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GENETIC DIVERSITY OF WINTER WHEAT (*Triticum aestivum* L.)

Andrea Pešková[1], Zdenka Gálová, Tímea Kuťka Hlozáková*

Address(es): Ing. Andrea Pešková,
[1]Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic, phone number: +421376414693.

*Corresponding author: peskova.andrea@gmail.com

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ABSTRACT

Cereals form an indispensable part of the human diet, and wheat is one of the most important agricultural commodities worldwide. Technological quality of wheat grain is determined mainly by the representation of gluten proteins. The aim of this work was to evaluate the technological quality of winter wheat grain based on genetic markers rested on polymorphism of storage proteins. We analyzed ten varieties of winter wheat (*Triticum aestivum* L.), in which we determined the content of crude proteins and fractional composition of proteins. Then we analyzed the samples by electrophoretic methods using SDS – PAGE and A – PAGE protocols, which allowed us to separate storage proteins and subsequently to detect the presence of individual HMW-GS as molecular markers of breadmaking quality of wheat. We calculated Glu-score based on the representation of HMW-GS. According to SDS – PAGE, the most common composition of HMW-GS was formed by alleles 0, 7+9, 5+10, which occurred in 30 % of analyzed varieties (Natanael, Silvanus, Genoveva). The highest Glu-score (10) was achieved by the variety Ilias. From fingerprinting of gliadins in A - PAGE we constructed a dendrogram of relatedness of individual analyzed varieties. In dendrogram, most genetically distant varieties according to gliadins polymorphism are the varieties Silvanus from the varieties Bardotka, Sophytra, Faustina and Genoveva; also Natanael from the variety Sophytra. Genetically closest from this point of view is the variety Federer to the variety Panonnicus, as well as Natanael to Silvanus.

Keywords: winter wheat, technological quality, protein markers, HMW-GS

INTRODUCTION

Storage proteins of cereals are highly polymorphic. Their use in the form of suitable markers for the characterization and differentiation of genotypes have many advantages over other genetic markers (**Žiarovská *et al.*, 2012**). Baking quality of wheat flour is largely determined by the accumulation, concentration and composition of proteins in the grain, which is influenced by genetic and environmental factors and their interaction (**Malik *et al.*, 2013**). Grain consists of two main components, water and dry matter. Water is an important component, it is necessary for all biological and physiological processes. Dry matter is mostly represented by polysaccharides, about 75 %, the proportion of proteins is between 10-15 % and content of lipids is about 2 % (**Kučerová, 2004**). Endosperm is the main source of flour, it contains the largest amount of grain proteins, carbohydrates, iron and B vitamins (riboflavin, niacin and thiamine). Outer layers of the endosperm and the aleurone layer have higher concentration of vitamins and phytic acid, in contrast to the inner endosperm, where is the majority of starch and proteins (**Kumar *et al.*, 2011**). Wheat storage proteins (gliadins and glutenins) with their properties differ from similar proteins in other cereals and that causes their exceptional position in cereal technologies. They are not soluble in water, but with the addition of water they swell to a limited extent, while insertion of mechanical energy by kneading in the presence of air form a solid gel, which is called gluten (**Kadlec *et al.*, 2009**). The quality of gluten plays a key role in shaping of the dough and also influences baking properties. Gluten proteins constitute in total about 80 % of the protein content in wheat grain (**Repka and Michalik, 1988**). Gliadins, wheat prolamins, a major fraction of the wheat storage proteins, are synthesized in the endoplasmic reticulum during development of the seed. Gliadins are stored in subcellular structures within immature endosperm – in the protein bodies (**Zhang, 2013**). The structure of the gliadins consists of a single polypeptide chain, in which short helical sections (α - helix) alterante with hydrophobic residues and straight sections with relatively high content of glutamic acid and proline. Gliadins are classified into structural types: α-, β-, γ-, ω-gliadins according to their electrophoretic mobility in acidic systems (A – PAGE). Unlike glutenins, gliadins are the monomer components, which contribute to the viscosity of the particular dough expansion (**Shewry and Halford, 2003; Kučerová, 2004**). The glutenins, wheat glutelins, are divided into high molecular weight subunits (HMW-GS) and low molecular weight subunits (LMW-GS). They form about 40 % of total proteins of grain, and are also among the largest molecules of the protein at all, achieved a molecular weight up to 20 milions Da (**Wrigley, 1996**). HMW-GS constitute of a stable complex of polymers and are linked mainly to the dough flexibility (**Shewry and Halford, 2003**). HMW-GS can be classified into the x- and y-type, which are coded by two genes and differ in molecular weight (**Kolster, 1992**). Bread wheat contains six HMW-GS genes, presented in each Glu-1A, Glu-1B and Glu-1D locus at the long arm of chromosomes 1A, 1B, and 1D (**Shewry and Halford, 2003**). Glu-score, calculated from presence of HMW-GS, is used to determine the breadmaking quality of wheat grain. The value of score is between 1 and 10. The evaluation of wheat over 7 means good technological quality for bakery use. The amount of HMW-GS is not important for the breadmaking quality, but their individual representation. The most significant effect on the quality of the flour has locus Glu-1D, where the subunits 5 + 10 affect the quality in a positive way and subunits 2 + 12 in a negative way (**Payne *et al.*, 1987**). Study of protein polymorphism by electrophoretic methods is one of the most important ways to get information about varieties. The principle of protein markers allow us to determine the origin of cultivated plants and their genome structure, accurately and quickly identify the variety and specify their total genomic analysis (**Gálová *et al.*, 2011a**).

MATERIAL AND METHODS

We analyzed grain of ten winter wheat (*Triticum aestivum* L.) varieties - Bardotka, Sophytra, Lukullus, Federer, Pannonicus, Faustina,

Genoveva, Ilias, Natanael and Silvanus, which were obtained from the Research and breeding station Malý Šariš NPPC VÚRV Piešťany and the Breeding station HORDEUM spol. s. r.o. Sládkovičovo. We received grains from the growing season 2010/2011. We determined total nitrogen content by Kjeldahl's method and fractional composition of proteins by Golenko **(Michalík, 2002)**, then we calculated crude proteins content (multiplying total nitrogen content with 5,7 – which is coefficient for wheat) and coefficient of nutritional quality ((albumins + globulins + residue)/prolamins) × 100) from fractional composition of proteins. Storage proteins were isolated from the endosperm of dry and mature single grains. Homogenization was carried out by grinding. Glutenins and gliadins were extracted by standard ISTA method. Next we performed electrophoresis in acid conditions **(Draper, 1987)** and with presence of sodium dodecylsulphate **(Wrigley, 1992)** using the electrophoretic unit Protean II (BioRad). Protein bands were coloured by Coomassie Brilliant Blue R – 250. Electrophoretic profiles were scanned by GS-800 Calibrated Densitometer (BioRad), which cooperates with program Quantity One. The separated gluten subunits were identified by the protocol of **Payne and Lawrence (1983)**. We evaluated the results by mathematical - statistical methods (average value, standard deviation and coefficient of variation). Dendrogram was constructed using Jaccard coefficient and the method UPGMA (Unweighted Pair - Group Method using Arithmetic Averages) in program, which is available free on http://usuaris.tinet.cat/debb/UPGMA/.

RESULTS AND DISCUSSION

Cereals are the most important plant source of proteins in the diet. The content and the quality of the individual protein fractions are the main factors, which affect the nutritional and technological quality of flour. On that basis, we determined total nitrogen content, content of crude proteins and also the fractional composition of proteins (Tab. 1). The content of nitrogen in the collection of analyzed varieties of winter wheat ranged from 1.40 % in the variety Natanael to 2.30 % in the variety Genoveva (average value was 1.73 %). We calculated the average value of crude proteins content at 9.88 %. The highest amount of crude proteins had the variety Genoveva (13.11 %) and the smallest the variety Natanael (7.98 %). **Socha *et al.* (2010)** determined the total nitrogen content of common wheat grains on 2.76 %, which is different from our results (about 1.03 %). The highest content of crude proteins was 15.75 %, which is about 2.64 % more from our calculated value. Albumins and globulins are important fractions from a nutritional point of view, they contain more essential amino acids than prolamins and glutelins. For these protein fractions high content of lysine, threonine, methionine, isoleucine, tryptophan and arginine is typical. In turn, prolamines and glutelins have higher content of non-essential amino acids such as glutamic acid, proline and glycine. However, they are significant factor in development of gluten and they are irreplaceable during technological use of wheat in bakery. We found the highest content of albumins and globulins in the variety Natanael (26.37 %), the lowest content showed the variety Silvanus (22.74 %). The average rate was 24.55 %, which corresponds to the results of **Gálová *et al.* (2011b)**, who determined the average value of 24.49 %, noting that the presence of albumins and globulins positively affects the nutritional value of wheat. **Socha *et al.* (2010)** found 22.62 % fraction of albumins and globulins in wheat grain. This value is lower from our in about 1.93 %. Most prolamins were detected in the variety Faustina (39.82 %), and least in the variety Ilias (28.56 %). Average value was 34.69 %. Silvanus variety showed the highest content of glutelins (35.03 %), the lowest value was detected in the variety Genoveva (28.04 %). Glutelins average content was 31.29 %.

Table 1 Crude proteins content, fractional composition of proteins and coefficient of nutritional quality in analyzed wheat varieties

Variety	CP (%)	alb + glo (%)	prolamins (%)	glutelins (%)	CNQ (%)
Bardotka	9,46	25,44	32,21	34,74	102,6
Sophytra	9,69	24,16	35,95	30,05	94,57
Lukullus	10,15	25,81	34,79	30,86	98,73
Federer	9,58	24,36	34,46	28,52	107,42
Pannonicus	10,55	25,92	35,64	30,24	95,73
Faustina	8,89	23,76	39,82	31,47	72,09
Genoveva	13,11	23,17	37,80	28,04	83,86
Ilias	10,09	23,81	28,56	33,31	133,5
Natanael	7,98	26,37	36,35	30,65	90,78
Silvanus	9,29	22,74	31,35	35,03	107,24
average value (%)	9,88	24,55	34,69	31,29	98,65
standard deviation (%)	1,34	1,25	3,27	2,39	16,30
coefficient of variation (%)	13,59	5,10	9,42	7,64	16,53

Legend: CP – crude proteins content, alb + glo – albumins and globulins, CNQ – coefficient of nutritional quality

Coefficient of nutritional quality indicates the quality of the grain in terms of its use for animal nutrition. From our varieties the highest value had the variety Ilias (133.5 %). This was also confirmed by its values of the sum and the ratio of gluten proteins, which are important from a technological point of view. Minimum nutritional quality was reflected by the variety Genoveva (83.86 %). All varieties showed average value of the coefficient of nutritional quality at the level of 98.65 %. These results are also approved by **Gálová *et al.* (2011b)**, who set the average value at 88.15 %. Difference between these values was 10.5 %, which means that our analyzed collection was on the higher level in regards to nutritional quality. Overall, we can conclude, that from our set of winter wheat, the most nutritionally valuable were varieties Ilias, Federer, Silvanus and Bardotka. From a technological point of view were the best varieties Faustina, Natanael, Bardotka and Silvanus. **Žilič *et al.* (2011)** analyzed the content of gliadins and glutenins in winter wheat genotypes and their content of these fractions were in the range of 58.17 % to 65.27 %. Our average value was still higher, in about 0.71 % (65.98 %).

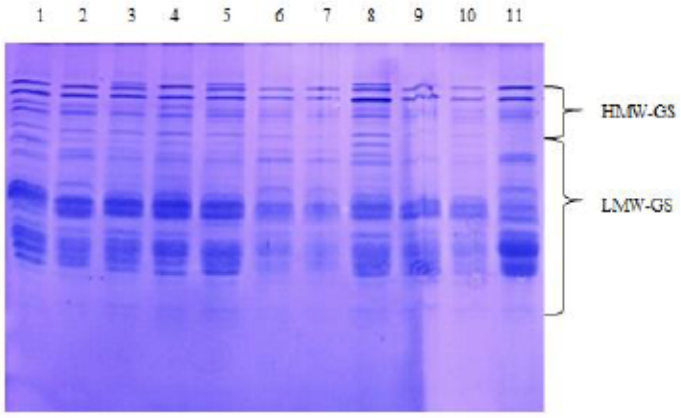

Figure 1 Electrophoretic profiles of storage proteins in grains of 10 winter wheat varieties using SDS – PAGE

Legend: 1 - Chinese Spring (marker), 2 – Bardotka, 3 – Sophytra, 4 - Federer, 5 - Lukullus, 6 – Natanael, 7 - Silvanus, 8 - Ilias, 9 – Pannonicus, 10 - Faustina, 11 - Genoveva, HMW-GS - high molecular weight glutenin subunit, LMW-GS - low molecular weight glutenin subunits

We used SDS – PAGE method to separate the storage proteins to HMW-GS and LMW-GS (Fig.1). Among analyzed varieties we detected the variability of presence HMW-GS and its influence to technological quality of grain. The most common HMW-GS composition (Fig. 2) in genetic resources was 0, 7 + 9, 5 + 10 (30 % varieties), the least common compositions were 0, 7 + 8, 2 + 12 (Federer); 0, 7 + 9, 2 + 12 (Pannonicus) and 1, 7 + 8, 10 + 5 (Ilias).

Our results are consistent with **Chňapek *et al.* (2014)**, who also found the most common composition of HMW-GS 0, 7 + 9, 5 + 10. Our results suggested that from the genes, encoded by Glu-1A locus, most commonly occurred null allele (70 % varieties), specifically in varieties Bardotka, Federer, Natanael, Silvanus, Pannonicus, Faustina and Genoveva, 30 % varieties - Sophytra, Ilias and Lukullus contained subunit 1. These results are confirmed by **Kuťka Hlozáková *et al.* (2015),** who detected three alleles at the locus Glu-1A - 0, 1, 2 *. This was probably due to the size of their studied collection - 108 genotypes of winter wheat. **Oslovičová *et al.* (2010)** in their collection of 67 genotypes of wheat from Slovakia managed highest proportion of alleles 0, which is consistent with our results. Locus Glu-1B was more common in the composition of HMW-GS 7 + 9 (60 % - Sophytra, Lukullus, Nathanael, Silvanus, Pannonicus, Genoveva) as in the composition 7 + 8 (40 % - Bardotka, Federer, Ilias, Faustina). **Kuťka Hlozáková *et al.* (2015)** at locus Glu-1B recorded high polymorphism, seven kinds of alleles, with the most frequent alleles 7 + 9, which corresponds with our results. According to **Oslovičová *et al.* (2010)** at the locus Glu-1B occurred three kinds of alleles, namely 6 + 8, 7 + 8 and 7+ 9. The most common pair was 7 + 9, which is also consistent with our results. Composition 5 + 10 prevailed at the locus Glu-1D (80 % varieties - Bardotka, Sophytra, Lukullus, Nathanael, Silvanus, Ilias, Faustina, Genoveva), in contrast to 12 + 2, which occurred only in 20 % of varieties (Federer, Pannonicus). According to the results of **Kuťka Hlozáková *et al.* (2015),** the presence of 5+10 positively correlates with the breadmaking quality. Most often composition 5 + 10 was also in the analysis of **Oslovičová *et al.* (2010)**, however they detected subunits 2 + 12 and 3 + 12, too.

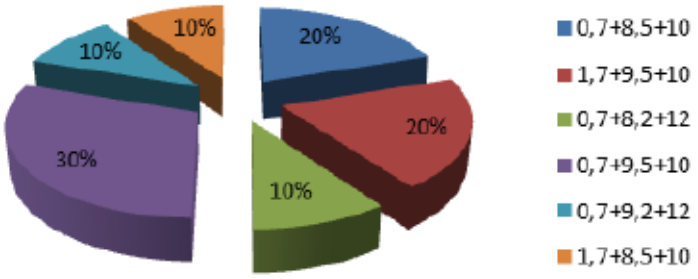

Figure 2 HMW-GS component composition in analyzed collection of winter wheat

In the collection of our samples, we calculated Glu-score, which ranged from 5 to 10. Highest Glu-score 10 was reached by the variety Ilias, Glu-score 9 was found in the varieties Sophytra and Lukullus (20 %), varieties Bardotka and Faustina (20 %) reached the score of 8, which means good technological quality of wheat grain. Still good Glu-score according to the criteria of **Payne *et al.* (1987)**, with a value of 7, was reached by the varieties Natanael, Silvanus and Genoveva (30 %). The varieties Panonnicus and Federer do not comply with this classification, they reached a value of 5 and 6. Average Glu-score was 7.6; which means satisfying technological quality of our collection of winter wheat. **Chňapek *et al.* (2014)** set in their collection of Czechoslovak and European wheat genotypes average Glu-score of 7.5; which agrees with our results. The largest percentage of their Glu-score was 9, while for us it was 7.

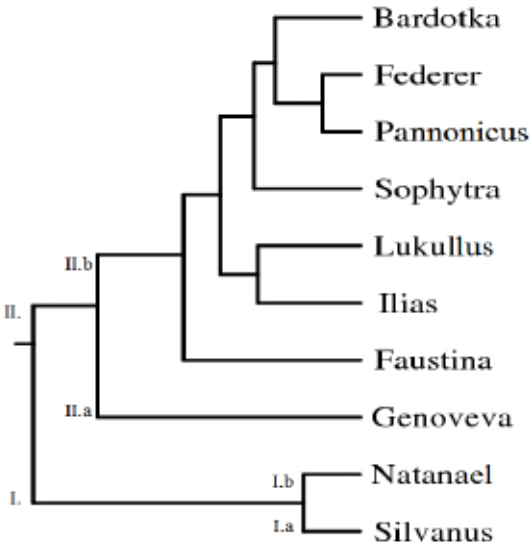

Figure 3 Dendrogram of genetic relatedness of varieties based on gliadins polymorphism

Electrophoretic analysis of gliadins from different wheat varieties received by A – PAGE method allowed us to use statistical methods in order to construct dendrogram of their relationships. On the basis of dendrogram (Fig. 3), common ancestors and degree of relatedness may be presumed. With variability analysis we divided the collection into two main clusters (I. and II.) of varieties. The first cluster (I.) contained only two varieties - Natanael and Silvanus (I.a and I.b), which indicate their close genetic relationship based on gliadins polymorphism. The second cluster (II.) was divided into two subclusters (II.a and II.b), the first (II.a) was characterized by the variety Genoveva, the second (II.b) was divided into two subgroups. The first subgroup was represented by the variety Faustina, the second was subdivided into two subclusters. The first subcluster contained the varieties Lukullus and Ilias, the second was separated into a group, which contained the variety Sophytra and the second group, which was next divided into clusters containing the varieties Federer and Pannonicus and cluster with the variety Bardotka. Genetic relatedness based on Jaccard similarity coefficient suggested, that gentically closest to the variety Federer is the variety Pannonicus (0,889), followed by the varieties Natanael and Silvanus (0,833) and a pair of the varieties Bardotka and Federer (0,800). On the contrary, most genetically different varieties based on Jaccard coefficient were Bardotka, Silvanus, Sophytra and Faustina from Genoveva (1,000), as well as Natanael from Sophytra (1,000).

CONCLUSION

Crude proteins content of the analyzed samples ranged from 7.89 % to 13.11 %, the highest proteins content had the variety Genoveva and the lowest had the variety Natanael. The nutritional quality of wheat grain is based on representation of albumins and globulins, which content was between 22.74 % in the variety Silvanus to 26.37 % in the variety Natanael. Gliadins and glutenins content affects the technological quality of grain. The content of storage proteins varied between 61.87 % in the variety Ilias to 71.29 % in the variety Faustina. From this perspective, the variety Faustina had the highest quality according to content of gluten proteins. The highest ratio of coefficient of nutritional quality was detected in the variety Ilias (133.5 %), the lowest nutritional quality was reflected by the variety Genoveva (83.86 %). In terms of the representation of HMW-GS in the samples, we found subunits 0 and 1 at locus Glu-1A, subunits 7 + 8 and 7 + 9 at locus Glu-1B, and subunits 5 + 10 and 2 + 12 at the locus Glu-1D. Most frequently was component composition 0, 7 + 9, 5 + 10, namely in the varieties Natanael, Silvanus and Genoveva. Glu-score can determine the technological quality of wheat grain. Our varieties reached Glu-score between 5 and 10. The highest score 10 was reached by the variety Ilias. The lowest rate had the

varieties Pannonicus (Glu-score 5) and Federer (Glu-score 6). Based on the acidic electrophoretic profile of wheat storage proteins we constructed dendrogram of genetic relatedness of different varieties. In this regard, we determined that the variety Silvanus is genetically furthest from varieties Bardotka, Sophytra, Faustina and Genoveva, as well as the variety Natanael from variety Sophytra. On the other hand, the most genetically related were varieties Federer and Pannonicus. Finally, we can say, that the analyzed set of winter wheat showed a satisfactory technological quality and we recommend it for use in bakery.

