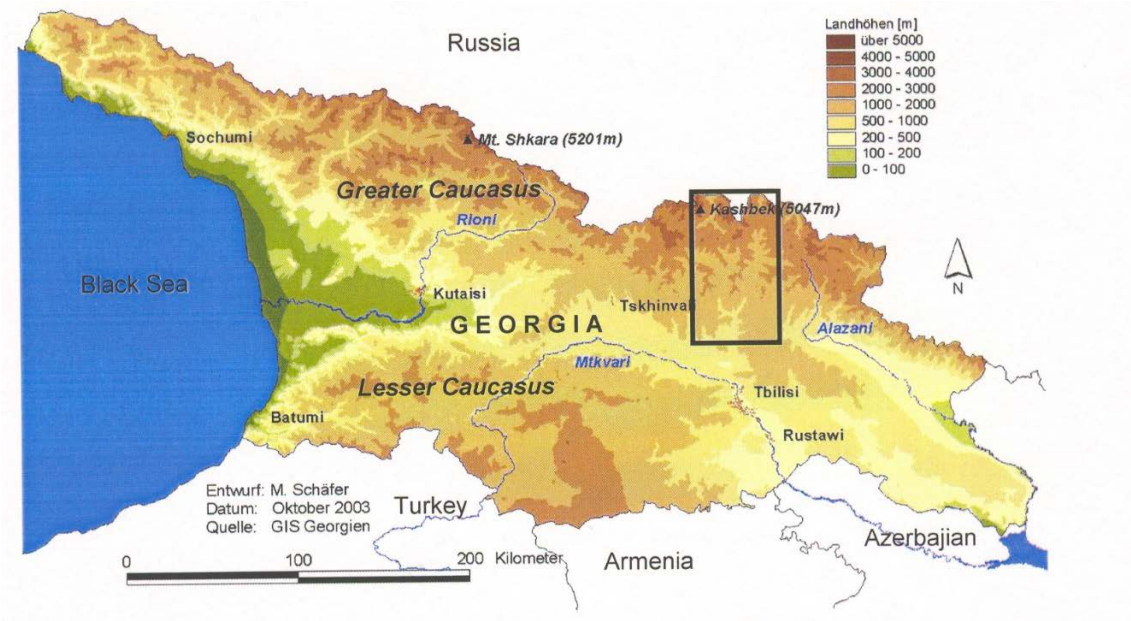


Fig. 1. *Study object*

The process of sedimentation of the Reservoir with solid drift takes place in the upper reaches where the drift surface level is at the height of 20 m from the water intake sill. As for the control of the water reservoir level, it is done continuously, with a 1 cm accuracy by means of automatic self-recording and as the calculation results suggest, the evaporation loss is 6,5 mln. cub.m. a year [6-9].

In order to predict the volumes of Zhinvali Reservoir, 3 field expeditions were organized: in April and May and September to December of 2018 and in April and May of 2019, in the periods of maximum and minimum flooding of water levels in the Reservoir and Figure 4 shows the views of Zhinvali Reservoir in a 3D format [10,11].

By considering the field-expedition and GIS technologies and by using the digital maps, the volumes of Zhinvali Reservoir are established by considering the area of the water level.

The field studies evidence that in terms of fluctuations of Zhinvali water horizon in the Reservoir water area, frequent changes of erosive processes observed, in particular, where the mudflow mass is transported by the Aragvi River and is then accumulated in the Reservoir water area, at places where no accumulation takes place, but bed erosion. Such locations are considered sensitive sites.