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REFERENCES

Draper, S. R. (1987). ISTA Variety Committee-Report of the working group for biochemical tests for cultivar identification 1983-1986. *Seed science and technology* (Switzerland).

Gálová, Z., Balážová, Ž., Michalík, I., Libantová, J., Moravčíková, J., Preťová, A., Matušíková, I. (2011a). Biotechnológie v rastlinnej produkcii. Nitra: SPU. ISBN 978-80-552-0682-0.

Gálová, Z., Trebichalský, A., Palenčárová, E. (2011b). Protein profil of Triticale (X Triticosecale Witt.), Wheat (Triticum aestivum L.) and Rye (Secale cereale). *Potravinárstvo*, *5*(mimoriadne číslo február), 274–277.

Chňapek, M., Tomka, M., Peroutková, R., Gálová, Z. (2014). Polymorphism of HMW-GS in collection of wheat genotypes. *International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering*, *8*(7), 652-657.

Kadlec, P., Melzoch, K., Voldřich, M. (2009). *Co by jste měli vědět o výrobě potravin? Technologie potravin*. Key Publishing: Ostrava. ISBN 978-80-7418-051-4.

Kolster, P. (1992). *High molecular weight glutenin subunits of wheat: qualitative and quantitative variation in relation to bread-making quality* (Doctoral dissertation).

Kučerová, J. (2004). *Technologie cereálií*. Brno: Mendelova zemědělská a lesnická univerzita. ISBN 80-7157-811-8.

Kumar, P., Yadava, R. K., Gollen, B., Kumar, S., Verma, R. K., Yadav, S. (2011). Nutritional contents and medicinal properties of wheat: a review. *Life Sci Med Res*, *22*, 1-10.

Kuťka Hlozáková, T., Gregová, E., Gálová, Z. (2015). Genetic diversity of Glu-1 in European wheat genetic resources and varieties. *The Journal of Microbiology, Biotechnology and Food Sciences*, *4*(2), 23 – 25. https://doi.org/10.15414/jmbfs.2015.4.special2.23-25

Malik, A. H., Kuktaite, R., Johansson, E. (2013). Combined effect of genetic and environmental factors on the accumulation of proteins in the wheat grain and their relationship to bread-making quality. *Journal of Cereal Science*, *57*(2), 170-174. http://dx.doi.org/10.1016/j.jcs.2012.09.017

Michalík, I. (2002). Unifikovaná metóda diskontinuálnej frakcionácie bielkovinového komplexu zrna obilnín. *Poľnohospodárstvo*, *48*(7), 333-341.

Oslovičová, V., Gálová, Z., Chňapek, M., Balážová, Ž. (2010). Identification of Triticum aestivum L., Triticum spelta L.and Triticum durum DESF. genotypes on the HMW-GS base. *Plant Soil Environ.*, *56*(2), 82–86.

Payne, P. I., Lawrence, G. J. (1983). Catalogue of alleles for the complex loci, Glu-A1, Glu-B1 and Glu-D1 which code for high-molecular-weight subunits of glutenin in hexaploid wheat. *Cereal Res. Commun.*, *11*, 29–35.

Payne, P.I., Seekings, J. A., Worland, A. J., Jarvis, M. G., Holt, L. M. (1987). Allellic variation of glutenin subunits and gliadins and its effect on breadmaking quality in wheat: analysis of F5 progeny from „Chinese Spring" x „Chinese Spring" (Hope 1A). *Journal of Cereal Science*, *6*(2), 103–118. http://dx.doi.org/10.1016/S0733-5210(87)80047-4

Repka, J., Michalík, I. (1988). *Biochemicko – fyziologické základy šľachtenia rastlín*. Nitra: VŠP – VES.

Shewry, P. R., Halford, N. G., Lafiandra, D. (2003). Genetics of wheat gluten proteins. *Advances in genetics*, *49*, 111-184. http://dx.doi.org/10.1016/S0065-2660(03)01003-4

Socha, P., Raždíková, A., Urminská, D. (2010). Optimalizácia stanovenia celiakálne aktívnych bielkovín v cereáliách a pseudocereáliách. Potravinárstvo, *4*(mimoriadne číslo február), 497-508.

Wrigley, C. W. (1992). Identification of cereal varieties by gel electrophoresis of the grain proteins. *Seed Analysis*. Berlin: Heilderberg, Springerverlag, 17 – 41. http://dx.doi.org/10.1007/978-3-662-01639-8_2

Wrigley, C. (1996). Giant proteins with flour power. *Nature*, *381*(6585), 738-739.

Zhang, W., Sangtong, V., Peterson, J., Scott, M. P., & Messing, J. (2013). Divergent properties of prolamins in wheat and maize. *Planta*, *237*(6), 1465-1473. http://dx.doi.org/10.1007/s00425-013-1857-5

Žiarovská, J., Ražná, K., Senková, S., Štefúnová, V., Bežo, M. (2012). Variability of Linum usitatissimum L. based on molecular markers. *ARPN Journal of Agricultural and Biological Science*, *7*(1), 50-58.

Žilić, S., Barać, M., Pešić, M., Dodig, D., Ignjatović-Micić, D. (2011). Characterization of proteins from grain of different bread and durum wheat genotypes. *International journal of molecular sciences*, *12*(9), 5878-5894. http://dx.doi.org/10.3390/ijms12095878

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PREPARATION OF PLANT VECTOR CONSTRUCT CONTAINING DEHYDRIN GENE At2g21490

*Mária Zimová [1,2], Eva Boszorádová[1], Zuzana Gregorová[1], Ildikó Matušíková[3], Jana Moravčíková[1]**

Address(es): Ing. Jana Moravčíková, PhD.,
[1]Institute of Plant Genetics and Biotechnology Slovak Academy of Sciences, Akademická 2, P.O. Box 39A, SK-950 07 Nitra, Slovak Republic.
[2]Department of Botany and Genetics, Faculty of Natural Sciences, The Constantine Philosopher University, Nábrežie mládeže 91, SK-94974 Nitra, Slovak Republic.
[3]Department of Ecochemistry and Radioecology, University of Cyril and Methodius, J. Herdu 2, Trnava, SK-917 01, Slovak Republic.

*Corresponding author: jana.moravcikova@savba.sk

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ABSTRACT

This work is focused on preparation of a plant transformation vector pDH2 containing the expression unit of the dehydrin gene At2g21490 under a control of the constitutive *dCaMV 35S* promoter and the selectable marker neomycin phosphotransferase gene controlled by the *nos* promoter. The gene At2g21490 is classified as a dehydrin of the type K_2SY_2. The gene was isolated from *Arabidopsis thaliana* by PCR approach. The plasmid pDH2 was transformed into the strain *Agrobacterium tumefaciens* LBA 4404. The stability of the plasmid pDH2 in *Agrobacterium* has been verified by restriction analysis after re-transformation of the pDH2 into *Escherichia coli*.

Keywords: *Agrobacterium tumefaciens*, *Arabidopsis thaliana*, At2g21490, binary vector, dehydrins

INTRODUCTION

Due to the changes in the environment, plants are increasingly exposed to different types of abiotic stresses such as drought, salinity, low temperature or the presence of heavy metals. Among the proteins that are significantly accumulated during the stress caused by dehydration, osmoticum and/or a low temperature are LEA proteins ("Late embryogenesis abudant proteins") (**Amara *et al.*, 2014**). They are hydrophilic molecules whose role probably is to protect biomolecules and cell membranes (**Candat *et al.*, 2014**). Dehydrins (Pfam PF00257) are one of the most characterized group of LEA proteins. The LEPdb database currently contains over 380 records on dehydrins from various plant species and their physicochemical properties. Most of dehydrins are associated mainly with the process of embryogenesis, but they can also be accumulated in vegetative parts during the plant lifecycle. Generally, dehydrins are enriched with glycine and lysine residues, but they lack cysteine and tryptophan (**Allagulova *et al.*, 2003**). The characteristic feature of all dehydrins is conservative sequences denominated as K-, S- and Y-segmets which are their structural attribute. Five different subclasses of dehydrins are defined on the basis of the conserved segments: Y_nSK_n, SK_n, K_n, Y_nK_n, and K_n (**Close, 1997**). The presence of K-segment is obligate for all groups of dehydrins.

Dehydrins are considered as multifunctional proteins that can bind to macromolecules such as nucleic acid, have a protective role, can bind free metals, and can act as chaperones or antioxidants (**Rorat *et al.* 2004; Hara, 2010**). In term of the structure and the presence of characteristic protein domains, they are divided into several groups. So far, 10 dehydrin genes were identified in *Arabidopsis* (**Hundertmark and Hincha, 2008**). All of these genes contain histidine rich regions (His). Since the structures His-X3-His and His-His show strong affinity to metals (**Hara *et al.*, 2005**), it was supposed they can reduce various types of cellular damage during metal stress. The regions rich in His may play a role in buffering and/or may serve as sensors of the levels of metals (**Hara, 2010**) or could be involved in the reduction of "reactive oxygen species" (ROS) (**Hara *et al.*, 2013**).

The *Arabidopis* gene At2g21490 is classified as a dehydrin of the type K_2SY_2. It is expressed in late stages of seed formation and completely absent in vegetative tissues. The gene possesses 14 His residues, two His-X3-His and one His-His sequences (**Hara, 2010**).

In this work we prepared a plant transformation vector pDH2 containing the gene At2g21490 under control of the constitutive double *dCaMV 35S* promoter. The gene At2g21490 was isolated from *Arabidopsis thaliana* by PCR approach. The binary vector pDH2 was transformed into the strain *Agrobacterium tumefaciens* LBA 4404. The stability of the plasmid pDH2 in *Agrobacterium* has been verified by restriction analysis after re-transformation of the pDH2 into *Escherichia coli*.

MATERIAL AND METHODS

Izolation of the gene At2g21490

The genomic DNA from *Arabidpopsis thaliana* was isolated according to the **Chen et al. (1992)**. The sequence of the gene At2g21490 was amplified using the combination of the primers P1 (5´-GGTAATAATACCATGGGGGATTTGAG-3´) and P2 (5´-GCTAGCAAAATACAGTTCCTTC-3´). The PCR reaction was carried out in 25 µl mixture containing 100 – 200 ng of DNA template, 20 pmol of each primer (forward, reverse), 200 µM dNTPs, 1x PCR buffer and 1 unit of *Taq* DNA polymerase. The first PCR step of 95°C for 4 minutes was followed by 35 cycles: 95°C – 45 s; 63°C – 45 s and 72°C for 2 minutes. The last step was performed at 72°C for 10 minutes. The PCR product was isolated from the gel using QIAquick Gel Extraction Kit (Qiagen). The identity of the PCR product was confirmed by sequencing.

Plant vector construct

The PCR product was ligated into the plasmid pGEm-T® Easy (Promega) to yield pZM1. Subsequently, a 1037 bp *EcoRI-EcoRI* fragment from pZM1 was ligated into *EcoRI*-*EcoRI*-digested plasmid pBSK+ to create pZM2. The plasmid pZM3 was created by cloning of *dCaMV35S* as *Hind*III-*Nco*I fragment from plasmid pBS4 and 762 bp *Nco*I-*Cla*I fragment from pZM9 into the vector pUN (**Vaculkova *et al.*, 2007**). The plant transformation vector pDH2 was obtained by ligation of *dCaMV35S/DH2/polyA* as *EcoRI-XbaI* fragment into *EcoRI-XbaI*-digested binary vector pBINPlus (**Van Engelen, 1995**). The binary vector pDH2 was introduced into *Agrobacterium tumefaciens* strain LBA 4404 using method „triparental matting" (**Matzke and Matzke**, 1986).

Stability of the plasmid pDH2 in *A. tumefaciens*

The stability was verified using restriction analysis after isolation of the plasmid pDH2 from *A. tumefaciens* and re-transformation into *E. coli*.

RESULTS AND DISCUSSION

To study the function of the dehydrin gene At2g21490 in tolerance to selected abiotic stresses using transgenosis, the plant transformation vector pDH2 was prepared. The T-DNA of the pDH2 contained the gene At2g21490 under control of constitutive *dCaMV 35S* promoter and the selectable neomycin phosphotransferase gene (*nptII*) under control of the *nos* promoter. The cloning strategy is given in Figure 1.

The specific primers P1/P2 were designed to amplify a PCR product P1-P2 of a size 1,037 kb that contained the sequence *DH2/polyA*. The plasmid pZM1 was created by ligation of the PCR product into the cloning vector pGEm-T® Easy. The identity of the amplified sequence was confirmed by sequencing. The sequence was compared with the sequence of the gene At2g21490 using the programe Clustal Omega (Figure 2).

The PCR product was ligated as *EcoR*I-*EcoR*I fragment of the plasmid pZM1 into the vector pBSK+ to yield plasmid pZM2. The identity of the inserted fragment was verified by restriction analysis (data not shown).

The next step of the cloning strategy was ligation of the sequence *DH2/polyA* from the pZM2 and the sequence of the *dCaMV 35S* promoter into the cloning vector pUN. Subsequently, the sequence of the expression unit *dCaMV 35S/DH2/polyA* was ligated into the binary vector pBinPlus. This vector contains the selectable *nptII* gene encoding resistance to antibiotic kanamycin. The identity of the plasmid pDH2 was confirmed by restriction analyses. In Figure 3a the position of restriction enzymes used in the analyses is given. The results of restriction analyses are in Figure 3b.

Based on our previous experiences with instability of some sequences in bacteria (**Vaculkova *et al.*, 2007**), the stability of the sequence of the T-DNA region was analysed using restriction analyses after re-transformation of the plasmid pDH2 from *A. tumefaciens* into *E.coli*. After retransformation, totally, 30 clones were isolated and subjected to restriction analyses. Restriction profiles of all analysed clones corresponded with expected (Figure 4).

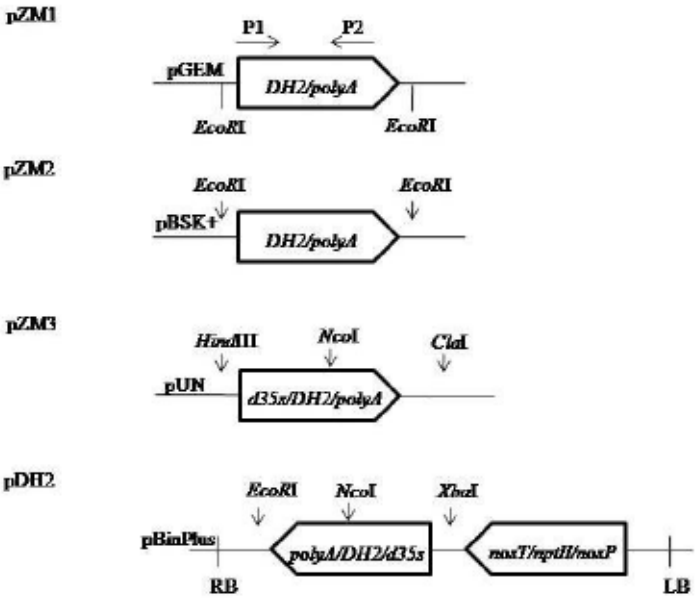

Figure 1 Cloning strategy. The arrows indicate restriction enzymes used in individual cloning steps. *d35s* – double *CaMV 35S* promoter, *dh2*- dehydrin gene, *polyA* – poly sequence of gene DH2, *nosT* – nos terminator, *npt*II – neomycin phosphotransferase gene, *nosP* – nos promoter.

Figure 2 The alignment of the sequence P1-P2 with the sequence of the gene At2g21490. Alignment was generated using the CLUSTAL OMEGA program. Nucleotides which are conserved in the sequences aligned are marked by asterisks. Dashes show sequence that is important for polyadenylation of mRNA. The arrows outline the position of primers P1 and P2. The sequence of the primers P1 and P2 are in the boxes.

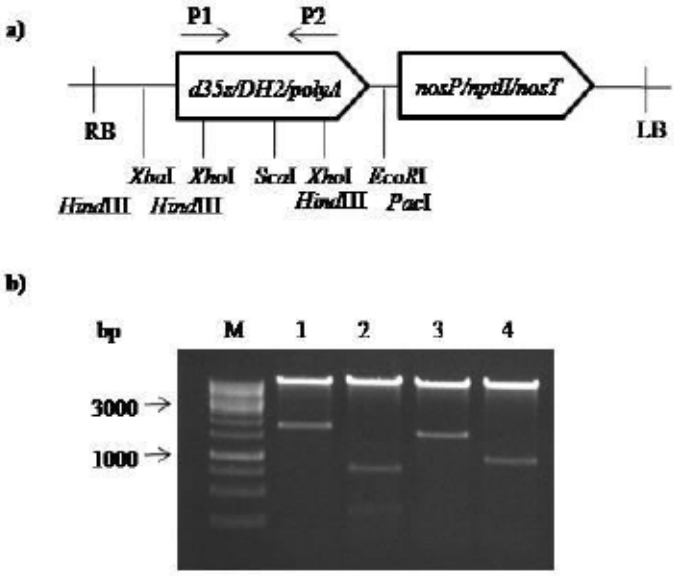

Figure 3 Restriction map and restriction analyses of the T-DNA-pDH2. **a)** T-DNA structure of the pDH2. The primers used for PCR analyses are indicated as P1, P2. The position of restriction enzymes used in restriction analyses is indicated **b)** Photograph of the ethidium bromide-stained 1 % agarose gel carrying in lane M - 1 kb DNA marker (Fermentas), lane 1 – pDH2 / *EcoR*I+*Xba*I, lane 2 – pDH2/ *Xho*I, lane 3 – pDH2 / *Hind*III, lane 4 – pDH2/ *Asc*I+*Sca*I.

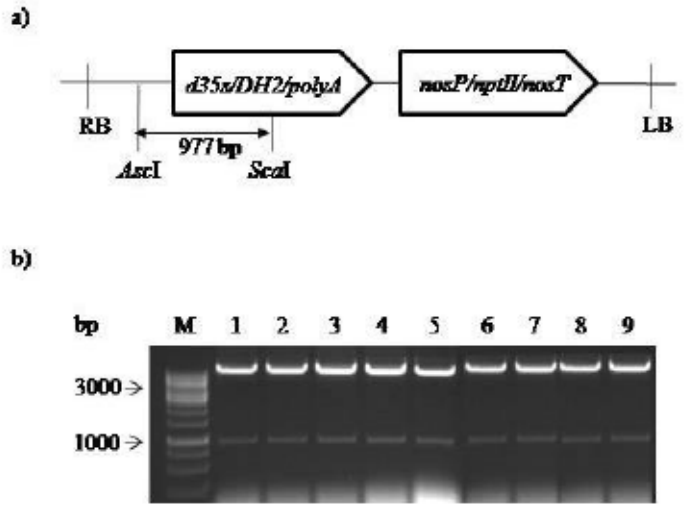

Figure 4 Restriction analyses of the T-DNA-pDH2 after re-transformation of plasmid into *E. coli*. **a)** The plasmid pDH2 with the position of restriction enzymes used in analyses. **b)** Photograph of the ethidium bromide-stained 1 % agarose gel carrying in lane M - 1 Kb DNA marker (Fermentas); lanes 1-9 – pDH2/*Asc*I + *Sca*I .

CONCLUSION

Using the techniques of recombinant DNA a plant transformation vector pDH2 was prepared. The T-DNA region of the pDH2 contained *Arabidopsis* gene At2g21490 under control of the constitutive *dCaMV 35S* promoter and selectable marker *npt*II gene providing resistance to the antibiotic kanamycin. The binary vector pDH2 has been shown to be stable in *A. tumefaciens* LBA 4404. In future, *A. tumefaciens* LBA 4404 carrying the plant transformation vector pDH2 will be used for transformation of tobacco leaf explants.

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REFERENCES

Allagulova, C. R., Gimalov, F. R., Shakirova, F. M., Vakhitov, V. A. (2003). The plant dehydrins: Structure and putative functions. *Biochemistry-Moscow*, 68 (9), 945-951. **http://dx.doi.org/10.1023/a:1026077825584**

Amara, I., Zaidi, I., Masmoudi, K., Ludevid, M. D., Pages, M., Goday, A., Brini, F. 2014. Insights into Late Embryogenesis Abundant (LEA) proteins in plants: from structure to the function. In *American Journal of Plant Sciences*. č. 5, s. 3440-3455. **http://dx.doi.org/10.4236/ajps. 2014.522360**

Candat, A., Paszkiewicz, G., Neveu, M., Gautier, R., Logan, D.C., Avelange-Macherel, M.E., Macherela, D. (2014). The Ubiquitous Distribution of Late Embryogenesis Abundant Proteins across Cell Compartments in Arabidopsis Offers Tailored Protection against Abiotic Stress. *The Plant Cell*, 26, 3148–3166. **http://dx.doi.org/10.1105/tpc.114.127316**

Close, T.J. (1997). Dehydrins: A commonalty in the response of plants to dehydration and low temperature. *Physiologia Plantarum*, 100, 291-296.

Hara, M., Fujinaga, M., Kuboi, T. 2005. Metal binding by citrus dehydrin with histidine-rich domains. *Journal of Experimental Botany*, 56 (420), 2695-2703. **http://dx.doi.org/10.1093/jxb/eri262**

Hara, M. (2010). The multifunctionality of dehydrins: an overview. *Plant signaling behavior*, 5 (5), 503-508. **http://dx.doi.org/ 10.4161/psb.11085**

Hara, M., Kondo, M., Kato, T. (2013). A KS-type dehydrin and its related domains reduce Cu-promoted radical generation and the histidine residues contribute to the radical-reducing activities. *Journal of Experimental Botany*, 64 (6), 1615-1624. **https://doi.org/10.1093/jxb/ert016**

Hundertmark, M., Hincha, D. K. (2008). LEA (Late Embryogenesis Abundant) proteins and their encoding genes in *Arabidopsis thaliana*. *Bmc Genomics*, 9 (119). **http://dx.doi.org/118 10.1186/1471-2164-9-118**

Chen, Z., Greenblatt, I. M., Dellaporta, S. L. 1992. Molecular analysis of *Ac* transposition and DNA replication. *Genetics*, 130 (3), 665-676. ISSN 1943-2631.

Matzke, M. A., Matzke, A. J. M. (1986). A set novel Ti plasmid-derived vectors for the production of transgenic plants. *Plant Molecular Biology*, 7 (5), 357-365.

Rorat, T., Grygorowicz, W. J., Irzykowski, W., Rey, P. (2004). Expression of KS-type dehydrins is primarily regulated by factors related to organ type and leaf developmental stage during vegetative growth. *Planta*, 218 (5), 878-885. **http://dx.doi.org/10.1007/s00425-003-1171-8**

Vaculková, E., Moravčíková, J., Matušíková, I., Bauer, M., Libantová, J. (2007). A modified low copy number binary vector pUN for *Agrobacterium*-mediated transformation. *Biologia Plantarum*, 51 (3), 538-540. ISSN 0006-3134.

Van Engelen, F. A., Molthof, J. W., Conner, A. J., Nap, J. P., Pereira, A., Stiekema, W. J. (1995). pBINplus: an improved plant transformation vector based on pBin19. *Transgenic Research*, 4, 288-290.

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POTENTIAL OF SELECTED SSR MARKERS FOR IDENTIFICATION OF MALTING BARLEY GENOTYPES

Marián Tomka, Dana Urminská, Milan Chňapek, Zdenka Gálová*

Address(es):
Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Biochemistry and Biotechnology, Tr. A. Hlinku 2, 949 76, Nitra, Slovak Republic.

*Corresponding author: ing.mariantomka@gmail.com

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ABSTRACT

Cereals are main part of human nutrition and strategic resources. Thousands of barley varieties are used in food industry and especially for the production of malt in the brewing industry. In the present study, we were focused on utilization of SSR markers for differentiation and characterization of different barley genotypes. Barley collection was analyzed by 4 pure and 1 compound markers. Using STMS method we have revealed significant polymorphism. A total of 27 alleles were detected among 24 varieties with an average of 5.4 alleles per locus. Average values for index of diversity (DI), polymorphic information content (PIC) were 0.767, 0.756 respectively. The highest level of polymorphism was detected with SSR marker *Bmag 0222* (7 alleles) which also revealed one heterozygous variety. Dendrogram was created by hierarchic cluster analysis using UPGMA algorithm on the basis of detected alleles. Analyzed genotypes were divided into three clusters so it can be used to study genetic relations among collection of analyzed barley varieties.

Keywords: Barley, Genetic diversity, Simple sequence repeat (SSR), DNA marker

INTRODUCTION

Cereals are grown across the globe for several thousand years, and after all these years have been subjected to various exogenous and endogenous factors which have formed their respective genomes. Barley (*Hordeum vulgare,* L.) is economically important crops mainly for food production and malting, but the value is increased due to use as a functional food with a variety of health benefits **Hua *et al.* (2015).** In order to increase the nutritional quality of grain, barley crop passed through the targeted selection and hybridization. The long process of barley breeding has resulted in increasing diversity of barley varieties **Ivandic *et al.* (2003).** Growers can select the best variety according to specific climate and end use of this crop such as human consumption, malt in brewing and distilling industry or animal feeding **Ferreira *et al.* (2016).** At present approximately hundred varieties of barley genotypes are registered in Slovakia, of which 20% have domestic origin. Wild barleys (*Hordeum spontaneum*) can be used as donors of important genes that could be used for improvement of different barley parameters **Shakhatreh *et al.* (2016).** Molecular markers such as SSR, SNP, STS **Kojima *et al.* (2007), Thormann *et al.* (2016)** or DArT **Lamara*et al.* (2013)** are often used for genotype identification and characterization and thus may provide more accurate genome assessment **Yadav *et al.* (2015).** Association mapping is a powerful tool in improving barley breeding via precise identification of markers significantly associated with important traits, which is vitally important for marker-assisted breeding **Abou-Elwafa (2016), Elakhdar *et al.* (2016).** Molecular markers are very useful for mapping and tagging the loci affecting malting quality **Han *et al.* (1997)** and largely fulfill most of the user requirements **Groven and Sharma (2016).** Using molecular markers to select for specific chromosome regions with potential positive yield contributions enhanced the breeding success for high yield while maintaining traditional malting quality **Schmierer *et al.* (2005).**
The aim of this study was to analyze genetic diversity among the set of 24 barley genotypes using 5 SSR markers and to evaluate their ability to identify malting barley genotypes.

MATERIAL AND METHODS

Collection of 24 barley genotypes contained 8 winter form (Tiffany, Monaco, Cedeco, Premuda, Graciosa, Barcelona, Metaxa, Heidi) and 16 spring form (Malz, Kangoo, Overture, Signora, Nadir, Laudis 550, Karmel, Antigone, Troon, Prodeum, Valis, Exalis, Kumran, Kompakt, Novum and Madonna) barleys. Some of these genotypes are commonly used in brewing industry. DNA was isolated from dry whole grain according to the methodology for a commercial kit GeneJET Genomic DNA Purification Plant Mini Kit from the Thermo Fisher Company.
Microsatellite analyses were carried out in 25 µl volume using Biorad C 1000 Thermocycler. As primers we used specific oligonucleotide sequences 18-23 bp, described by **Ramsay *et al.* (2000).** The amplification conditions for each microsatellite marker were set according to **Ramsay *et al.* (2000)** and **Varshney *et al.* (2007).** Primers (Table 1) were selected on the basis of our previous research **Tomka *et al.* (2013),** on the basis of best values for DI and PIC due to ability of differentiation.

Table 1 List of used SSR markers

Markers	Chromosome	Primer sequence (5´-3´)	Repetition
Bmac 0040	6H	AGCCCGATCAGATTTACG TTCTCCCTTTGGTCCTTG	$(AC)_{20}$
Bmac 0134	2H	CCAACTGAGTCGATCTCG CTTCGTTGCTTCTCTACCTT	$(AC)_{28}$
Bmag 0125	2H	AATTAGCGAGAACAAAATCAC AGATAACGATGCACCACC	$(AG)_{19}$
Bmag 0211	1H	ATTCATCGATCTTGTATTAGTCC ACATCATGTCGATCAAAGC	$(CT)_{16}$
Bmag 0222	5H	ATGCTACTCTGGAGTGGAGTA GACCTTCAACTTTGCCTTATA	$(AC)_9(AG)_{17}$

Amplified alleles were separated in the 6 % polyacrylamide gels denatured with urea. Preparation of the gel solution to a total volume of 100 ml: 18.5 ml 40 % acrylamid / bisacrylamid in ratio 19:1; 5 ml 10 x TBE (107.8 g Tris-base, 7.44 g EDTA and 55 g H_3BO_3 in 1L solution, pH 8.3); 20 ml redistilled water; 55.82 ml 66.3 % urea; 180 µl TEMED and 500 µl 10 % APS solution.

The separation of amplified alleles was conducted at OWLTM electrophoresis unit at maximum current 90 mA in a buffer system 1 x TBE for 3-4 hours, depending on the expected size of fragments. After electrophoretic separation of DNA, bands were visualized by silver staining followed **Benbouza *et al.* (2006):**

5 minutes in fixative solution (10 % ethanol; 0.5 % acetic acid),

6-7 minutes in a dye solution (1.5 g $AgNO_3$, 1.5 ml 37 % H_2CO, H_2O to 1000 ml),

1 second washing in ultrapure water,

3-5 minutes in develop solution (15 g NaOH, 2 ml 37 % H_2CO, H_2O to 1000 ml),

2 minutes in fixative solution (10 % ethanol; 0.5 % acetic acid).

The PCR products separated in polyacrylamide gels were scanned using ChemiDoc Imaging System followed by evaluation using the GelAnalyzer 2010 software **(Lazar 2010).** On the basis of allele frequency diversity index (DI) **(Weir, 1990)** and polymorphic information content (PIC) **(Weber, 1990)** were calculated.

RESULTS AND DISCUSSION

Number of alleles per locus is an important indicator of genetic diversity. For some authors it is the only and main parameter for study of genetic relations. **Kalinowsky (2002)** statistically confirmed that it is not required to examine highly polymorphic loci or large numbers of loci. The only requirement is that a sufficient number of alleles have been detected. Overall, we have identified 27 alleles at five loci which were located on chromosomes 1H, 2H, 5H and 6H. Number of allele per locus varied from 4 (*Bmag 0211*) to 7 (*Bmag 0222*) with average value of 5.4. Frequencies of detected alleles ranged from 4.0 % to 50.0 % (figure 1). **Shakhatreh *et al.* (2016)** analyzed comparable collection of cultivated barleys (27 genotypes) but have used 11 markers and therefore identified more alleles (95) with an average 8.6 allele per locus. **Yadav *et al.* (2015)** used even more markers (47 SSR´s) but his collection of barley contained just 10 barley cultivars. Number of alleles per locus varied from 2 to 7 with an average 3.52 alleles per locus. Using markers *Bmac 0040* and *Bmag 0222* they have identified 5 and 6 alleles respectively, which is very close to our results. Similar results obtained **Nandh and Singh (2014)** who analyzed 27 wild and 20 cultivated barley accessions and identified 5 alleles at loci *Bmag 0125* and also *Bmag 0211* in cultivated barley collection. **Khodayari *et al.* (2014)** analyzed 40 wild barley genotypes and have identified from 1 to 17 alleles but SSR marker *Bmag 0125* amplified only 4 alleles. **Hua *et al.* (2015)** identified 204 alleles with mean value of 5.83 in collection of 277 colored barley varieties. Due to very large collection of genotypes, number of alleles detected in the same loci were much higher *Bmac 0040* (42 alleles), *Bmag 0134* (35 alleles), *Bmag 0222* (19 alleles). **Ferreira *et al.* (2016)** analyzed 64 Brazilian barley genotypes and in 34 different loci they have identified from 1 up to 18 alleles. Using marker *Bmag 0211* they have identified 6 alleles but in the case of marker *Bmag 0125* number of detected alleles (10) was double compared to our results.

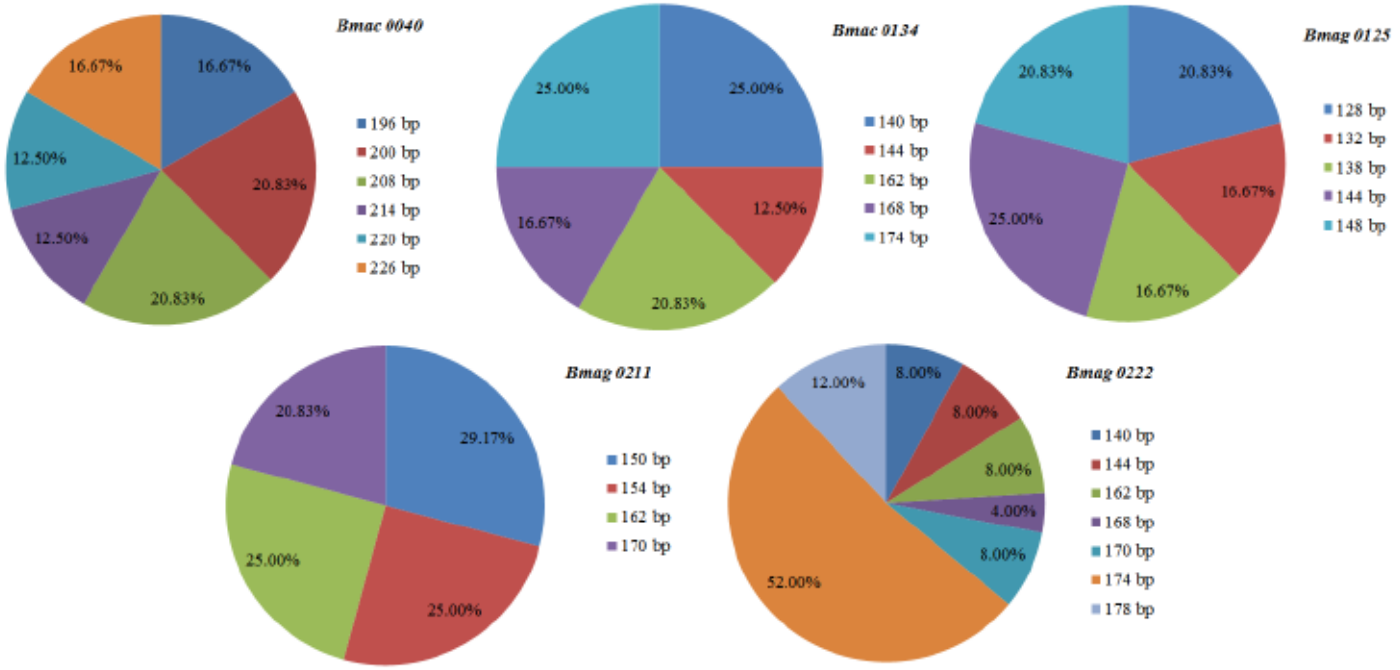

Figure 1 Preview of detected alleles in 5 SSR loci and their frequencies

Calculated indexes of DI, PIC are used to evaluate the level of polymorphism in analyzed collections of different species. The higher value for these indexes is calculated the better identification they provide (table 2). PIC values are used as a measure of a marker's usefulness for linkage analysis. Values of index diversity for our SSR markers were calculated and varied from 0.688 to 0.826 with a mean value of 0.767. For the next index (PIC) values ranged from 0.677 to 0.817 with an average value of 0.756. These values indicate that these markers are able to detect high level of polymorphism because markers with DI and PIC values over 0.6 are considered as useful tool for genotype differentiation. **Nandh and Singh (2014)** in comparable collection of 20 cultivated barleys estimated average value of diversity index of 0.729, what is very close to our result. Polymorphic information content indexes for SSR markers *Bmac 0134* (0.835), *Bmag 0125* (0.685) and *Bmag 0211* (0.760) were comparable to our values. **Khodayari *et al.* (2014)** computed values for PIC index from 0.304 to 0.913 with mean value of

0.711. Also **Lamara *et al.* (2013)** calculated similar average PIC index (0.690). Other authors **Yadav *et al.* (2015)** and **Hua *et al.* (2015)** acquired lower mean values of PIC 0.551 and 0.549 but this was affected with some SSR markers with low level of polymorphism and thus lower value of PIC 0.286 and 0.407.

Table 2 Characteristics ofindividual SSR markers

Markers	Numberofalleles	Allele size	DI	PIC
Bmac 0040	6	196 - 226 bp	0.826	0.817
Bmac 0134	5	140 - 174 bp	0.788	0.779
Bmag 0125	5	128 - 148 bp	0.795	0.782
Bmag 0211	4	150 - 170 bp	0.747	0.723
Bmag 0222	7	140 - 178 bp	0.688	0.677
Average	**5.4**	**-**	**0.767**	**0.756**

Legend: DI- diversity index, PIC- polymorphic information content,

DNA fragments amplified by 5 SSR markers were evaluated and on the basis of their presence or absence binary matrix was created. In the next step on the basis of this matrix dendrogram (figure 2) was created which revealed genetic relations between barley genotypes. We have successfully differentiated all 24 genotypes but we have failed to separate spring and winter forms as **Pillen *et al.* (2000)** in their study but they have used almost 40 markers. We can get better results by choosing different collection of SSR markers or by using more markers. On the other hand our set of markers showed some potential to differentiate malting barleys when 4 of them created own cluster, moreover genotypes Laudis 550, Overture and Kangoo are considered as varieties with premium malting quality, what is claimed by producers.

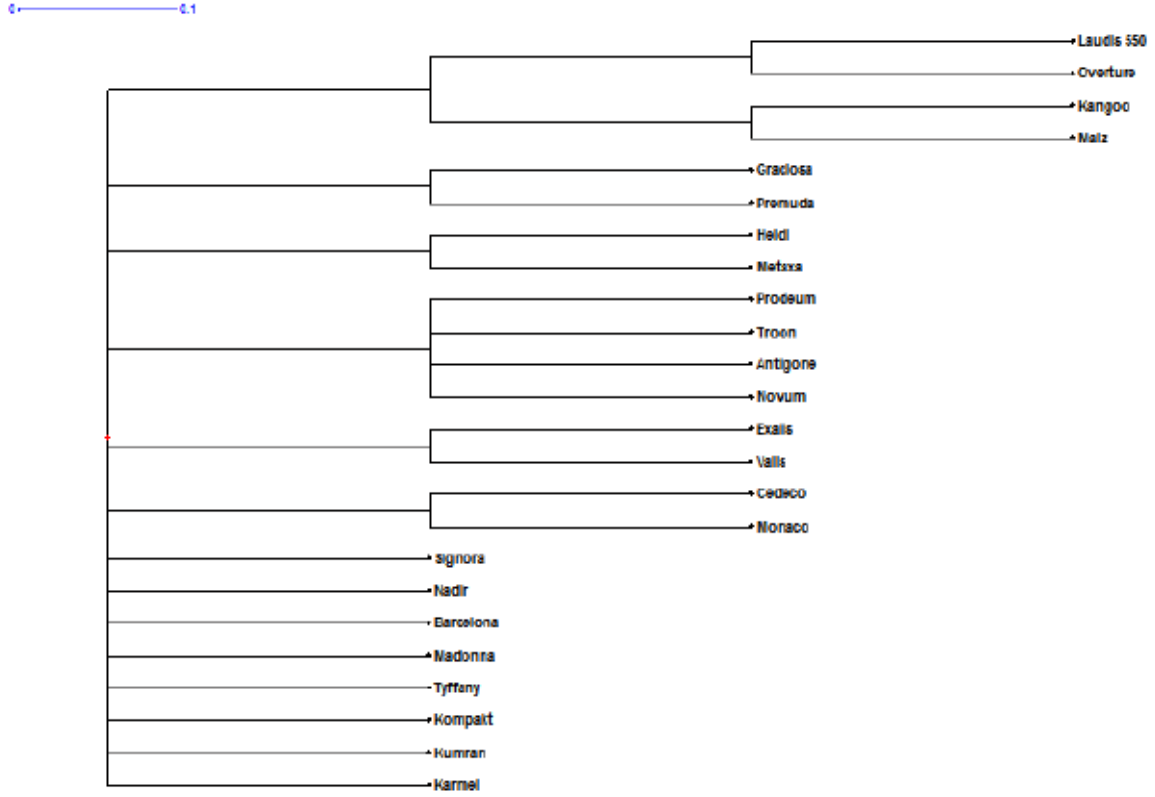

Figure 2 Dendrogram of 24 barley genotypes on the basis of 5 SRR markers

CONCLUSION

Molecular markers have been used many years for different applications mainly genotype and gene identification and genome mapping. According to obtained results, they are still very popular tool which helps growers and breeders to select the elite genotypes for their needs. Our results demonstrate the effectiveness of used SSR marker collection for differentiation of barley varieties and proved that SSR markers from our collection are still used worldwide for different applications. Comparable results with other authors confirmed potential of analyzed SSR markers for barley genotypes identification and characterization. Created dendrogram revealed relations between barley varieties and showed that this collection of SSR markers have some potential to differentiate malting barley cultivars.