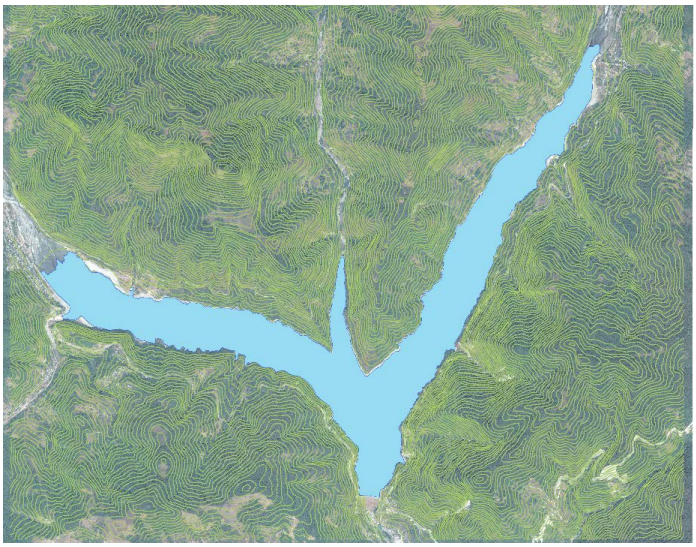


Fig. 2. *Plan of Zhinvali Reservoir*

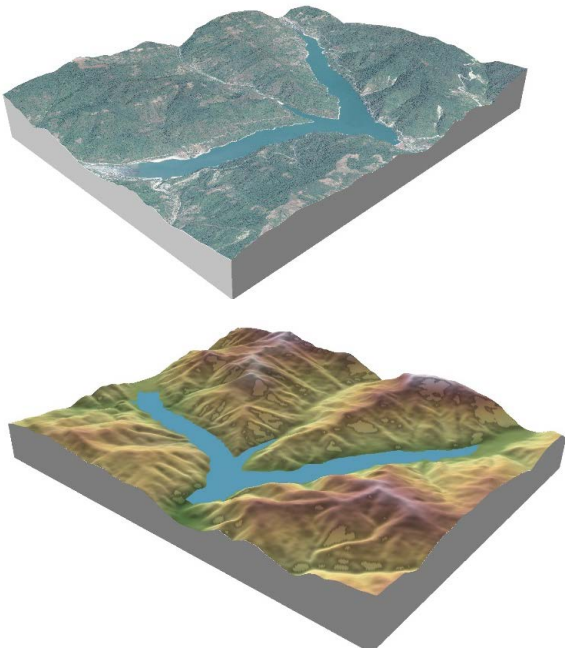


Fig. 3. *Views of Zhinvali Reservoir in 3D format*

3. Establishing the flood risk zone in case of possible accident of Zhinvali Earth Dam

A Critical Asset and Portfolio Risk Analysis (CAPRA) model, whose author is Bilal M. Ayyub, professor of the University of Maryland (USA), considers the quantitative assessment, testing and introduction of all expected risks [12].

A risk is the probability of the outcome, which is a deviation from the planned/expected result and has a negative impact on the achievement of the set goals on the study object.

The risk is defined with the combination of the following properties:

a) Probability;

- The probability is the possibility of a concrete outcome to occur, by considering the frequency of the outcome.

b) Impact (if any).

An impact is an effect in case of a concrete outcome and considers four elements: 1) time, 2) quality, 3) benefit, 4) human resource and other resources.

The combination of the probability and the impact determines the level of a concrete risk value and following the set goals, allows classifying the risks depending on their priorities.

In the first instance, the risks with the highest probability and impact must be considered and managed. In the row, every next risk must have less probability and impact than the previous risk. In practice, this process is much more complex, as there are risks with high probability, but low impact and/or vice versa. In such cases, in order to avoid mistakes, the risks must be classified by priority following the goals and objectives of an organization (Table 2).

As for the risk management, it must be permanent and be done by the study object manager in line with the risk management strategy proved annually.

Risk management helps and promotes the study object, as it allows efficient realization of its objectives, including:

- The formulation of the general direction of the study object that allows running the business in a trouble-free and controlled manner;
- The improvement of processes – making, planning and prioritizing the decisions;
- The protection and improvement of the reputation of the study object;
- The development and strengthening a human resources and institutional knowledge base.
- Operation optimization, etc.

Table 1. *Volumes of Zhinvali Reservoir 20-meter levels Maximum flooding (level) – 840, minimum flooding (level) (thalweg) – 712*

Water horizon absolute level (m)	Water level surface area (m^2)	Water volume in the Reservoir, (m^3)
840	10939612	884724026
832	10879099	799599514
812	10847811	586982276
792	10509107	376098980

Table 2. *Risk Assessment Scale*

Probability	High	A priority
Impact	High	
Probability	High	Ranking must be based on the goals and strategies of the organization
Impact	Low	
Probability	Low	Ranking must be based on the goals and strategies of the organization
Impact	High	
Probability	Low	Less priority
Impact	Low	

The process of risk management is a set of coordinated and consistent continuous actions [1,2].

A common practice of risk analysis is to develop a risk matrix what allows risk ranking and identification. The matrix is compiled by associating the risk probability and the impact that is used as a reference point to rate and categorize the risk.

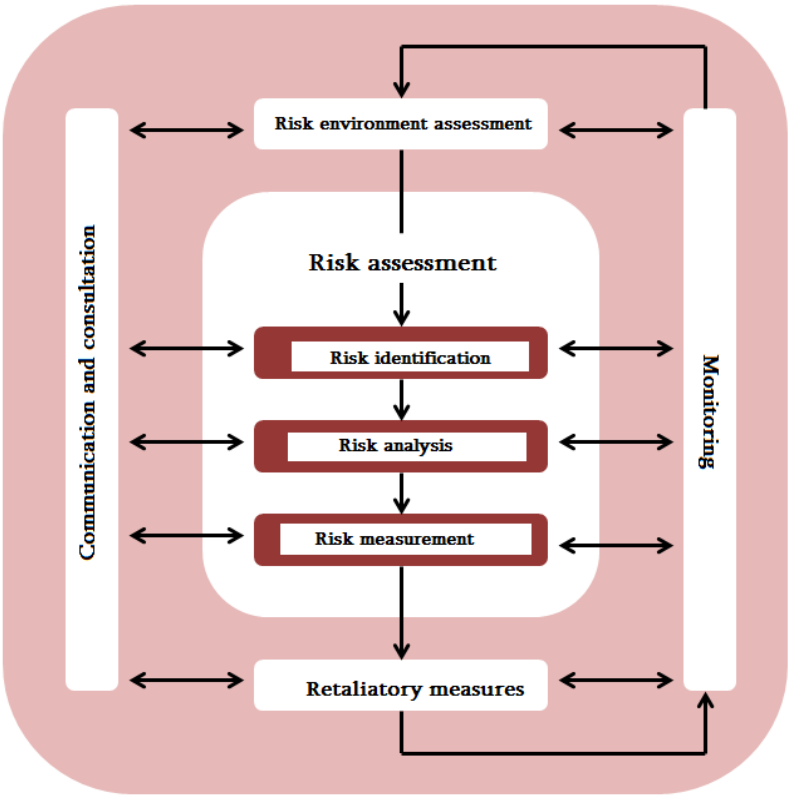
The volume of water in the Reservoir (W_0) was calculated with the following dependence [12]:

$$W = \frac{H_B S_B}{3} \quad (\text{mln. m}^3) \qquad (1)$$

Where: H_B is the water depth at a normal dam flooding level (m); S_B is the area of the Reservoir water surface (mln. m^3);

The length of the river is taken from topographic maps. As for the number of points, it must not exceed 3 points on each side from the river axis, mak-

Table 3. *Risk management process (under ISO 31000)*



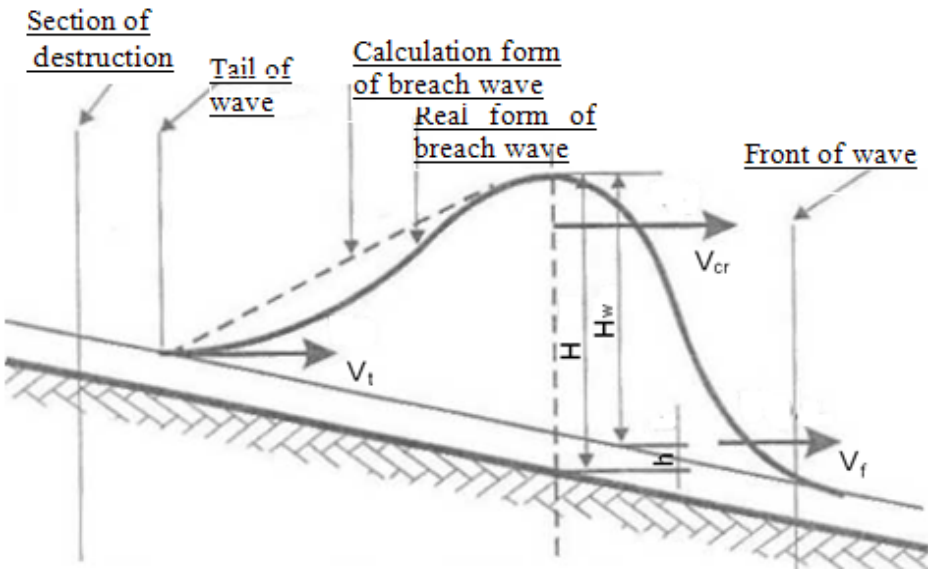


Fig. 4. *A longitudinal profile of a tsunami wave*

ing total 6 points, and must cover the whole water catch area. The number of sections from the dam needed to determine the area of the flooded territory must not exceed 8 sections, and the distance between them must be plotted on a topographic map in advance.

During the flood, a wave velocity (V) in the tailrace of the structure is calculated by formula [13, 14]:

$$V = V_0 (H_1 / H_0)^{2/3}, \text{ (m/sec)} \quad (2)$$

Degree of the dam failure (E_p) was calculated with the following dependence [13]:

$$E_p = \frac{F_B}{F_0}, \quad (3)$$

Where: F_B is the area of the bank ruptured area (m^2); F_0 is the surface area (m^2).