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REFERENCES

Abou-Elwafa, S. F. (2016). Association mapping for yield and yield-contributing traits in barley under drought conditions with genome-based SSR markers. *Comptes rendus biologies*, 339(5), 153-162. http://dx.doi.org/10.1016/j.crvi.2016.03.001

Benbouza, H., Jacquemin, J. M., Baudoin, J. P., &Mergeai, G. (2006). Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. *Biotechnologie, agronomie, société et environnement*, 10(2), 77-81.

Elakhdar, A., EL-Sattar, M. A., Amer, K., Rady, A., & Kumamaru, T. (2016). Population structure and marker–trait association of salt tolerance in barley (Hordeum vulgare L.). *Comptes Rendus Biologies*, 339(11), 454-461. http://dx.doi.org/10.1016/j.crvi.2016.06.006

Ferreira, J. R., Pereira, J. F., Turchetto, C., Minella, E., Consoli, L., & Delatorre, C. A. (2016). Assessment of genetic diversity in Brazilian barley using SSR markers. *Genetics and molecular biology*, 39 (1), 86-96. http://dx.doi.org/10.1590/1678-4685-GMB-2015-0148

Grover, A., & Sharma, P. C. (2016). Development and use of molecular markers: past and present. *Critical reviews in biotechnology*, 36 (2), 290-302. http://dx.doi.org/10.3109/07388551.2014.959891

Han, F., Romagosa, I., Ullrich, S. E., Jones, B. L., Hayes, P. M., & Wesenberg, D. M. (1997). Molecular marker-assisted selection for malting quality traits in barley. *Molecular Breeding*, *3*(6), 427-437. http://dx.doi.org/10.1023/A:1009608312385

Hua, W., Zhang, X., Zhu, J., Shang, Y., Wang, J., Jia, Q., Li, Ch., & Yang, J. (2015). A study of genetic diversity of colored barley (*Hordeum vulgare* L.) using SSR markers. *Genetic Resources and Crop Evolution*, 62 (3), 395-406. http://dx.doi.org/10.1007/s10722-014-0165-7

Ivandic, V., Thomas, W. T. B., Nevo, E., Zhang, Z., & Forster, B. P. (2003). Associations of simple sequence repeats with quantitative trait variation including biotic and abiotic stress tolerance in Hordeum spontaneum. *Plant Breeding*, 122 (4), 300-304. http://dx.doi.org/10.1046/j.1439-0523.2003.00841.x

Kalinowski, S. T. (2002). How many alleles per locus should be used to estimate genetic distances?. *Heredity*, 88(1), 62-65. http://dx.doi.org/10.1038/sj.hdy.6800009

Khodayari, H., Saeidi, H., Akhavan, A., Pourkheirandish, M., & Komatsuda, T. (2014). Microsatellite analysis of genetic diversity of wild barley (*Hordeum vulgare* subsp. *spontaneum*) using different sampling methods in Iran. *Iranian Journal of Botany*, *20*, 41-50.

Kojima, L., Yang, F., Donovan, L. M., &Dawson, K. J. (2007). QTL analysis for some quantitative traits in Hulless barley. *Journal of Plant Genetics*, *20*, 71-83.

Lamara, M., Zhang, L. Y., Marchand, S., Tinker, N. A., & Belzile, F. (2013). Comparative analysis of genetic diversity in Canadian barley assessed by SSR, DarT, and pedigree data. *Genome*, *56*(6), 351-358. http://dx.doi.org/10.1139/gen-2013-0048

Lazar, I. (2010). GelAnalyzer software. Avalaible on: http://www.gelanalyzer.com

Nandha, P. S., &Singh, J. (2014). Comparative assessment of genetic diversity between wild and cultivated barley using SSR and EST-SSR markers. *Plant breeding*, 133(1), 28-35. http://dx.doi.org/10.1111/pbr.12118

Pillen, K., Binder, A., Kreuzkam, B., Ramsay, L., Waugh, R., Förster, J., & Leon, J. (2000). Mapping new EMBL-derived barley microsatellites and their use in differentiating German barley cultivars. *Theoretical and Applied Genetics*, 101(4), 652-660. http://dx.doi.org/10.1007/s001220051527

Ramsay, L., Macaulay, M., Ivanissevich, S. D., MacLean, K., Cardle, L., Fuller, J., Edwards, K. J., Tuvesson, S., Morgante, M., Massari, A., Maestri, E., Marmiroli, N., Sjakste, T., Ganal, M., Powell, W., & Waugh, R. (2000). A simple sequence repeat-based linkage map of barley. *Genetics*, 156 (4), 1997 – 2005.

Schmierer, D. A., Kandemir, N., Kudrna, D. A., Jones, B. L., Ullrich, S. E., &Kleinhofs, A. (2005). Molecular marker-assisted selection for enhanced yield in malting barley. *Molecular Breeding*, 14(4), 463-473. http://dx.doi.org/10.1007/s11032-004-0903-1

Shakhatreh, Y., Baum, M., Haddad, N., Alrababah, M., & Ceccarelli, S. (2016). Assessment of genetic diversity among Jordanian wild barley (Hordeum spontaneum) genotypes revealed by SSR markers. *Genetic Resources and Crop Evolution*, 63(5), 813-822. http://dx.doi.org/10.1007/s10722-015-0285-8

Thormann, I., Reeves, P., Reilley, A., Engels, J. M., Lohwasser, U., Börner, A., Pillen, K., & Richards, C. M. (2016). Geography of Genetic Structure in Barley Wild Relative *Hordeum vulgare* subsp. *spontaneum* in Jordan. *PloSone*, *11*(8), http://dx.doi.org/10.1371/journal.pone.0160745

Tomka, M., Chnapek, M., Balážová, Z., & Gálová, Z. (2013). Differentiation Of Barley Genotypes Based On DNA Polymorphism. *The Journal of Microbiology, Biotechnology and Food Sciences*, 2, 1677-1684.

Varshney, R.K., Marcel, T.C., Ramsay, L., Russell, J., Röder, M.S., Stein, N., Waugh, R., Langridge, P., Niks, R.E., & Graner, A. (2007). A high density barley microsatellite consensus map with 775 SSR loci. *Theoretical and Applied Genetics*, 114(6), 1091-1103. http://dx.doi.org/10.1007/s00122-007-0503-7

Weber, J. L. (1990). Informativeness of human (dC-dA) n·(dG-dT) n polymorphisms. *Genomics*, *7*(4), 524-530.

Weir, B. S. (1990). *Genetic data analysis. Methods for discrete population genetic data*. Sinauer Associates, Inc. Publishers.

Yadav, S. K., Singh, A. K., & Malik, R. (2015). Genetic diversity analysis based on morphological traits and microsatellite markers in barley (Hordeum vulgare). *The Indian Journal of Agricultural Sciences*, *85*(10), 37-44.

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THE FAMILY OF CHITINASES IN COTTON *G. raimondii*

Akmal M. Asrorov[1], Ildikó Matušíková[2], Zdenka Gálová[3], Zuzana Gregorová[4], Patrik Mészáros[4], Surayyo Dalimova[5], Shavkat Salikhov[1]*

Address(es):
[1] Laboratory of Proteins and Peptides Chemistry, A.S.Sadykov Institute of Bioorganic chemistry, Academy of Sciences of Uzbekistan, M.Ulughbek Str., 83, Tashkent, Uzbekistan.
[2] Department of Ecochemistry and Radiology, Faculty of Natural Sciences, University of Ss. Cyril and Methodius in Trnava, Hlavná 418, SK-91951 Špačince, Slovakia.
[3] Department of Biochemistry and Biotechnology, Slovak University of Agriculture in Nitra, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovakia.
[4] Department of Botany and Genetics, Faculty of Natural Sciences, Constantine the Philosopher University in Nitra, Nábrežie mládeže 91, 949 74 Nitra, Slovakia.
[5] Department of Biology and Soil Sciences, National University of Uzbekistan, Talabalar Shaharchasi, Tashkent, Uzbekistan.

*Corresponding author: akmal84a@gmail.com

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ABSTRACT

Chitinases are enzymes widely spread in plants with no endogenous substrate and play significant role to lyse the cell walls of many pathogens. Their role in defense is firmly established. Recently, their functions in plants have been extended to response to abiotic stresses and various developmental plant processes. The gene family of chitinases is well characterized in some model species. Few researches on their involvement in cotton defense against pathogens have been reported. Here were investigated *Gossypium raimondii* genome in the Phytozome database for the presence of homologues. BLAST similarity search, using five chitinase representatives from Arabidopsis belonging to different classes I-V, identified a total of 41 non-redundant chitinase gene sequences in cotton. We analysed them *in silico* using available bioinformatics software and characterized their basic molecular structures. Signal peptides, carbohydrate-binding domains, hinge regions were predicted, molecular weights and expected isoelectric points were calculated. Genes were divided into different chitinase classes based on homology clustering with genes from Arabidopsis. The individual family members were further linked to expression data and/or literary knowledge, which is in relation to cotton rather scarce. Knowledge on chitinase gene family members in cotton provides a basic for further basic- as well as applied cotton research as they are functionally validated.

Keywords: *G. raimondii*, chitinase classes, carbohydrate-binding domain, glycosyl hydrolase domain, chitinase clusters

INTRODUCTION

Plants evolve mechanisms to counter biotic and abiotic stresses. Accumulation of pathogenesis-related (PR) proteins is one of the developed mechanisms highly expressed by plants during the attack by pathogens. Among PR members, chitinases (PR-3) can hydrolyse chitin in the exoskeleton of insects and cell wall of pathogenic fungi, consequently play an important role in the plant defense against those organisms. Chitinases have extensively been studied for their use in different biotechnological programs for plant protection. A number of crops such as tomato, potato, maize, rapeseed, wheat, rice and others have been successfully engineered for resistance either with chitinase alone or in combination with other PR proteins (**Cletus *et al.* 2013**). In recent 10 years was observed an increasing amount of evidence on the role of these enzymes in plant adaptation to abiotic stresses, including drought (**Gregorová *et al.* 2015**), toxic metals (**Mészáros *et al.* 2014**) and even pesticides (**Asrorov *et al.* 2013**). Besides, chitinases play pivotal role in plant morphogenesis and development with impact on growth and stress tolerance. Therefore, research on chitinases in important crop species is of crucial importance.

Breeding stress-tolerant and high-yield cotton might exploit chitinases and other PR proteins since a couple of researchers proved their importance in cotton defense against aphids and mites. For example, three acidic chitinase isozymes (pI in the range 3.7-4.2) from *G. hirsutum* leaves were gradually elicited by *V. dahliae* for 120 hours (**Dubery and Slater, 1997**), some of them probably inhibited the germination of conidia (**Liu *et al.* 1995**). PRs including chitinases likely contribute to restriction of wilting in cotton infected by *Verticillium* (**Bu *et al.* 2014**). Furthermore, elevated activities of chitinases and other PR proteins resulted in reduced population of aphids in cotton (**Rajendran *et al.* 2011**), and their inhibition by insecticide treatment resulted in the population growth of spider mites both in greenhouse and field experiments (**Szczepaniec *et al.* 2013**). On the other hand, chitinases in cotton are believed to play a role in fiber formation, too (**Wiweger *et al.* 2003**).

Plant chitinases are structurally well characterized and are divided into several groups. The class I chitinases have an N-terminal cysteine-rich regions involved in chitin-binding (**Iseli *et al.* 1993**). These regions are separated from the catalytic domain by a short proline-rich variable hinge region and the catalytic domain is often followed by a C-terminal extension which is involved in vacuolar targeting (**Neuhaus *et al.* 1991**). Class II chitinases have a catalytic domain with a high sequence and structural similarity to those of class I chitinases. However they possess neither the N-terminal cystein-rich region nor the C-terminal extension. The main structure of class IV chitinases resembles class I chitinases, but is reduced by few deletions along the carbohydrate-binding domain (CBD) and the catalytic region (**Passarinho and deVries 2002**). The class III chitinases are similar to class V chitinases of plant origin and fungal/bacterial chitinases (**Graham and Sticklen, 1994**). Class V chitinases have a C-terminal extension for vacuolar targeting and may contain CBD as well (**Heitz *et al.* 1994; Ponstein *et al.* 1994**). Class V and III chitinases belong to the family 18 of glycosyl hydrolases whereas all other classes belong to family 19. Chitinases of families 19 and 18 do not share sequence similarity, they have completely different 3-D structures and molecular mechanisms (**Suzuki *et al.* 1999**).

Inspite of cotton genome sequence available, the family of chitinases in cotton is low explored. The objective of this work to identify and describe the chitinase genes and their families in *G. raimondii* and link them with corresponding knowledge available in literature.

MATERIAL AND METHODS

Searching the cotton genome for chitinases

The cotton genome in the Phytozome database was searched for chitinases using the BLAST program (Table 1). Five chitinase gene representatives from

Arabidopsis thaliana AT1G56680, AT1G02360, AT3G12500, AT4G19720 and AT5G24090, belonging to classes I-V were used as queries (Passarinho and de-Vries, 2002). Redundant sequences were removed at ≥ 98% similarity in amino acid translations. All identified putative chitinases in cotton were back-searched in the NCBI database to prove similarity with chitinases (E value ≤ 10^{-20}). Subsequently, different databases and softwares (Table 1) were used to predict their structural and molecular characteristics such as molecular weight and putative isoelectric points, the presence of individual protein domains and signal peptides (Table 1). Sequence alignment of chitinases from cotton and Arabidopsis was done using ClustalW (Table 1) and evolutionary relationships were observed by viewing Cladogram or Phylogram in the program. The phylogenetic tree was built using software (www.ebi.ac.uk/Tools/msa). Maximum likelihood method was used. Available data on chitinase gene expression in cotton were obtained from the database plex (Table 1).

Table 1 List of used databases and bioinformatic softwares

Phytozome	http://www.phytozome.jgi.doe.gov/
National Center for Biotechnological Information	http://www.ncbi.nlm.nih.gov/
Molecular Weight / Isoelectrical Point	http://web.expasy.org/compute_pi/
Signal peptide	http://www.cbs.dtu.dk/services/SignalP/
Decrease redundancy	http://web.expasy.org/decrease_redundancy/
ClustalW/Phylogenetic tree	http://www.ebi.ac.uk/Tools/msa/clustalw2/
Expression data/PLEXdb	www.plexdb.org/modules/tools/plexdb_blast.php
Arabidopsis Genome database	www.arabidopsis.org

Protein Extractions and Analysis

We studied total chitinase activity of cotton plant leaves in comparison with other plant materials. For that lyophilized, fully developed mature leaves were collected from *Gossypium hirsutum*, *Malva sylvestris*, *Morus multicaulis*, *Populus*, *Ligustrum vulgare* and *Rumex obtusifolius*, growing in close distance with the same ecological parameters in the locality of Tashkent, Uzbekistan (GPS coordinates: 41° 15′ 52.7400" N and 69° 12′ 58.5720" E). Tissue material (500 g) was ground with liquid nitrogen using a mortar and pestle. After grinding, proteins were isolated and assayed for chitinase activity as described previously (Reissig *et al.* 1955). The activity of the enzyme was expressed as mmol of N-acetyl glucoseamine amount hydrolyzed for 2 hours.

RESULTS AND DISCUSSION

Mature cotton leaves contain proteins with chitinolytic activity. This activity is comparable with that measured in leaves of malva (*Malva sylvestris*), white mulberry (*Morus multucaulis*) and poplar (*Populus*), but lower than in wild privet (*Ligustrum vulgare*) or bitter dock (*Rumex obtusifolius*) (Fig. 1).

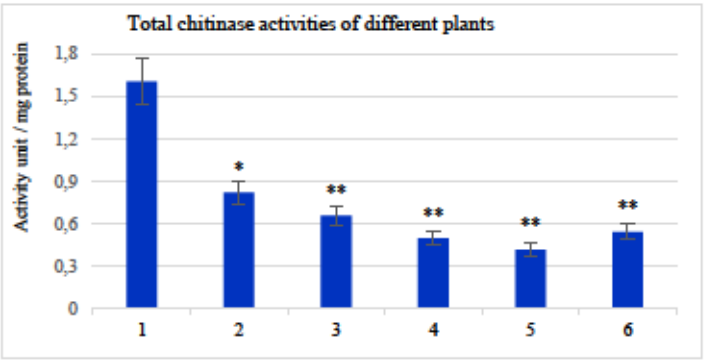


Figure 1 Variability of total chitinase activity in mature leaves of different plant species such as *Rumex obtusifolius* (1), *Ligustrum vulgare* (2), *Populus alba* (3), *Morus multicaulis* (4), *Malva Sylvestris* (5) and *Gossypium hirsutum* (6). (The error bars were calculated based on the four replicates with standard deviation, "*": significant difference at P < 0.1; "**": significant difference at P < 0.05)

We have measured the overall activity of chitinases in cotton. The obtained values are, however, hardly indicative since results are from the activities of several individual isoforms of different activity, regulation and function. The activity of chitinases varies among plant species, but also depends on developmental stage and even plant organ (**Gregorová *et al.* 2015**). Nevertheless, their total activity in pea was shown to coincide with metal tolerance (**Metwally *et al.* 2005**). Moreover, not only absolute activity values but kinetics of individual chitinase isoforms can coincide with plant tolerance to stresses (**Mészáros *et al.* 2013**), hence detailed study of corresponding gene family might be very useful for plant improvement.

The measured activities are the results of action of several different isoforms encoded by the members of chitinase family. Here, the genome of *G. raimondii* and *G. hirsutum* were searched with chitinases from *Arabidopsis thaliana* for presence of expressed and putative gene homologues using the BLAST program in Phytozome and NCBI database. This search revealed a total of 43 gene sequences annotated as chitinases, of which 2 were removed as redundant using a software. A total of 41 non-redundant putative chitinases were detected in cotton. Their calculated molecular weights varied in the range of 24.9-49.8 kDa. More than half of them (29 out of 41) were predicted as acidic with the pI lower than 6.5, and only four chitinases were found to have pI in neutral range and 9 chitinases are possibly basic. Clustering with chitinase representatives from *A. thaliana* and the cotton chitinase class VII (**Li and Liu 2003**) sorted the cotton chitinases into six classes (Figure 2).

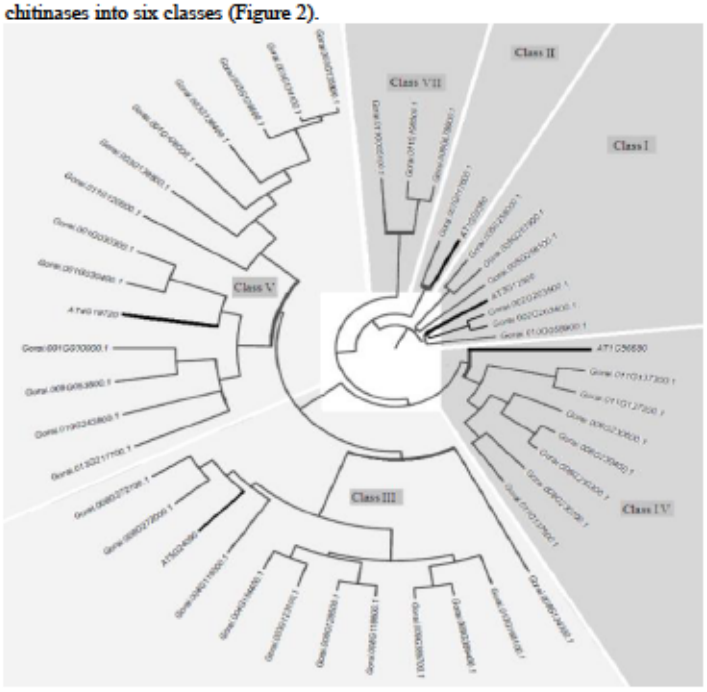
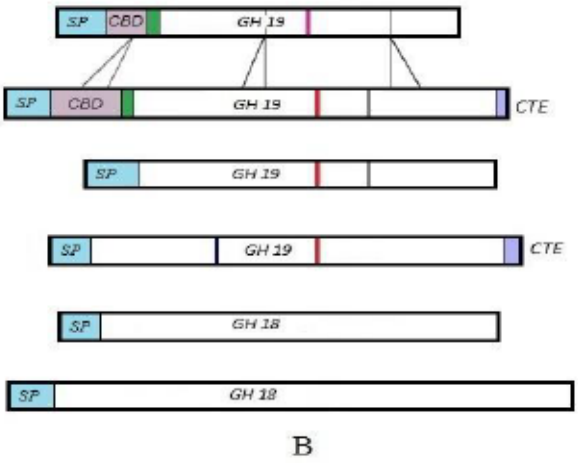


Figure 2 Dendrogram of cotton chitinases based on amino acid sequence alignment with representatives of genes belonging to classes I-V from *A. thaliana*. Representatives of genes from *A. thaliana* are described in bold. In light grey background, genes of Family GH 18 are shown. Genes of Family GH 19 are indicated in dark grey background.