In addition to the above-mentioned, the algorithm considers: the height of the river bank sill (m), number of the sections along the length of the river, distance between the sections (km), width of the riverbed (m), water velocity in the riverbed (m/sec), width of the River Nogha bed (m), values of riverbed levels (m), etc.

In case of a possible rupture of Zhinvali earth dam in the bed of the Aragvi River when tsunami waves are formed, the only means to protect the people is an organized evacuation. However, Zhinvali Dam rupture will not occur abruptly, but will be preceded by the accumulation of defects in the structure what is a prognostic sign. Besides, the technical parameters and hydrological values of the Dam and the topographic indices of the Aragvi River must be taken into account, as they allow predicting the area of the territory flooded following Zhinvali Dam accident.

By means of field studies, GPS, GIS and relevant software, the maximum widths of the Aragvi River bed where areas may be flooded as a result of a flood formed in case of possible Zhinvali Dam accident what will bring a great damage to our country, including great human and animal losses, were identified.

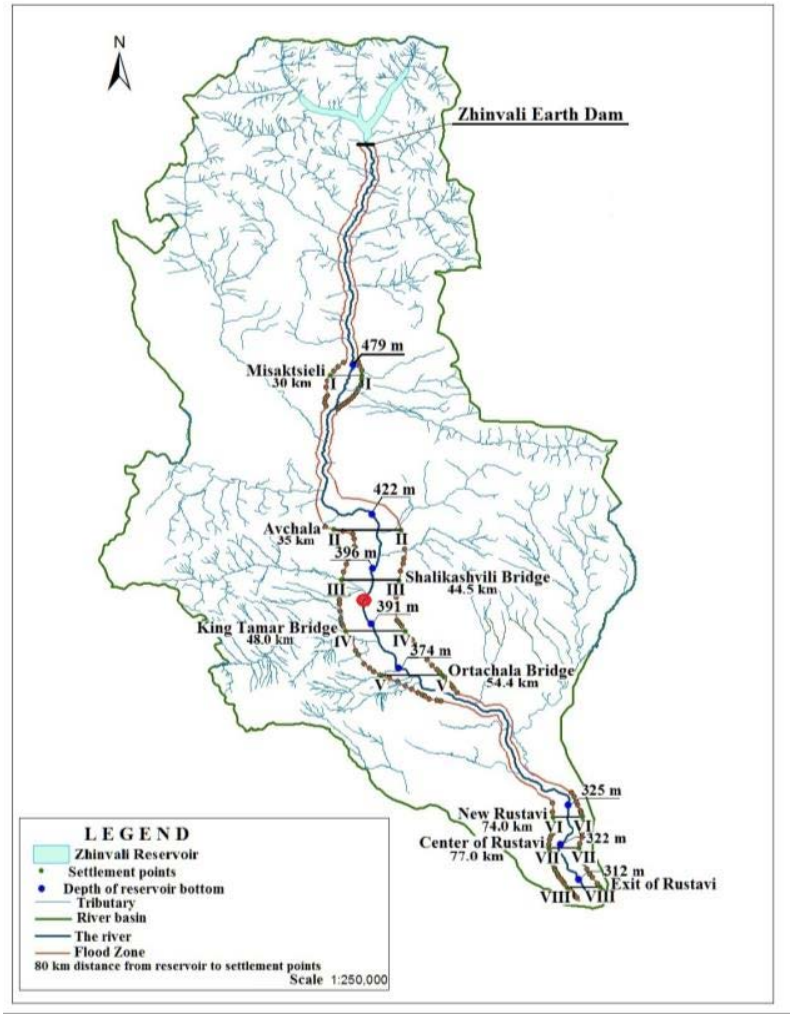


Fig. 5. *A digital, map of the flooded areas, GIS system*

As a result of the analysis of the accomplished studies, it was established that in case of a possible Zhinvali Earth Dam accident, a number of villages in Dusheti and Mtskheta Municipalities up to Tbilisi, the capital of Georgia, with their population of 14 823, will be in a high risk zone of flooding. This is similarly true for the population of Tbilisi and Rustavi living in high flooding risk zone.

Thus, the areas of villages of Dusheti and Mtskheta Municipalities in a high risk zone flooded as a result of possible Zhinvali Earth Dam accident and their population facing a great danger is identified based on the initial prediction data.

5. Increasing ecological awareness of the population living in flood risk zones

In case of emergency, including floods (Fig. 7), the awareness of each resident of the rules of behavior and action living in the high-risk zone of natural calamity, in addition to determining the degree of awareness of specific methods and rational actions, leads to the psychological stability and better self-confidence of people in extreme conditions [15-18].