This distribution fits with expected structural data. Translations of 17 identified cotton chitinases contain a Glyco_hydro_19 domain (GH19) (Family 19 chitinases), and 24 chitinases possess a Glyco_hydro_18 domain (GH18) typical for the family 18 chitinases. The family 19 chitinases comprised classes I, II, IV, VII and included 17 putative genes, and the family 18 chitinases possessed 11 genes of class III and 13 of class V chitinases (Figure 2 and 3). A putative signal peptide (SP) is present in 37 chitinase genes (Figure 3). A carbohydrate-binding domain was identified in only some of Family 19 chitinases, namely in six class IV and three class I chitinase genes (Figure 3). Among them, a proline rich, so-called hinge region was found in two out of three class I, and four of six class IV chitinases (Figure 3).



B

Class	Number of genes *A.thaliana*	*P.trichocarpa*	*G.raimondii*	*Abundance of classes in G.raimondii*
IV	9	5	7	0.171
I	1	10	6	0.146
II	4	3	1	0.024
VII	-	-	3	0.073
III	1	13	11	0.268
V	9	5	13	0.317

C

Figure 3 Primary structural maps of chitinase (class I - VII) genes found in cotton (*G. raimondii*) genome and the number of their representants in different plant species. SP - Signal peptide; CBD - carbohydrate-binding domain; ■ - Hinge region; *GH 18* and *GH 19* - Glycohydrolase domain 18/19, CTE- C-terminal extension; △ Deletions; with red lines YNYG motifs are represented in classes I, II, and VII; dark grey lines in class I and class II chitinases stands for WFWM motifs; CRGP motif in class IV chitinases is noted in pink line; EVAAF motifs are marked with a violet line in class VII chitinases

We identified and characterized to some extent *in silico* a total of 41 chitinase genes in the genome of cotton. This number is higher comparing with 24 genes described in the model plant *A. thaliana* **(Passarinho and de-Vries, 2002)** or the 37 genes in poplar (*P.trichocarpa)* **(Jiang *et al.* 2013)** (Figure 3). Since the number of chitinase genes among dicotyledonous and monocotyledonous plant species is expected to be comparable (Yokoyama and Nishitani 2004), the observed discrepancy is likely because of available rapidly developing bioinformatic tools (Figure 3). Consequently, the currently described gene families in individual species might in reality still be larger and incomplete.

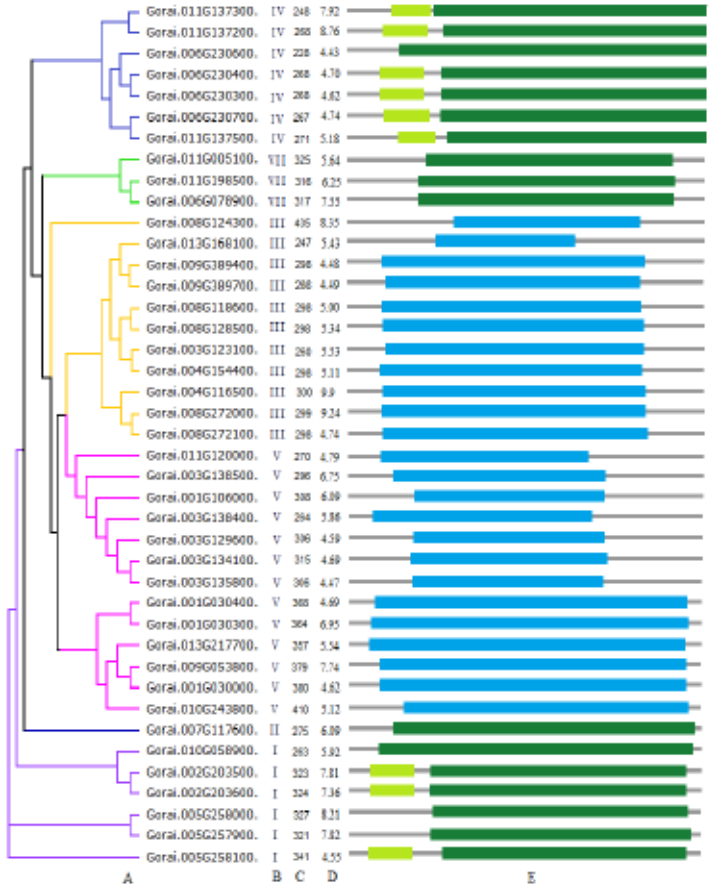


Figure 4 Phylogenetic relationships among *G. raimondii* chitinase genes and their protein structures: A - Phylogenetic tree; B – Chitinase class; C - number of amino acids in the enzyme molecule: D – Putative isoelectric points of enzymes E - Domains: ■ - Glycosyl hydrolase Family 18; ■ - Glycosyl hydrolase Family 19; ■ - Carbohydrate binding domain;

Calculated molecular weights of all encoded cotton chitinases were in the range of 24.9-49.8 kDa which are similar to those found in Arabidopsis 23.3-46.9 kDa **(Passarinho and deVries 2002)**.

Intron-exon structures of cotton chitinases resemble some similarities with respect to enzyme classification (Figure 4). All class I chitinases have 2 introns and 3 exons. All class IV chitinases (except for the Gorai.011G137300) have 1 intron and 2 exons. In contrast, gene structures of Family 18 chitinases were more variable. Seven of eleven class III members lack any intron; 3 genes possess one intron and one gene (Gorai.008G124300) contains 7 introns. Seven of thirteen cotton class V chitinase genes possess one intron. Five of them have a relatively conserved position of intron at the beginning of the gene. The other six class V family members lack any intron.

The chitinase genes in the Phytosome database were assigned to the five typical structural classes, and clustering with chitinases from well-described Arabidopsis confirmed this distribution (Figure 2). In addition to these five typical classes, a class VII chitinase has been described and characterized by **Li and Liu (2003)**. We identified two more chitinases showing high sequence similarity to the class VII chitinase found in the cotton genome (Figure 2 and 4). Typically, these enzymes lack the CBD **(Li and Liu 2003)**. While two of them (Gorai011G005100 and Gorai006G078900) show responsiveness to abiotic stresses **(Christianson *et al.* 2002; Padmalatha *et al.* 2012**; Table 2), the third (Gorai.011G198500) has been suggested to play a role in fiber development **(Zhang D *et al*, 2004)**.

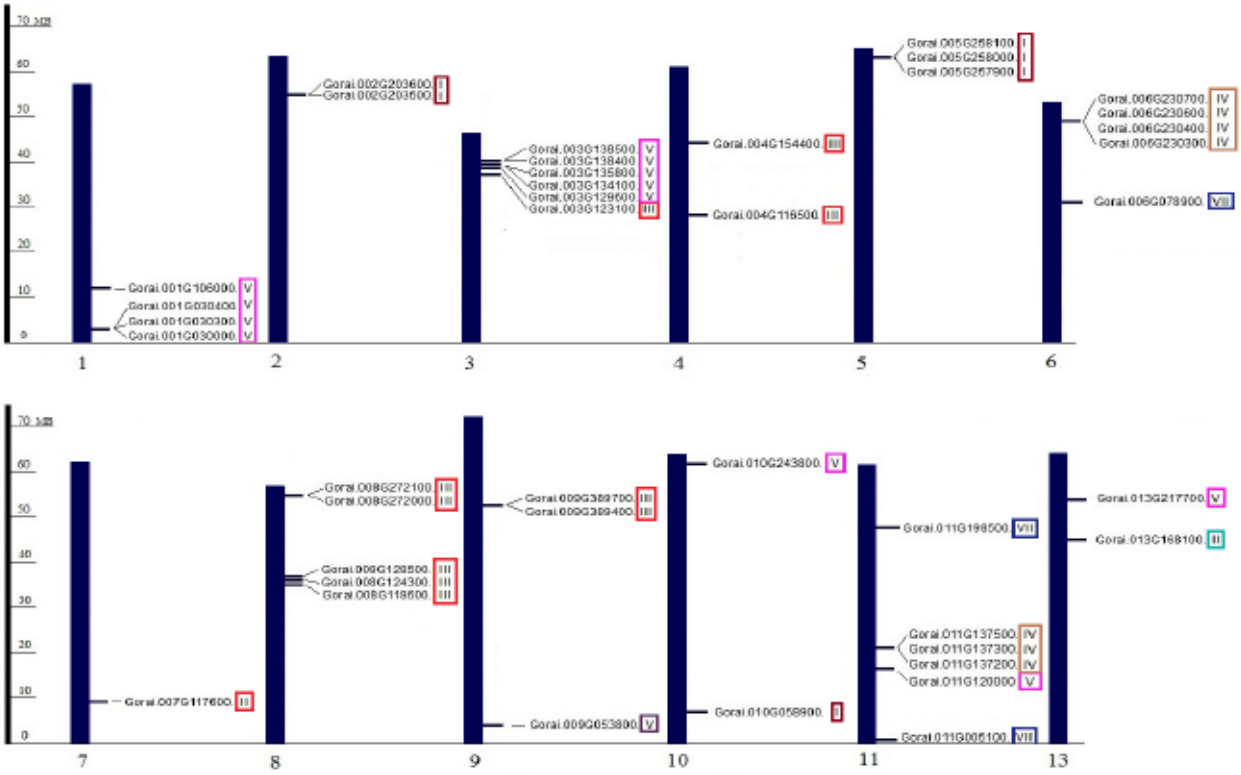


Figure 5 Genomic localization of *G. raimondii* chitinase genes. Transcript names and chitinase classes (in Roman digits) are given in front of localization. Numbers of chromosomes are indicated under the columns. Classes of chitinases are shown in quadrangles.

We retrieved data on the localization of chitinase genes in chromosomes of *G. raimondii* from the Phytozome database (Figure 5). The largest numbers of chitinase genes were located on the chromosomes 3, 11 (6 genes) and 6, 8 (5 genes). Many genes belonging to similar classes are co-localized. Only class III chitinases are located on the chromosomes 4 and 8, class I chitinases are only present on the chromosomes 2 and 5, while only class V chitinases were identified on the chromosome 1. On the other hand, clusters of chitinase genes (small regions of chromosomes where several genes are co-located) were identified on the cotton chromosomes 1, 5, 6 and 11. On the chromosomes 6 and 11 there were two clusters of class IV chitinases, three class I chitinases were clustered on the chromosome 5, and three class V chitinases are clustered on the chromosome 1.

The chitinase genes in cotton are distributed along the almost entire genome, except for the single chromosome 12 (Figure 5). This distribution is relatively even comparing with other species. For instance in *A.thaliana* all the 24 chitinases were found in five chromosomes **(Passarinho and de Vries, 2002)**, while the largest number of chitinases belonged to chromosomes 2 (6 chitinases) and 4 (9 chitinases). The majority (30 of 37) of chitinase genes in *P. trichocarpa* were positioned on the chromosomes 8 of 19 **(Jiang *et al.* 2013)**. On the other hand, co-localisation of similar class chitinases in cotton appears as a common feature. Similar pattern was observed in *P. trichocarpa* chitinases, where the class I chitinases were localized on chromosomes 4 and 9, and class V chitinases were located on chromosomes 6 and 18 **(Jiang *et al.* 2013)**. Similarly, in *A. thaliana* the class IV chitinases were found on chromosome 2 and Class V genes were positioned on chromosome IV **(Passarinho and de Vries, 2002)**.

Since expression data are still unavailable for cotton in the Phytozome database, the chitinase genes identified were used as queries for similarity search of the PLEXdb database (Cotton PLEX). Expression data could be retrieved for a total of 8 cotton chitinase genes (Table 2).

Microarray gene expression data were obtained on leaves and roots of experimental cotton plants in the context of flooding **(Christianson *et al.* 2009)** and drought conditions **(Rodriguez *et al.* 2014; Padmaltaha *et al.* 2012)** (Table 2). A clone with high sequence similarity to the class IV chitinases Gorai.006G230700 was strongly responsive to drought in greenhouse condition **(Padmalatha *et al.* 2012)** and significantly lower value was true in field experiments **(Rodriguez *et al.* 2014)**, however remained unaffected in flood-exposed leaves and roots in a field study **(Christianson *et al.* 2009)**.

Table 2 Expression data of chitinases in the PLEX*db* (www.plexdb.org/modules/tools/plexdb_blast.php)

Phytozome name		Christianson *et al.* 2009				Rodriguez *et al.* 2014		Padmalatha *et al.* 2012	
		Root		Leaf		Leaf (field experiment)		Leaf (Greenhouse)	
	Class	Control	Flooded	Control	Flooded	Control	Drought	Control	Drought
Gorai.005G257900.1	I	**4.8**	**2.03**	1.22	1.39	4.9	4.31	**4.09**	**2.78**
Gorai.005G258100.1	I	10.05	11.82	7.98	6.29	7.03	7.49	7.4	9.0
Gorai.008G272000.1	III	11.45	11.68	5.47	5.87	7.37	7.04	8.16	8.9
Gorai.008G272100.1	III	5.95	6.95	5.86	6.5	7.34	6.82	9.49	11.78
Gorai.008G124300.1	III	8.42	7.58	7.02	6.68	7.16	7.88	8.85	8.76
Gorai.006G230700.1	IV	4.29	4.92	3.35	3.1	**5.48**	**3.95**	**2.72**	**5.07**
Gorai.011G005100.1	VII	5.98	5.73	**3.85**	**2.43**	5.84	4.01	4.9	3.97
Gorai.006G078900.1	VII	**3.54**	**2.27**	1.62	1.26	3.44	4.16	**5.17**	**0.8**

* Genes identified as responsive to given stress types are indicated bold

We detected six class I chitinase gene in cotton (Figure 4). One of them (Gorai.005G257900) has been reported as affected in flooded roots and in drought stressed leaves (Table 1; **Padmalatha *et al.* 2012**). The other two class I chitinases (Gorai.010G058900 and Gorai.002G203600) have been described as defensive and inducible by SA and ethylene **(Hudspeth *et al.* 1996; Levorson and Chlan, 1997**, respectively). Previously, relatively low number of class I chitinase was identified in Arabidopsis as active in roots and at lower levels in leaves and flowers of aging plants **(Passarinho *et al.* 2002)**.

A single class II chitinase gene in cotton contradicts the relatively higher number of identified class II chitinases in other plant species (Figure 2 and 3). This class of genes might have evolutionary evolved from class I chitinases and was also described for role during defense **(Kirsch *et al.* 1993; Ponath *et al.* 2000; Meins *et al.* 1994)**. On the contrary, surprisingly high number of class III chitinases was identified in cotton, similarly as in poplar but unlike in *A. thaliana*. The three experimentally studied class III cotton genes are probably involved in the processes other than adaptation to abiotic stresses (Table 2). The single class III

chitinase in Arabidopsis was induced by fungi, but not wounding, ethylene or SA (**Samac and Shah 1991**).

Seven class IV chitinases were identified in cotton. One of them (Gorai.006G230700) was induced in leaves exposed to drought (Table 2, **Padmalatha *et al.* 2012**), and another (Gorai.011G137500) was found induced in wilting leaf (**Wang *et al.* 2011**). Class IV chitinases in other plant species were proposed to be associated with plant resistance against fungi (**Lange *et al.* 1996; Nielsen *et al.* 1994**), viruses, and also abiotic stresses such as heavy metals and UV irradiation (Margis Pinhero *et al.* 1993). **However Passarinho and de-Vries (2002)** suggested that class IV chitinases are involved in developmental processes rather than in defense.

Thirteen cotton chitinases belong to class V enzymes. Specific function for this rather diverse group of chitinases has not yet been attributed. They are suggested to play a role in plant defense as well as development (**Heitz *et al.* 1994; Melchers *et al.* 1994**). Two class V chitinase genes in cotton together with class II chitinase were significantly upregulated in cotton roots in response *V. dahliae* and significantly increased following treatment with jasmonic acid and H_2O_2. Authors suggested that these genes may be involved in plant resistance to stress (**Xu *et al.* 2016**).

For some of the chitinase classes a typical role has been assigned. Most typically, antifungal activity has been attributed to class I and II chitinases (**Neuhaus *et al.* 1191**) by hydrolyzing the cell walls of plant pathogenic fungi (**Wargo PM 1975**), and/or by releasing elicitors of defense reactions (**Hadwiger and Beckman 1980**). Furthermore, class I and class II chitinases were linked with antifreeze activity in *Secale cereale* (**Pihakaski-Maunsbach *et al.* 2001; Nakamura *et al.* 2008**), and salt-adaptation in tobacco (*Nicotiana tabacum*) (**Yun *et al.* 1996**). The functional differences between individual (cotton) chitinase classes are, however, not clear since chitinases of separate classes can reflect different chitin recognition levels. For example, **Sasaki *et al.* (2006)** demonstrated that class I and class III chitinases recognize three contiguous N-acetyl glucose amine residues in different subsites. Furthermore, available expression data indicate that some cotton chitinases of different classes might have overlapping functions. For instance, class I, IV and VII chitinases might be defense-related due to responsiveness to several different abiotic stresses, ethylene or the stress hormone salicylic acid (**Chlan and Bourgeois 2001; Nielsen *et al.* 1994; Li and Liu 2003**). In the past decade increasing amount of evidence indicates involvement of chitinases in abiotic stress like metals (**Békésiová *et al.* 2008**), cold and spring hardening (**Zur *et al*, 2014**), drought (**Gregorová *et al.* 2015**). However, these functions have not been attributed to a particular chitinase class (Stintzi *et al.* 1993). Researches that are more recent prove that chitinases are activated in cotton upon application of commonly used pesticides (**Asrorov *et al.* 2013; Rajendran *et al.* 2011; Szczepaniec *et al.* 2013**). On the other hand, class IV chitinases have been suggested as markers of embryogenesis (**Wiweger *et al.* 2003**). In cotton the chitinase gene corresponding to Gorai.011G198500 has been identified (**Li and Liu 2003**) as not similar to any of the typical plant chitinase classes I-VI (**Neuhaus *et al.* 1996**). This unique chitinase, similar to class I and class II chitinases to only 30%, was defined as belonging into a separate class (class VII chitinase), and is abundant in fibers and in the seedling roots (**Li and Liu 2003**). More detailed structural and expression analyses suggested that the two closely related cotton genes, GhCTL1 and GhCTL2, can be preliminarily classified as chitin binding lectins. They likely lack hydrolytic activity, while the chitinase activity in fiber is attributable to the expression of authentic chitinases along with putatively non-hydrolytic GhCTL1/GhCTL2 (**Zhang *et al.* 2004**). These specific cotton chitinase initiates fiber wall thickening via the deposition of helical cellulose microfibrils in secondary walls (**Zhang *et al.* 2004**), but inducibility with salicylic acid and ethylene indicates additional role in defense (**Li and Liu 2003; Hudspeth *et al.* 1996; Levorson and Chlan *et al.* 1997**). The exact mechanism of action of these genes in fiber formation, however, remains to elucidate.

CONCLUSION

In summary, we identified the members of chitinase gene family in cotton using bioinformatics tools. The available genome data enable a more detailed analysis of cotton chitinase genes both *in silico* as well as experimentally. Especially the gene expression data have to be extended in near future to improve our knowledge on the pivotal role of chitinases not only in processes of adaptation to environment, but also in important developmental processes like fiber formation. The obtained results can be a valuable basis for the functional studies of these gene family members in basic- as well as applied cotton research.

REFERENCES

Asrorov A, Sultanova E, Veshkurova O, Sattarov N, Khodjayev Sh, Salikhov Sh. 2013. Effects of different classes of insecticides on the activity of hydrolases in cotton plant leaves. *American-Eurasian Journal of Agricultural & Environmental Sciences*, 13(3), 296-300. http://dx.doi.org/10.5829/idosi.aejaes.2013.13.03.1926

Bu, B., Qiu, D., Zeng, H., Guo, L., Yuan, J., & Yang X. 2014. A fungal protein elicitor PevD1 induces *Verticillium* wilt resistance in cotton. *Plant Cell Reports*, 33(3), 461-470. http://dx.doi.org/10.1007/s00299-013-1546-7

Chlan, C. A., & Bourgeois, R. P. 2001. Class I chitinases in cotton (*Gossypium hirsutum*): characterization, expression and purification. *Plant Science*, 161(1), 143-154. http://dx.doi.org/10.1016/S0168-9452(01)00398-3

Christianson, J. A., Llewellyn, D. J., Dennis, E. S., & Wilson, I. W. 2009. Global gene expression responses to waterlogging in roots and leaves of Cotton (*Gossypium hirsutum L.*). *Plant Cell Physiology*, 51(1), 21-37. http://dx.doi.org/10.1093/pcp/pcp163

Cletus, J., Balasubramanian, V, Vashisht, D., & Sakthivel, N. 2013. Transgenic expression of plant chitinases to enhance disease resistance. *Biotechnology Letters*, 35(11), 1719-1732. http://dx.doi.org/10.1007/s10529-013-1269-4

Dubery, I. A., Slater, V. 1997. Induced Defence Responses in Cotton Leaf Disks by Elicitors from *Verticillium dahliae*. *Phytochemistry*, 44(8), 1429-1434. http://dx.doi.org/10.1016/S0031-9422(96)00635-8

Ebrahim, S., Usha, K., & Singh, B. 2011. Pathogenesis Related (PR) Proteins in Plant Defense Mechanism. In: A. Méndez-Vilas (eds.) Science against microbial pathogens: communicating current research and technological advances. 3rd edn. Badajoz, Spain, Formatex Research Centre Publisher. p. 1043-1054.

Graham, L. S., Sticklen, M. B. 1994. Plant chitinases. *Canadian Journal of Botany, 72*, 1057-1083.

Gregorová, Z., Kováčik, J., Klejdus, B., Maglovski, M., Kuna, R., Hauptvogel, P., & Matušíkova, I. 2015. Drought-Induced Responses of Physiology, Metabolites, and PR Proteins in *Triticum aestivum*. *Journal of Agricultural and Food Chemistry*, 63(37), 8125-8133, http://dx.doi:10.1021/acs.jafc.5b02951

Hadwiger, L. A., Beckman, J. M. 1980. Chitosan as a component of pea-*Fusarium solani* interactions. *Plant Physiology*, 66(2), 205-211. http://dx.doi:10.1104/pp.66.2.205

Hamid, R., Khan, M. A., Ahmad, M., Ahmad, M. M., Abdin, M. Z., Musarrat, J., & Javed, S. 2013. Chitinases: An update. *Journal of Pharmacy and Bioallied Sciences*, 5(1), 21-29. http://dx.doi:10.4103/0975-7406.106559

Heitz, T., Segond, S., Kauffmann, S., Geoffroy, P., Prasad, V., Brunner, F., Fritig, B., Legrand, M. 1994. Molecular characterization of a novel tobacco pathogenesis-related (PR) protein: a new plant chitinase/lysozyme. *Molecular and General Genetics*, 245(2), 246-254. http://dx.doi.org/10.1007/BF00283273

Hudspeth, R. L., Hobbs, S. L., Anderson, D. M., & Grula, J. W. 1996. Characterization and expression of chitinase and 1,3-beta-glucanase genes in cotton. *Plant Molecular Biology*, 31(4), 911-916. http://dx.doi.org/10.1007/BF00019478

Iseli, B., Armand, S., Boller, T., Neuhaus, J. M., & Henrissat, B. 1996. Plant chitinases use two different hydrolytic mechanisms. *FEBS Letters*, 382(1-2), 186-188.

Jiang, G., Huang, R. F., Song, J. L., Huang, M. R., & Xu, L. A. 2013. Genomewide analysis of the chitinase gene family in *Populus trichocarpa*. *Journal of Genetics*, 92(1), 121-125. http://dx.doi.org/10.1007/s12041-013-0222-6

Keen, N. T., Dawson, W. O. 1192. Pathogen avirulence genes and elicitors of plant defense. In: Boller T., Meins F. (eds.) Genes Involved in Plant Defense, New York, Springer-Verlag. p. 85-114.

Kim, Y. S., Lee, J. H, Yoon, G. M., Cho, H. S., Park, S. W., Suh, M. C., Choi, D., Ha H. J., Liu, J. R., Pai, H. S. 2000. CHRK1, a chitinase-related receptor-like kinase in tobacco. *Plant Physiology*, 123(3), 905-916. http://dx.doi.org/10.1104/pp.123.3.905

Lange, J., Mohr, U., Wiemken, A., Boller T., & Vogeli-Lange, R. 1996. Proteolytic processing of class IV chitinase in the compatible interaction of bean roots with *Fusarium solani*. *Plant Physiology*, 111(4), 1135-1144. http://dx.doi:10.1104/pp.111.4.1135

Levorson, J. P., Chlan, C. A. 1996. Isolation of a genomic DNA clone from Gossypium hirsutum with high similarity to Class I endochitinase plant sequences (Accession No.U60197)(PGR96-054). *Plant Physiology*, 111, 1354.

Levorson, J. P., Chlan, C. A. 1997. Cloning of an Ethylene-Responsive Chitinase from Cotton (Accession No.U78888) (PGR97-034). *Plant Physiology*, 113, 665.

Li, J., Liu, J. Y. 2003. A Novel cotton gene encoding a new class of chitinase. *Acta Botanica Sinica*, 45(12), 1489-1496.

Liu, R. J., Li, H. F., Shen. C. Y., & Chiu, W. F. 1995. Detection of pathogenesis-related proteins in cotton plants. *Physiological and Molecular Plant Pathology*, 47(6), 357-363.

Margis-Pinheiro, M., Martin, C., Didierjean, L., & Burkard, G. 1993. Differential expression of bean chitinase genes by virusinfection, chemical treatment and UV irradiation. *Plant Molecular Biology*, 22(4), 659-668.

Meins, F., Fritig, B., Linthorst, H. J. M., Mikkelsen, J. D., Neuhaus, J. M., & Ryals, J. 1994. Plant chitinase genes. *Plant Molecular Biology Reporter*, 12, 22-28.

Metwally, A., Safronova, V. I, Belimov, A. A., & Dietz, K. J. 2005. Genotypic variation of the response to cadmium toxicity in *Pisum sativum*. *Journal of Experimental Botany*, 56, 167-178.

Nakamura, T., Ishikawa, M., Nakatani, H., & Oda, A. 2008. Characterization of cold-responsive extracellular chitinase in bromegrass cell cultures and its

relationship to antifreeze activity. *Plant Physiology*, 147(1), 391-401. http://dx.doi.org/10.1104/pp.106.081497

Neuhaus, J. M., Sticher, L., & Meins, F (Jr)., Boller, T. 1991. A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. *Proceedings of the National Academy of Sciences USA*, 88(22), 10362-10366. http://dx.doi.org/10.1073/pnas.88.22.10362

Nielsen, K. K., Bojsen, K., Roepstorff, P., & Mikkelsen, J. D. 1994. A hydroxyproline-containing class IV chitinase of sugar beet is glycosylated with xylose. *Plant Molecular Biology*, 25(2), 241-257.

Padmalatha, K. V., Dhandapani, G., Kanakachari, M., Kumar, S., Dass, A., Patil, D. P., Rajamani, V., Kumar, K., Pathak, R., Rawat, B., Leelavathi, S., Reddy, P. S., Jain, N., Powar, K. N., Hiremath, V., Katageri, I. S., Reddy, M. K., Solanke, A. U., Reddy, V. S., & Kumar, P. A. 2012. Genome-wide transcriptomic analysis of cotton under drought stress reveal significant down-regulation of genes and pathways involved in fibre elongation and up-regulation of defense responsive genes. *Plant Molecular Biology*, 78(3), 223-246. http://dx.doi.org/10.1007/s11103-011-9857-y

Passarinho, P. A., deVries, S. C. 2002. Arabidopsis Chitinases: a Genomic Survey. The Arabidopsis Book, American Society of Plant Biologists. p. 1-25.

Pihakaski-Maunsbach, K., Moffatt, B., Testillano, P., Risueno, M., Yeh, S., Griffith, M., & Maunsbach, A.B. 2001. Genes encoding chitinase-antifreeze proteins are regulated by cold and expressed by all cell types in winter rye shoots. *Physiologia Plantarum*, 112(3), 359-371. http://dx.doi.org/10.1034/j.1399-3054.2001.1120309.x

Ponath, Y., Vollberg, H., Hahlbrock, K., Kombrink, E. 2000. Two differentially regulated class II chitinases from parsley. *Biological Chemistry*, 381(8), 667-678. http://dx.doi.org/10.1515/BC.2000.087

Ponstein, A. S., Bres-Vloemans, S. A., Sela-Buurlage, M.B., vanden Elzen, P. J., Melchers, L. S., & Cornelissen, B. J. 1994. A novel pathogen- and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. *Plant Physiology*, 104(1), 109-118. http://dx.doi.org/10.1104/pp.104.1.109

Rajendran, L., Ramanathan, A., Durairaj, C., & Samiyappan, R. 2011. Endophytic Bacillus subtilis enriched with chitin offer induced systemic resistance in cotton against aphid infestation. *Archives of Phytopathology and Plant Protection*, 14, 1375-1389.

Reissig, J. L., Strominger, J. L., & Leloir, L. F. 1955. A Modified Colorimetric method for the estimation of N-acetylamino sugars. *Journal of Biological Chemistry*, 217(2), 959-966.

Rodriguez-Uribe, L., Abdelraheem, A., Tiwari, R., Sengupta-Gopalan, C., Hughs, S. E., & Zhang, J. 2014. Identification of drought-responsive genes in a drought tolerant cotton (*Gossypium hirsutum L.*) cultivar under reduced irrigation field conditions and development of candidate gene markers for drought tolerance. *Molecular Breeding*, 34(4), 1777-1796. http://dx.doi.10.1007/s11032-014-0138-8

Samac, D. A., Hironaka, C. M., Yallaly, P. E., & Shah, D. M. 1990. Isolation and characterization of the genes encoding basic and acidic chitinase in *Arabidopsis thaliana*. *Plant Physiology*, 93(3), 907-914.

Samac, D. A., Shah, D. M. 1991. Developmental and pathogen-induced activation of the Arabidopsis acidic chitinase promoter. *Plant Cell*, 3, 1063-1072. http://dx.doi.org/10.1105/tpc.3.10.1063

Sasaki, C., Varum, K. M., Itoh, Y., & Fukamizo, T. 2006. Rice chitinases: sugar recognition specificities of the individual subsites. *Glycobiology*, 16(12), 1242-1250. http://dx.doi.org/10.1093/glycob/cwl043

Stintzi, A., Heitz, T., Prasad, V., Wiedemann-Merdinoglu, S., Kauffmann, S., Geoffroy, P., Legrand, M., & Fritig, B. 1993. Plant 'pathogenesis related' proteins and their role in defense against pathogens. *Biochimie*, 75(8), 687-706. http://dx.doi.org/10.1016/0300-9084(93)90100-7

Suzuki, K., Taiyoji, M., Sugawara, N., Nikaidou, N., Henrissat, B., & Watanabe, T. 1999. The third chitinase gene (chiC) of Serratia Marcescens 2170 and the relationship of its product to other bacterial chitinases. *Biochemical Journal*, 343(3), 587-596.

Szczepaniec, A., Raupp, M. J., Parker, R. D., Kerns, D., & Eubanks, M. D. 2013. Neonicotinoid insecticides alter induced defenses and increase susceptibility to Spider Mites in distantly related crop plants, *PLoS ONE. 8(5)*. http://dx.doi.org/10.1371/journal.phone.0062620

Wang, F. X., Ma, Y. P., Yang, Ch. L, Zhao, P. M., Yao, Y., Jian, G.L., Luo, Y.M., & Xia, G.X. 2011. Proteomic analysis of the sea-island cotton roots infected by wilt pathogen *Verticillium dahliae*. *Proteomics*, 22, 4296-4309. http://dx.doi.org/10.1002/pmic.201100062

Wargo, P. M. 1975. Lysis of the cell wall of Armillaria mellea by enzymes from forest trees. *Physiological Plant Pathology*, 5, 99-105.

Wiweger, M. M., Farbos, I., Ingouff, M., Lagercrantz, U., & von Arnold, S. 2003. Expression of Chi4-Pa chitinase genes during somatic and zygotic embryo development in Norway spruce (Picea abies): similarities and differences betwen gymnosperm and angiosperm class IV chitinases. *Journal of Experimental Botany*, 54, 2691-2699. http://dx.doi.org/https://doi.org/10.1093/jxb/erg299

Xu, J., Xu, X., Tian, L. Wang, G., Zhang, X., Wang, X., & Guo, W. 2016. Discovery and identification of candidate genes from the chitinase gene family for *Verticillium dahliae* resistance in cotton. *Scientific Reports*, 6: 1-12. http://dx.doi.org/10.1038/srep29022

Yun, D. J, D'Urzo, M. P., Abad, L., Takeda, S., Salzman, R., Chen, Z., Lee, H., Hasegawa, P. M., & Bressan, R. A. 1996. Novel osmotically induced antifungal chitinases and bacterial expression of an active recombinant isoform. *Plant Physiology*, 111(4), 1219-1225.

Zhang, D., Hrmova, M., Wan, C. H., Wu, C., Balzen, J., Cai, W., Wang, J., Densmore, L. D., Fincher, G. B., Zhang, H., & Haigler, C. H. 2004. Members of a new group of chitinase-like genes are expressed preferentially in cotton cells with secondary walls. *Plant Molecular Biology*, 54, 353-372. http://dx.doi.org/10.1023/B:PLAN.0000036369.55253.dd

Zur, I., Golebiowska, G., Dubas, E., Golemiec, E., Matušíková, I., Libantová, J., & Moravčíková, J. 2013. β-1,3-glucanase and chitinase activities in winter triticales during cold hardening and subsequent infection by *Microdochium nivale*. *Biologia*, 68, 241-248. http://dx.doi.org/10.2478/s11756-013-0001-0

Yokoyama, R., Nishitani, K. 2004. Genomic basis for cell-wall diversity in plants. A comparative approach to gene families in rice and *Arabidopsis*. *Plant Cell Physiology*, 45, 1111-1121. http://dx.doi.org/10.1093/pcp/pch151

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IN VITRO EFECTS OF SELECTED BIOLOGICALLY ACTIVE COMPOUNDS ON RABBIT SPERMATOZOA MOTILITY BEHAVIOUR

*Michal Ďuračka, Marek Halenár, Eva Tvrdá**

Address(es): MSc. Eva Tvrdá, PhD.,
Slovak university of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Animal Physiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia, +421-37-641-4918.

*Corresponding author: evina.tvrda@gmail.com

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ABSTRACT

The aim of this study was to evaluate the *in vitro* effects of selected pure naturally occuring biologically active compounds (resveratrol-RES, quercetin-QUE, curcumin-CUR, epicatechin-EPI, isoquercitrin-ISO) on rabbit sperm motility using the IDENT staining method and the computer-aided sperm (CASA) analysis. Semen samples were collected regularly from 10 male rabbits, pooled, the seminal plasma was removed and the sperm fraction was exposed to different concentrations (1, 5, 10, 50 and 100 µmol/L) of chosen biomolecules. At culture times of 0h, 2h, 4h, 6h and 8h, the spermatozoa motility was assessed using the TOX IVOS II. CASA system and the IDENT fluorescent staining method. The motility assessment revealed different behavior patterns, specific and unique to each of the studied biomolecules. After 8h of *in vitro* culture, the highest sperm motility was detected in experimental groups subjected to 10 µmol/L RES ($P<0.05$); 1-10 µmol/L QUE ($P<0.01$ with respect to 1 µmol/L QUE; $P<0.001$ in case of 5 and 10 µmol/L QUE); 1 µmol/L CUR ($P<0.01$); 1-100 µmol/L EPI ($P<0.01$ in relation to 50, 10 and 5 µmol/L EPI; $P<0.001$ with respect to 100 and 1 µmol/L EPI) and 10 µmol/L ISO ($P<0.05$) when compared to the untreated Control. Our data provide evidence on specific toxic and beneficial concentrations as well as exposure periods applicable for each biomolecule, which may be useful for future *in vitro* farmagological or toxicological studies on male reproduction in rabbits.

Keywords: Spermatozoa, rabbit, resveratrol, quercetin, curcumin, epicatechin, isoquercitrin

INTRODUCTION

Current evidence suggests that oxidative stress (OS) represents an important factor in male reproductive dysfunction. An increased concentration of abnormal spermatozoa generating free radicals (FRs) and a reduced antioxidant capacity of semen, which has been reported in animals and humans, are two dominant factors underlying seminal OS (**Aitken *et al.*, 1991; Tvrdá *et al.*, 2011**). At the same time, a specific cellular structure leaves spermatozoa to be uniquely vulnerable to oxidative insults. Sperm plasmatic membranes contain large quantities of polyunsaturated fatty acids, while their cytoplasms lack significant amounts of FR scavengers (**Buettner, 1993**), leading to an icreased risk of oxidative damage, and subsequently a decreased semen quality (**de Lamirande and Gagnon, 1993**). Furthermore, FR overproduction may result in increased morphological defects, alterations to sperm capacitation and acrosome reaction, all of which are related to a compromised fertility (**Agarwal *et al.*, 2014**).

Recently, a number of reports have emphasized on the positive effects of oral antioxidant administration on male fertility in animals and humans (**Donnely *et al.*, 1999**; **Agarwal and Sekhon, 2010**). On the other hand, studies focused on the *in vitro* effects of antioxidants on spermatozoa are still very sparse, controversial or contradictory. The in vitro data are nevertheless important, as it has been shown on numerous occasions that external antioxidants may protect spermatozoa against oxidative injury and a subsequent dysfunction. Such knowledge is essential for spermatozoa processing protocols performed in medical or veterinary andrology for spermatozoa cryopreservation or assisted reproductive technologies (**Saleh and Agarwal, 2002**).

Administration of synthetic antioxidants to cell cultures is an efficient strategy to prevent OS. However, the safety of synthetic additives has been under a constant debate, raising the potential of naturally occurring compounds with antioxidant properties because of their chemical diversity, structural complexity, availability, lack of significant toxic effects and intrinsic biologic activity (**Alarcón de la Rastra, 2008**).

In this study, we followed a systematic approach to assess the *in vitro* effects of a wide range of pure flavonoid (quercetin, epicatechin, isoquercitrin) and polyphenolic (resveratrol, curcumin) compounds on rabbit sperm motility, one of the physiological manifestations of their fertilizing ability. Furthermore, we have validated the fluorescent-based rabbit sperm motility assessment using the IDENT staining method and the computer-aided sperm (CASA) analysis.

MATERIAL AND METHODS

Ten male rabbits (New Zealand white broiler line) were used in the experiment. The animals were 4 months old, with a weight of 4.0±0.2 kg and kept at an experimental farm of the Animal Production Research Centre Nitra, Slovak Republic. The rabbits were housed in a partially air-conditioned rabbit house under a photoperiod of 16L:8D (a minimum light intensity of 80 lux), kept in individual cages and fed with a commercial diet. Water was provided *ad libitum*. The air temperature of 20-24 °C and relative humidity of 65% were maintained in the rabbit house. Institutional and national guidelines on the care and use of animals were followed, and all the experimental procedures were approved by the State Veterinary and Food Institute of Slovak Republic (no. 3398/11-221/3) and Ethics Committee.

One ejaculate was collected from each rabbit on a regular collection schedule (twice a week for two consecutive weeks) using an artificial vagina. Immediately upon collection, the sperm concentration and motility were assessed in each ejaculate. Only samples with a minimum motility of 60% were used in the experiments. Individual ejaculates were mixed together in order to acquire a pooled sample of rabbit semen.

The resulting semen sample was centrifuged (300 x g) at 25°C for 5 min, seminal plasma was removed and the sperm pellet was washed twice with PBS (Dulbecco's phosphate-buffered saline without calcium chloride and magnesium chloride; Sigma-Aldrich, St. Louis, MO, USA), resuspended in a culture medium consisting of PBS, 5% glucose (Centralchem, Bratislava, Slovak Republic) and 4% BSA (bovine serum albumin, Sigma-Aldrich). Each experimental group was exposed to a different concentration (1, 5, 10, 50 and 100 µmol/L) of a chosen biomolecule (resveratrol, quercetin, curcumin, epicatechin, isoquercitrin; Sigma-Aldrich) using a dilution ratio of 1:20. The Control group carried no supplementation. The samples were cultured at 37 °C.