If the people live in the Dam tailrace, then they live in a high-risk zone and must be aware of the following things [19-21]:

- Possible boundaries of flooding, as well as elevated locations in immediate vicinity of their houses, which rarely get flooded, and the shortest ways to them. The practice has evidenced that in case of rupture of big reservoirs, tsunami waves are formed, and the best protective means from them is the organized evacuation of people.
- All members of the families must be aware of the evacuation plan and rules of conduct in case of abrupt and rapidly progressing floods. They must remember where the boats, rafts and building materials to make them are stored.
- They must have a list of documents, things and medications to take with them in case of evacuation. They must put all necessary warm clothes, products, water and medications in their bags or rucksacks.
- They must urgently leave a possible zone with the danger of catastrophic flooding with the mandated rule immediately after they receive the message about flooding hazard and evacuation and move to a safe region or elevated area, take with them the documents, jewelry, necessary items and supply of products sufficient for two days.
- As they leave home, they must turn off the electricity and gas, put out the fire in an oven, fix all swimming items outside the building or put them in auxiliary storerooms. If there is a time, they must move the family's valuable things to the upper floors of the house or garret. They must close doors and windows and, if needed and if time allows, they must

Table 4. *Flooded villages at high risk in case of Zhinvali Earth Dam accident*

#	Name of the settlement	Number of the population (People)
1	Chinti	188
2	Zhinvali	121
3	Bichagnauri	424
4	Aragvispiri	907
5	Bodorna	140
6	Tsiteli sopheli	546
7	Navazi	677
8	Misaqtsieli	2100
10	Natakhtari	1234
11	Mtskheta	7 940
12	Zahesi	546
	Total:	14 823

board up the doors and windows of the first floor with planks from the outside.

- In case of a sudden formation of a catastrophic flooding, they must quickly move to an elevated area, climb a large tree or get on upper floors of solid structures to protect themselves from the beat of a breakdown wave. If they are in water, they must not be confused and scared at an approaching wave, they must deeply dive near the bottom of the wave, and swim up onto the water surface after some delay in water (by swimming underwater). In case they are in water, they must try to reach a dry place (preferably, an embankment or dam) by swimming or by using swimming items what will allow them to easily reach non-flooded area.
- Self-evacuation of people on foot or by using swimming items is permitted in the following cases: if the non-flooded area can be seen straightforward; if the foodstuff is over; if it is hopeless to wait for the external help or if the people are in need of urgent first aid.
- During the flood, they must take self-control and not panic. They must put the shipping equipment in order, but in case there is none, they must do it by using locally available materials. If they find themselves in water, they must try to take off heavy clothes and footwear and swim to non-flooded sites. They must try to avoid contact with the items swimming on the water surface to avoid possible trauma.
- If no organized evacuation is organized, they must go to the upper floors of a building, roofs, trees or other elevated places before the lifeguards arrive or before the water level lowers. At the same time, they must give out the signal of disaster continuously: by waving a stick with a well visible cloth on it during the day and by using a light signal and calling out periodically as it is gets dark. When the lifeguards are near, they must get on the swimming vessels calmly and carefully and without a panic. At the same time, they must fulfill the lifeguards' requirements and must not overload the swimming vessels. They must stay in place on their swimming vessel during the transportation, must not sit on outer borders and must carefully fulfill the instructions of the swimming crew.
- During the flood, apart from disruption of the existing medical service system in the area of disaster, a number of other serious problems occur as well. As the buildings ruin, such vitally important objects, as power supply and drinking water supply structures, get out of order. Insanitation may be the case what is followed by the hazard of spreading infectious diseases. Therefore, the given issues must be permanently supervised by health service provides of the relevant regions.

6. Conclusion

Based on the field-scientific and theoretical studies, the article establishes the number of people living in the risk zone of flood caused by possible accident of Zhinvali Earth Dam and measures to improve their ecological awareness and preliminary preventive measures, in particular:

- By using the theoretical and experimental studies, Global Positioning System (GPS) and Geographical Information Systems (GIS) software, the territories occupied by settled areas and infrastructure damaged (flooded) as a result of floods and mudflows in case of water current flow over Zhinvali Earth Dam were studied and their contours were plotted on the digital maps.
- In case of a possible accident of Zhinvali Earth Dam, the area of the flooded territory and the number of the local population in the zone of natural calamity were specified.
- The rules of behavior of the population living in the risk zone during the flood before, during and after the calamity were developed and the necessary rules of behavior of people during emergencies were specified.

Acknowledgement

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