At culture times of 0h, 2h, 4h, 6h and 8h, the spermatozoa motility (percentage of motile spermatozoa; motility > 5 μm/s; %) was assessed using the computer-aided sperm analysis (CASA; Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA). In order to standardize a fluorescent assessment of sperm activity, the samples were stained using the IDENT stain, a DNA-specific dye based on Hoechst bisbenzimide (Hamilton-Thorne Biosciences). The IDENT dye provided in Eppendorf tube was diluted with 1 mL of the culture medium and mixed with the sample using a ratio of 1:1. Following a 10 min incubation in the dark, the sample was analyzed under fluorescent illumination. The system was set up as follows: frame rate - 30 at 60 Hz, dark field; minimum contrast - 50; static head size - 0.28-4.30; static head intensity - 0.12-2.92; static elongation - 8-97; minimum cell size - 7 pixels; default cell intensity - 70, magnification - 1.75, illumination intensity - 2198. Ten μL of each sample were placed into the Makler counting chamber (depth 10 μm, 37 °C; Sefi Medical Instruments, Haifa, Israel) and immediately assessed. 10 microscopic fields were subjected to each analysis in order to include at least 300 cells.

All the data were subjected to statistical analysis using the GraphPad Prism program (a 3.02 version for Windows, GraphPad Software incorporated, San Diego, California, USA, http://www.graphpad.com/). The results are quoted as the arithmetic mean ± standard error of mean (SEM). The comparative analysis was carried out by a one-way ANOVA with the Dunnett's post test. The level of significance for the analysis was set at * P<0.05; ** P<0.01; *** P<0.001.

RESULTS AND DISCUSSION

Resveratrol (RES)

The CASA assessment revealed a continuous decrease of spermatozoa motility and in all groups over the course of a 8h *in vitro* culture (Table 1). The initial (Time 0h) MOT was higher in the experimental groups supplemented with 5 and 50 μmol/L RES when compared to the Control group, although without any statistical significance (P>0.05). Despite being statistically insignificant, a motion-promoting effect of RES remained notable after 2h, specifically following the addition of 1, 5 and 50 μmol/L RES. A significantly higher (P<0.05) MOT was detected in case of 1 and 5 μmol/L RES following a 4h culture. After 6h, the decline of spermatozoa MOT was significantly slowed down following the supplementation of a concentration range of 1-10 μmol/L RES (P<0.001). At the end of the experiment (8h), elevated motility was observed in the experimental group supplemented with 5 μmol/L RES, being significantly higher in comparison with the Control (P<0.05) (Table 1).

Table 1 Time- and dose-dependent effects of resveratrol (RES) on rabbit spermatozoa motility [%]

	Ctrl	100 μmol/L RES	50 μmol/L RES	10 μmol/L RES	5 μmol/L RES	1 μmol/L RES
0h	80.55±2.33	68.97±6.59	81.90±8.21	73.98±5.55	82.98±8.32	73.95±6.87
2h	69.03±4.02	61.09±7.00	72.99±6.98	69.09±4.76	78.99±7.89	73.56±8.00
4h	59.12±3.33	53.98±4.44	58.90±6.09	68.89±3.44	73.98±6.02*	69.98±5.99*
6h	37.55±2.99	43.98±3.99	46.97±3.98	56.09±5.55**	63.79±5.96***	60.98±5.99***
8h	20.04±1.88	11.98±2.09	15.04±0.67	14.90±1.23	29.99±2.09*	23.19±2.00

Mean±SEM. * P<0.05; ** P<0.01; *** P<0.001

RES has recently emerged as a complex biomolecule due to its wide range of cardiovascular, anticancer, antiinflamatory and protective effects (**Calabrese *et al.*, 2010**). Although no distinct toxicity was reported *in vivo* (**Cottart *et al.*, 2010**), *in vitro* experiments show that low RES doses improve cell survival, while high doses increase cell death (**Brown *et al.*, 2009**).

Our CASA results are contradictory to **Collodel *et al.* (2010)** who evaluated the effects of RES on human spermatozoa. Unlike our results, 100 μmol/L RES exerted cytotoxic activities against spermatozoa in a dose dependent manner. The LD50 was defined as 50 μmol/L RES, spermatocytes were more sensitive to the harmful effect of RES with a LD50 between 30 and 50 μmol/L. While at 100 μmol/L RES the rabbit sperm motility was decreased in a non-significant manner, human sperm motility in the above mentioned study was absent. Moreover, **Tvrdá *et al.* (2015b)** reported a significant decrease of bull sperm motility following exposure to 100 or 200 μmol/L RES during a 24 *in vitro* culture. The differences in our results may be explained by the sample type which was collected from rabbits as opposed to humans or bulls respectively, as well as a shorter exposure to the biocompound. On the other hand, the stimulating effects of lower (1-5 μmol/L) RES concentrations detected by our IDENT CASA technique are in agreement with **Tvrdá *et al.* (2015a;b)** who emphasize on the beneficial and protective effects of a concentration range of 5-50 μmol/L RES on the motion behavior, mitochondrial activity and intracellular superoxide production by bovine sperm cells. Furthermore, in an *in vitro* human study, progressive motility reached high values between 6 and 15 μmol/L RES (**Collodel *et al.*, 2010**).

Our CASA records are in agreement with **Mojica-Villegas *et al.* (2014)** who reported that a pretreatment with 15 μmol/L RES 15 min prior to incubation with ferrous ascorbate (FeAA) showed an 8.0-fold increase in murine spermatozoa motility. On the other hand, spermatozoa motility diminished in spermatozoa cryopreserved with RES comparably to the control in the studies by **Pasqualotto *et al.* (2006)** and **Garcez *et al.* (2010)** Furthermore **Silva *et al.* (2012)** reported that the addition of RES before cryopreservation did not significantly affect progressive motility, vigor, acrosome integrity, or plasma membrane integrity. In the meantime, **Sarlos *et al.* (2002)** recorded a higher motility and acrosome integrity of ram spermatozoa supplemented with RES after their storage at 5 C for 6 days. Similar results were reported in studies focused on swim-up selected human spermatozoa where low RES doses led to a higher progressive motility (**Collodel *et al.*, 2010**).

Quercetin (QUE)

The CASA examination revealed an immediate (Time 0h) but insignificant (P>0.05) motion-promoting effect of QUE, specifically in experimental groups exposed to 1 and 10 μmol/L of this flavonoid (Table 2). Beneficial effects of QUE remained visible although non-significant (P>0.05) at Time 2h, and covering all the concentrations applied to the experimental groups. After 4h, spermatozoa motion was significantly higher in the experimental groups supplemented with 1, 5 (P<0.05) and 100 (P<0.01) μmol/L QUE. Nevertheless, motility evalution at 6h revealed a rapid decline of rabbit sperm motion exposed to the highest QUE concentration, resulting in a significantly lower MOT in comparison with the Control (P<0.01). At the same time, sperm motility promoting properties of low QUE concentrations were confirmed, leading to a higher MOT (P<0.001 in relation to 1 μmol/L QUE; P<0.05 with respect to 5 μmol/L QUE; Table 2) when compared to the Control. At the end of the experiment (8h), a significantly higher spermatozoa MOT was observed in experimental groups supplemented with a range of 1-10 μmol/L QUE (P<0.01 with respect to 1 μmol/L QUE; P<0.001 in case of 5 and 10 μmol/L QUE). Meanwhile, the MOT assessment revealed a decreased bias in the experimental group supplemented with the highest concentration of QUE (100 μmol/L), after a comparison with the Ctrl group (P<0.001).

Table 2 Time- and dose-dependent effects of quercetin (QUE) on rabbit spermatozoa motility [%]

	Ctrl	100 μmol/L QUE	50 μmol/L QUE	10 μmol/L QUE	5 μmol/L QUE	1 μmol/L QUE
0h	80.55±2.33	76.16±6.09	79.98±5.55	80.80±9.01	77.55±3.23	81.45±2.90
2h	69.03±4.02	74.55±4.55	77.34±3.99	77.77±6.09	74.23±2.90	77.64±3.39
4h	59.12±3.33	73.22±3.99**	68.09±5.22	67.67±5.02	73.89±3.22*	72.76±4.44*
6h	37.55±2.99	16.90±1.20**	20.90±3.33	29.12±2.31	47.66±1.99*	60.77±4.44***
8h	20.04±1.88	9.09±0.35***	18.07±1.77	41.21±2.33***	42.24±2.12***	39.03±2.22**

Mean±SEM. * P<0.05; ** P<0.01; *** P<0.001

Quercetin is a common dietary flavonoid, reported to exhibit a broad variety of favorable biological effects (**Aherne and O'Brien, 2000**), hence it is not a surprise to find reports focused on assessing its impact on male reproductive performance.

Improved motility recorded after QUE administration to the rabbit sperm medium in our study disagrees with earlier reports, according to which QUE compromised human sperm motility (at 5–200 μmol/L QUE) and viability (50-100 μmol/L QUE), coinciding with Ca^{2+}-ATPase downregulation, and leading to the loss of sperm motion (**Khanduja *et al.*, 2001**). In addition, this biomolecule exhibited a significant inhibitory impact on the hyaluronidase activity and sperm penetration ability of non-capacitated, capacitated and acrosome-reacted *Cynomolgus* monkey sperm in a dose-dependent manner (**Li *et al.*, 1997**). Talking in favor of

our results, **Tvrdá *et al.* (2014; 2016b)** did suggest protective effects of QUE on bovine sperm motion activity when incubated over a period of 24 h (**Tvrdá *et al.*, 2014**) or without the presence of seminal plasma (**Tvrdá *et al.*, 2016**), although we must acknowledge that in case of a broader concentration range, QUE may act dose dependently as either a stimulant at low concentrations or as an inhibitor at high doses (**Taepongsorat *et al.*, 2008**). This controversy was validated *in vivo* when **Aravindakshan *et al.* (1985)** revealed that treatment with higher QUE doses (300 mg/kg body weight) reduced the fertility rate of male rats, while **Taepongsorat *et al.* (2008)** showed that intramuscular administration of 90 and 270 mg QUE/kg body weight/day led to significant improvements in the spermatozoa concentration, motility and viability.

Mazzi *et al.* (2011) and **Moretti *et al.* (2012)** compared the potential of quercetin and resveratrol on human sperm incubated with tert-butylhydroperoxide (TBHP). On the one hand, QUE showed a higher toxicity with respect to the sperm motility and viability than RES, and on the other hand, its antioxidant activity was stronger, as evidenced by fluorescent and electron microscopy. QUE was particularly active in preserving sperm membranes, chromatin texture and acrosomes, which were compromised by TBHP. Similarly, **Tvrdá *et al.* (2016b)** found that QUE was capable of preventing the decline of spermatozoa vitality and functional activity as a consequence of FeAA-associated oxidative damage. Quercetin concentrations ranging between 50 and 100 µmol/l were particularly effective in protecting the spermatozoon against the damage caused by free radical overgeneration through prevention of lipid peroxidation, protection and maintenance of spermatozoa metabolism as reflected by the motility and mitochondrial activity.

Curcumin (CUR)

In case of CUR, the MOT evaluation revealed that the initial (Time 0h) MOT was higher in experimental groups supplemented with 5 and 100 µmol/L CUR when compared to the Control group (0 µmol/L CUR), although without any statistical significance (P>0.05). Although statistically insignificant, a motion-promoting effect of CUR was visible after 2h, specifically in experimental groups exposed to 5 and 10 µmol/L CUR. At the same time, 50 and 100 µmol/L CUR caused a significant decrease of the spermatozoa motility (P<0.001). After 4h, the decline of spermatozoa motion was significantly decreased following the administration of 50 and 100 µmol/L CUR in comparison with the Control. Furthermore, a significantly increased sperm MOT was observed in experimental groups supplemented with 1, 5 (P<0.01) and 10 µmol/L CUR (P<0.05). Examination at 6h of *in vitro* culture showed that the spermatozoa motility was significantly increased following exposure to 1 and 5 µmol/L CUR (P<0.001) when compared to the Control. At the same time, an insignificantly higher (P>0.05) motion was recorded in the group supplemented with 10 µmol/L CUR, while a significant (P>0.001) decline was observed in the case of 50 and 100 µmol/L CUR. At the end of the experiments (8h), the highest motility was observed in the experimental group supplemented with 1 µmol/L CUR, being significantly higher in comparison with the Control (P<0.01), while MOT was significantly decreased (P<0.001) in the groups supplemented by the highest concentrations of CUR, after a comparison with the Ctrl group (P<0.001; Table 3).

Table 3 Time- and dose-dependent effects of curcumin (CUR) on rabbit spermatozoa motility [%]

	Ctrl	100 µmol/L CUR	50 µmol/L CUR	10 µmol/L CUR	5 µmol/L CUR	1 µmol/L CUR
0h	80.55±2.33	82.31±7.56	77.88±6.78	79.98±6.77	85.98±5.98	75.33±5.32
2h	69.03±4.02	0.00±0.00***	44.45±3.33***	76.77±7.07	78.86±4.44	75.65±3.33
4h	59.12±3.33	0.00±0.00***	26.24±2.11***	70.09±5.55*	74.98±6.08**	75.77±4.67**
6h	37.55±2.99	0.00±0.00***	1.09±0.09***	42.43±3.99	74.57±5.45***	57.87±5.78***
8h	20.04±1.88	0.00±0.00***	0.00±0.00***	17.56±1.90	17.90±2.02	37.09±1.11**

Mean±SEM. * P<0.05; ** P<0.01; *** P<0.001

Previous reports on the impact of CUR on male fertility are debatable. **Naz (2011)** revealed that exposure of human and murine sperm to CUR caused a concentration-dependent decrease of sperm motility, capacitation and acrosome reaction. At high CUR concentrations, a complete inhibiton of spermatozoa motility and function was observed. Studies on the impact of CUR on the spermatozoa activity have indicated its role in the energy metabolism of male reproductive cells. **Reddy and Aggarwal (1994)** suggest that CUR has the ability to inhibit protein kinase C (PKC), which is believed to have a role in modulating spermatozoa flagellar movement (**Rotem *et al.*, 1990a,b**). As such, the CUR-induced PKC inhibition may be responsible for its possible spermatozoa-immobilizing activities (**Rithaporn *et al.*, 2003**). On the other hand, our results agree with **Salahoor *et al.* (2012)** and **Tvrdá *et al.* (2016b)** suggesting stimulating and protective effects of CUR on spermatozoa motility and antioxidant status. Moreover **Salahoor *et al.* (2012)** reported that increasing doses of CUR significantly increased the sperm concentration, motility and testosterone content in rats.

The first report on the useful effects of CUR (**Bucak *et al.*, 2008**) on the motility, morphology and antioxidant activities of cooled ram spermatozoa has given rise to its use as an antioxidant additive to the freezing extender. Motility parameters recorded by our IDENT CASA technique complement previous findings by **Bucak *et al.* (2008; 2010)** demonstrating a significant improvement in the motion of cryopreservad ram spermatozoa supplemented with CUR. Interestingly, their later study focused on CUR administration to a cryopreservation extender for bovine semen led to non-significant differences in the sperm motion characteristics (**Bucak *et al.*, 2012**). Nevertheless, our results correlate with the report by **Soleimanzadeh and Saberivand (2013)** as well as **Tvrda *et al.* (2016c)** on frozen-thawed semen, where CUR addition had a positive impact on both motility and viability.

Epicatechin (EPI)

In the case of EPI, the initial MOT assessment (Time 0h) revealed a non-significantly increased sperm motion in all experimental groups supplemented with EPI (P>0.05) in comparison to the Control group (Table 4). After 2h, a significant motion-promoting effect was noted in case of 1 µmol/L and 50 (P<0.01) µmol/L EPI (P<0.001). This beneficial effect remained visible after 4h and furthermore included a broader concentration range when compared to the Control (P<0.05 with respect to 50 µmol/L EPI; P<0.01 in terms of 100 and 5 µmol/L EPI; P<0.001 in case of 1 µmol/L EPI). After 6h, the decline of MOT associated with the *in vitro* culture was slowed down significantly in all experimental groups exposed to EPI (P<0.05 with respect to 5 µmol/L EPI; P<0.001 in case of 100, 50, 10 and 1 µmol/L EPI), and this MOT-stimulating and protective effect extended throughout the end of the *in vitro* experiment (Time 8h; P<0.01 in relation to 50, 10 and 5 µmol/L EPI; P<0.001 with respect to 100 and 1 µmol/L EPI).

Table 4 Time- and dose-dependent effects of epicatechin (EPI) on rabbit spermatozoa motility [%]

	Ctrl	100 µmol/L EPI	50 µmol/L EPI	10 µmol/L EPI	5 µmol/L EPI	1 µmol/L EPI
0h	80.55±2.33	83.89±8.01	80.77±7.65	81.98±7.43	80.98±7.89	83.24±67
2h	69.03±4.02	75.67±7.07	80.08±6.78**	76.77±5.67	77.09±5.09	82.76±7.77***
4h	59.12±3.33	74.67±4.78**	73.56±8.00*	68.65±3.21	76.67±6.56**	80.98±8.00***
6h	37.55±2.99	72.22±6.65***	60.97±5.56***	58.46±4.44***	49.79±3.67*	73.98±7.44***
8h	20.04±1.88	42.77±3.33***	36.09±6.07**	38.98±3.87**	38.33±2.99**	40.48±7.09***

Mean±SEM. * P<0.05; ** P<0.01; *** P<0.001

Epicatechin is a flavonoid and antioxidant commonly found in green tea and, most of all, in cocoa (**Katz *et al.*, 2011**). Since it is known that spermatozoa are sensitive to OS, several studies have examined potential roles of *in vitro* EPI supplementation in protecting sperm from ROS overproduction. **Jamalan *et al.* (2016)** evaluated the effects of different flavonoids including EPI on the recovery of sperm motility and prevention of membrane damage from aluminum chloride, cadmium chloride, and lead chloride. The study revealed that catechin behaved in an unexpected manner as it did not protect spermatozoa from heavy metal-mediated damage, nor it did not exhibit any protective effects, rather, it showed inhibitory effects on the sperm MOT associated with a co-incubation with selected heavy metals. Following the application of increased catechin concentrations from 0 to 1000 µmol/L, a gradual and dose-dependent decrease in sperm motility compared to the untreated control group was observed. Similarly, **Moretti *et al.* (2012)** showed that none of the selected EPI concentrations selected (20, 30, 50, 100, 200, 400 µmol/L) was not effective as an antioxidant to protect swim-up selected human sperm against tert-butylhydroperoxide induced lipid peroxidation. Furthermore, the study showed that sperm progressive motility and viability were significantly reduced after incubation with EPI at 400

µmol/L. Recently, differential protective effects of epicatechin gallate against induced mitochondrial dysfunction were explored in intestinal epithelial Caco-2 cells and even in this case no protection was observed (**Carrasco-Pozo *et al.*, 2011**).

On the contrary to the above mentioned studies our results revealed stimulating effects of all EPI concentrations on the motility behavior following its supplementation to rabbit spermatozoa. Such data are in agreement with **Purdy *et al.* (2004)** who aimed to determine whether supplementing diluents with catechin, would aid in maintaining the viability of caprine sperm during cooling and storage at 5 °C. The study revealed significant differences ($P<0.05$) in motility between the control (34%) and the 25, 50, 75 and 100 µmol/L concentrations of catechin (57, 53, 55 and 64% motile cells, respectively) at 96h. These results demonstrated that catechin may aid in maintaining the motility of cooled goat sperm in a dose dependent manner.

Isoquercitrin (ISO)

According to the CASA analysis the initial MOT (Time 0h) was insignificantly increased in experimental groups subjected to treatment with 1, 5 and 50 µmol/L ISO when compared to the Control (Table 5). This non-significant but motility-promoting effect of 1, 5, 50 and 100 µmol/L ISO remained notable after 2h of *in vitro* culture. After 4h, a significantly higher MOT was recorded in the group exposed to 5 µmol/L ISO when compared to the Control ($P<0.01$). At 6h the spectrum of ISO concentrations exhibiting beneficial effects on the sperm motility expanded, as a significantly higher MOT was recorded in groups administered with 1, 5, 10 ($P<0.05$) and 50 µmol/L ISO ($P<0.001$). Nevertheless, at the end of the *in vitro* culture, a significantly higher MOT was detected in the experimental group supplemented with 10 µmol/L ISO ($P<0.05$) when compared to the Control. Interestingly, a notble however non-significant ($P>0.05$) decrase of sperm MOT was recorded following exposure to the highest ISO concentration (100 µmol/L ISO). Administration of the rest of the selected concentrations led to an insignificantly higher rabbit sperm MOT ($P>0.05$) in comparison with the Control (Table 5).

Table 5 Time- and dose-dependent effects of isoquercitrin (ISO) on rabbit spermatozoa motility [%]

	Ctrl	100 µmol/L ISO	50 µmol/L ISO	10 µmol/L ISO	5 µmol/L ISO	1 µmol/L ISO
0h	80.55±2.33	79.89±6.67	81.98±7.99	79.56±4.90	84.89±9.02	81.99±7.02
2h	69.03±4.02	76.77±4.42	76.56±6.01	67.97±5.09	77.87±7.02	76.54±4.01
4h	59.12±3.33	63.54±5.09	67.87±5.55	66.67±6.09	76.87±6.98**	67.22±5.98
6h	37.55±2.99	44.87±3.99	48.35±4.78*	73.78±8.00***	73.77±4.78***	65.56±3.33***
8h	20.04±1.88	11.09±1.89	20.09±1.98	30.99±4.09*	21.34±2.06	17.97±2.11

Mean±SEM. * P<0.05; ** P<0.01; *** P<0.001

Isoquercitrin is found in foods such as apple and onion, as well as in a variety of medicinal plants, likely contributing to the pharmacological qualities of a large number of botanical medicines. Some of the most commonly used plants containing these flavonoids include Horse chestnut, Ginkgo, Horsetail, Fennel, Rooibos, Hops, Sundew, Bilberry and Mulberry (**Dok-Go *et al.*, 2003; Appleton, 2010**). ISO is one of the naturally occurring glucosides of quercetin. Isoquercitrin is also sometimes called isoquercetin, a nearly identical quercetin-3-monoglucoside. Technically the two are different (isoquercetin has a pyranose ring whereas isoquercitrin has a furanose ring), but functionally the two molecules are indistinguishable. The literature often considers them as one and uses the names interchangeably (**Appleton, 2010**). To our knowledge, no study is currently available on the impact of isoquerctrin on male reproduction. According to our data, although being structurally similar to QUE, the molecule did not exhibit significant negative effects on the sperm MOT when compared to QUE. On the other hand, potential beneficial effects of lower concentrations of ISO were less notable and significant in comparison with QUE. Although experiments based on the administration of pure ISO have not been done yet, numerous animal studies emphasize on potential ameliorative and antioxidant effect of plant extracts containing ISO on the testicular structure and function, as well as sperm concentration, motility and morphology in sickness and health (**Awoniyi *et al.*, 2011; Ayeleso *et al.*, 2014; Ďuračka *et al.*, 2016**). As such, we may suggest that more specific experiments on the roles of ISO are to be done in the future in order to elucidate its beneficial and/or harmful roles in male reproduction.

CONCLUSION

A relatively broad spectrum of positive as well as negative effects has been linked to the treatment of individual biologically active compounds in experimental in vitro and in vivo models. Their mechanisms of action have only recently started to be uncovered, particularly in relation to male reproduction. Our study has unraveled a specific behavior of each biomolecule, providing evidence on their toxic and beneficial concentrations as well as exposure periods adequate for the exhibition of their protective or harmful impact on rabbit sperm survival.

Identification of a normal physiological range of biologically active compounds and their metabolites in mammalian tissues is of utmost importance if researchers aim to determine if the effects observed with respect to a certain dose are physiologically relevant. Furthermore, determining the clinical relevance of results obtained from animal or in vitro studies may be difficult as these studies often use doses which may exceed physiological concentrations. As such, these aspects must be taken into account in the design of future experimental studies, irrespective of whether they are aimed at evaluating beneficial or adverse effects of natural biomolecules.

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REFERENCES

Agarwal, A. & Sekhon, L. (2010). The role of antioxidant therapy in the treatment of male infertility. *Human Fertility*, 13(4), 217–225. http://dx.doi.org/10.3109/14647273.2010.532279.

Agarwal, A., Tvrda, E. & Sharma, R. (2014). Relationship amongst teratozoospermia, seminal oxidative stress and male infertility. *Reproductive Biology and Endocrinology*, 12, 45. http://dx.doi.org/10.1186/1477-7827-12-45.

Aherne, S.A. & O'Brien, N.M. (2000). Mechanism of protection by the flavonoids, quercetin and rutin, against tert-butylhydroperoxide- and menadione-induced DNA single strand breaks in Caco-2 cells. *Free Radicals in Biology and Medicine*, 29, 507-514. http://dx.doi.org/10.1016/S0891-5849(00)00360-9.

Aitken, R. J., Irvine, D. S. & Wu, F. C. (1991). Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *American Journal of Obstetrics and Gynecology*, 164, 542-551. https://doi.org/10.1016/S0002-9378(11)80017-7.

Alarcón de la Rastra, C. (2008). Curcumin: a promising spice for therapeutics. *Molecular Nutrition & Food Research*, 52, 985. http://dx.doi.org/10.1002/mnfr.200890036.

Appleton, J. (2010). Evaluating the bioavailability of isoquercetin. *Natural Medicine Journal*, 2(1), 1-4.

Aravindakshan, M., Chauhan, P.S. & Sundaram, K. (1985). Studies on germinal effects of quercetin, a naturally occurring flavonoid. *Mutation Research*, 144, 99-106.

Awoniyi, D.O., Aboua, Y.G., Marnewick, J.L., du Plesis, S.S. & Brooks, N.L. (2011). Protective effects of rooibos (*Aspalathus linearis*), green tea (*Camellia sinensis*) and commercial supplements on testicular tissue of oxidative stress induced rats. African Journal of Biotechnology, 10(75), 17317-17322. http://dx.doi.org/10.5897/AJB11.2210.

Ayeleso, A.O., Oguntibeju, O.O., Aboua, Y.G. & Brooks, N.L. (2014). Effects of red palm oil and rooibos on sperm motility parameters in streptozotocin-induced diabetic rats. *African Journal of Traditional, Complementary, and Alternative Medicines*, 11(5), 8-15.

Brown, L., Kroon, P.A., Das, D.K., Das, S., Tosaki, A. & Chan, V. (2009). The biological responses to resveratrol and other polyphenols from alcoholic beverages. *Alcoholism: Clinical & Experimental Research*, 33, 1513– 23. http://dx.doi.org/10.1158/0008-5472.

Bucak, M.N., Atessahin, A. & Yuce, A. (2008). Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze-thawing process. *Small Ruminant Research*, 75, 128–134. http://dx.doi.org/10.1016/j.cryobiol.2010.09.001.

Bucak, M.N., Sariozkan, S., Tuncer, P.B., Sakin, F., Atessahin, A., Kulaksız, R. & Cevik, M. (2010). The effect of antioxidants on post-thawed Angora goat (*Capra hircus ancyrensis*) sperm parameters, lipid peroxidation and antioxidant

activities. *Small Ruminant Research*, 89, 24–30. http://dx.doi.org/10.1016/j.smallrumres.2009.11.015.
Bucak, M.N., Basspinar, N., Tuncer, B.P., Coyan, K., Sariozkan, S., Akalin, P.P. & Buyukleblebici, S., Kucukgunay, S. (2012). Effects of curcumin and dithioerythritol on frozen-thawed bovine semen. *Andrologia*, 44, 102-109. http://dx.doi.org/10.1111/j.1439-0272.2010.01146.x.
Buettner, G. R. (1993). The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol and ascorbate. *Archives of Biochemistry and Biophysics*, 300, 535-543.
Calabrese, E.J., Mattson, M.P. & Calabrese, V. (2010). Dose response biology: the case of resveratrol. *Human Experimental Toxicology*, 29(12), 1034-1037. http://dx.doi.org/10.1177/0960327110383625.
Carrasco-Pozo, C., Mizgier, M.L., Speisky, H. & Gotteland, M. (2011). Differential protective effects of quercetin, resveratrol, rutin and epigallocatechin gallate against mitochondrial dysfunction induced by indomethacin in Caco-2 cells. *Chemico-Biological Interactions*, 195, 199-205. http://dx.doi.org/10.1016/j.cbi.2011.12.007.
Collodel, G., Federico, M.G., Geminiani, M., Martini, S., Bonechi, C., Rossi, C., Figura, N. & Moretti, E. (2010). Effect of trans-resveratrol on induced oxidative stress in human sperm and in rat germinal cells. *Reproductive Toxicology*, 31(2), 239-246. http://dx.doi.org/10.1016/j.reprotox.2010.11.010.
Cottart, CH. H., Antoine, V. N., Laguillier-Morizot, CH. & Beaudeux, J. L. (2010). Resveratrol bioavailability and toxicity in human. *Molecular Nutrition & Food Research*, 54:7-16. http://dx.doi.org/ 10.1002/mnfr.200900437.
de Lamirande, E. & Gagnon, C. (1993). Human sperm hyperactivation and capacitation as parts of an oxidative process. *Free Radicals in Biology and Medicine*, 14, 157-166.
Dok-Go, H., Lee, K.H. & Kim, H.J. (2003). Neuroprotective effects of antioxidative flavonoids, quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether, isolated from *Opuntia ficus indica* var. saboten. *Brain Research*, 965(1-2), 130-136.
Donnelly, E.T., McClure, N. & Lewis, S.E.M. (1999). The effect of ascorbate and α-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis*, 14(5), 505-512. http://dx.doi.org/10.1093/mutage/14.5.505.
Ďuračka, M., Michalko, J., Matušíková, I. & Tvrdá, E. (2016). Antioxidant effects of schizandra and bilberry extracts on male gametes. In *Zborník referátov z vedeckej konferencie: „Dendrologické dni v Arboréte Mlyňany SAV 2016", 05.10.2016*. Vieska nad Žitavou: Arborétum Mlyňany SAV, p. 41-46. ISBN 978-80-89408-26-9.
Garcez, M.E., dos Santos Branco, C., Lara, L.V., Pasqualotto, F.F. & Salvador, M. (2010). Effects of resveratrol supplementation on cryopreservation medium of human semen. *Fertility and Sterility*, 94, 2118-2121. http://dx.doi.org/ 10.1016/j.fertnstert.2010.01.058.
Jamalan, M., Ghaffari, M.A., Hoseinzadeh, P., Hashemitabar, M. & Zeinali, M. (2016). Human sperm quality and metal toxicants: protective effects of some flavonoids on male reproductive function. *International Journal of Fertility and Sterility*, 10(2), 215-223.
Katz, D.L., Doughty, K. & Ali, A. (2011). Cocoa and chocolate in human health and disease. *Antioxidants and Redox Signalling*, 15, 2779-2811. http://dx.doi.org/10.1089/ars.2010.3697.
Khanduja, K.L., Verma, A. & Bhardwaj, A. (2001). Impairment of human sperm motility and viability by quercetin is independent of lipid peroxidation. *Andrologia*, 33, 277-281.
Li, M.W., Yudin, A.I., VandeVoort, C.A., Sabeur, K., Primako , P. & Overstreet, J. W. (1997). Inhibition of monkey sperm hyaluronidase activity and heterologous cumulus penetration by flavonoids. *Biology of Reproduction*, 56, 1383-1389.
Mazzi, L., Geminiani, M., Collodel, G., Iacoponi, G., Martini, S., Boenchi, C., Rossi, C. & Moretti, E. (2011). Quercetin and rutin: effects of two flavonoids on induced oxidative stress in human ejaculated sperm. *Journal of the Sienna Academy of Science*, 3, 22-26.
Mojica-Villegas, M.A., Izquierdo-Vega, J.A., Chamorro Cevallos, G. & Sanchez-Guiterrez, M. (2014). Protective effect of resveratrol on biomarkers of oxidative stress induced by iron/ascorbate in mouse spermatozoa. *Nutrients*, 6, 489-503. http://dx.doi.org/10.3390/nu6020489.
Moretti, E., Mazzi, L., Terzuoli, G., Bonechi, C., Iacoponi, F., Martini, S., Rossi, C. & Collodel, G. (2012). Effect of quercetin, rutin, naringenin and epicatechin on lipid peroxidation induced in human sperm. *Reproductive Toxicology*, 34, 651-657. http://dx.doi.org/10.1016/j.reprotox.2012.10.002.
Naz, R.J. (2011). Can curcumin provide an ideal contraceptive? *Molecular Reproduction and Development*, 78, 116-123. http://dx.doi.org/10.1002/mrd.21276.
Pasqualotto, F.F., Garcez, M., Borges, E., Gupta, S., Salvador, M. & Agarwal, A. (2006). Lipid peroxidation, superoxide dismutase, cata- lase levels and sperm motility: protective role of vitamin C and resveratrol prior to sperm cryopreservation. *Fertility and Sterility*, 86,
S446.
Purdy, P.H., Ericsson, S.A., Dodson, R.E., Sternes, K.L. & Garner, D.L. (2004). Effects of the flavonoids, silibinin and catechin, on the motility of extended cooled caprine sperm. Small Ruminant Research, 55(1-3), 239-243. http://dx.doi.org/10.1016/j.smallrumres.2004.02.005.
Reddy, S. & Aggarwal, B.B. (1994). Curcumin is a non-competitive and selective inhibitor of phosphorylase kinase. *FEBS Letters*, 341 (1), 19-22.
Rithaporn, T., Monga, M. & Rajasekaran, M. (2003). Curcumin: a potential vaginal contraceptive. *Contraception*, 68, 219-223.
Rotem, R., Paz, G.F., Homonnai, Z.T., Kalina, M. & Naor, Z. (1990a). Protein kinase C is present in human sperm: possible role in flagellar motility. *Proceedings of the National Academy of Sciences*, 87, 7305-7308.
Rotem, R., Paz, G.F., Homonnai, Z.T., Kalina, M. & Naor, Z. (1990b). Further studies on the involvement of protein kinase C in human sperm flagellar motility. *Endocrinology*, 127, 2571-2577.
Salahshoor, M.R., Jalili, C., Khazaei, M. & Khani, F. (2012). Effects of curcumin on reproductive parameters in male mice. *Journal of Clinical Research in Paramedical Sciences*, 1(3), 1-3.
Saleh, R.A. & Agarwal, A. (2002). Oxidative stress and male infertility: from research bench to clinical practice. *Journal of Andrology*, 23(6), 737-752.
Sarlos, P., Molnar, A., Kokai, M., Gabor, G. & Ratky, J. (2008). Comparative evaluation of the effect of antioxidants in the conservation of ram semen. *Acta Veterinaria Hungarica*, 50, 235–245.
Silva, E.C., Cajueiro, J.F., Silva, S.V., Soares, P.C. & Guerra, M.M. (2012). Effect of antioxidants resveratrol and quercetin on in vitro evaluation of frozen ram sperm. *Theriogenology*, 77, 1722-1726. http://dx.doi.org/10.1016/j.theriogenology.2011.11.023.
Soleimanzadeh, A. & Saberivand, A. (2013). Effect of curcumin on rat sperm morphology after the freeze-thawing process. *Veterinary Research Forum*, 4(3), 185-189. http://dx.doi.org/10.1016/S0093-691X(02)01231-1.
Taepongsorat, L., Tangpraprutgul, P., Kitana, N. & Malaivijitnond, S. (2008). Stimulating effects of quercetin on sperm quality and reproductive organs in adult male rats. *Asian Journal of Andrology*, 10, 249-258.
Tvrdá, E., Halenár, M., Greifová, H., Mackovich, A., Hashim, F. & Lukáč, N. (2016a). The effect of curcumin on cryopreserved bovine semen. *Intrenational Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering*, 10(11), 669-673.
Tvrdá, E., Kňažická, Z., Bárdos, L., Massányi, P. & Lukáč N. (2011). Impact of oxidative stress on male fertility - a review. *Acta Veterinaria Hungarica*, 59, 465-484. https://doi.org/10.1556/AVet.2011.034.
Tvrdá, E., Kováčik, A., Tušimová, E., Massányi, P. & Lukáč, N. (2015a). Resveratrol offers protection to oxidative stress induced by ferrous ascorbate in bovine spermatozoa. *Journal of Environmental Science and Health Part A*, 50, 1440-1451. http://dx.doi.org/10.1080/10934529.2015.1071153.
Tvrdá, E., Lukáč, N., Lukáčová, J., Hashim, F. & Massányi, P. (2015b). In vitro supplementation of resveratrol to bovine spermatozoa: effects on motility, viability and superoxide production. *Journal of Microbiology, Biotechnology and Food Sciences*, 4, 336–341. http://dx.doi.org/10.15414/jmbfs.2015.4.4.336-341.
Tvrdá, E., Lukáč, N., Lukáčová, J., Jambor, T., Hashim, F. & Massányi, P. (2014). Dose-and time-dependent in vitro effects of quercetin on bovine spermatozoa activity and superoxide production. *Folia Veterinaria*, 58, 224-231.
Tvrdá, E., Tušimová, E., Kováčik, A., Paál, D., Greifová, H., Abdramanov, A. & Lukáč, N. (2016b). Curcumin has protective and antioxidant properties on bull spermatozoa subjected to induced oxidative stress. *Animal Reproduction Science*, 172, 10-20. http://dx.doi.org/10.1016/j.anireprosci.2016.06.008.
Tvrdá, E., Tušimová, E., Kováčik, A., Paál, D., Libová, Ľ. & Lukáč, N. (2016c). Protective effects of quercetin on selected oxidative biomarkers in bovine spermatozoa subjected to ferrous ascorbate. *Reproduction in Domestic Animals*, 51(4), 524-537. http://dx.doi.org/10.1111/rda.12714.

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IMMOBILIZTION OF ORGANOPHOSPHORUS HYDROLASE ENZYME ON FERRIC MAGNETIC NANOPARTICLES AND INVESTIGATION OF IMMOBILIZED ENZYME STABILITY

Seyed Mortaza Robatjazi, Mohammadmostafa Reihani, Sanaze Mahboudi, Seyed mohammad Hasanpour, Mohammad Ali Nasiri Khalili

Address(es): Seyed Mortaza Robatjazi,
Department of Bioscience and Biotechnology, Malek Ashtar University of Technology, Tehran, Iran, Tel/Fax: +9822974605.

*Corresponding author: mailto:s_m_robatjazi@mut.ac.ir

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ABSTRACT

In the present study, organophosphorus hydrolase enzyme on Functionalized ferric magnetic nanoparticles was immobilized by the covalent binding method. The Optimized amount for parameters of mg $_{EDAC}$/mg $_{nanoparticles}$, enzyme units (U)/mg $_{nanoparticles}$, reaction time, and pH were determined to be 6.125, 0.1341, 3 h and 6.15 respectively. The amount of immobilization yield according to the enzyme activity was obtained to be 70% and also the amount of immobilized enzyme on nanoparticles was 0.25 U/mg $_{nanoparticles}$. Stability studies showed significant increase in immobilized enzyme stability at 4, 25 and 45°C. The stability of Immobilized enzyme showed a 6.3-fold increase in comparison to free enzyme at 4°C. The results demonstrated that the pH stability of the immobilized enzyme significantly increased in comparison with free enzyme. The immobilized enzyme was usable and recoverable for seven cycles. The results depicted that 80% of enzyme activity was retained after fifth cycle. FTIR test showed the covalent binding of enzyme to magnetic nanoparticles' surface and the modified enzyme magnetic nanoparticles property was superparamagnetic by vibrating sample magnetometer test.

Keywords: Magnetic nanoparticles, immobilization, organophosphorus hydrolase, stability

INTRODUCTION

Organophosphorus compounds with respect to their widespread use in agriculture, expose food resources, water and even air to the pollution. Organophosphate compounds such as insecticide parathion (*o,o*-diethyl-*o*-4-nitrophenyl phosphorothioate), methyl parathion and diazinon, in spite of their extreme toxicity have been used in production of plant pesticides. Compared with the potential disadvantage of conventional methods, bioremediation would appear to be more attractive because it is far less disruptive and more cost-effective (**Lei *et al.*, 2005**). Some reports have shown that organophosphorus compounds are degraded by some bacteria. *Pseudomonas diminuta* MG (**Serda *et al.*, 1985**) and a *Flavobacterium* sp. (ATCC 27551) (**Sethunathan *et al.*, 1973**) have the ability to degrade a broad spectrum of organophosphorus triesters by virtue of a constitutively expressed organophosphorus hydrolase (**Harper *et al.*, 1988**). Organophosphorus hydrolase enzyme called OPH (E.C 3.1.8.1) is able to hydrolyze the ester bond of organophosphate compounds such as parathion and paraoxon but due to the instability of this enzyme in free mode bioremediation process or its application in hydrolysis or materials detection has faced many problems. The enzyme activity is dependent on the environmental variables, so the OPH enzyme after 5 hours incubation at room temperature in the aquatic environment loses about 50% of its activity (**Obare *et al.*, 2010; Robatjazi *et al.*, 2010**). Biocatalysts immobilization processes are economically efficient and lead to development of the biological continuous processes. The unique physical properties of nanoparticles allow their application in many fields such as biomedicine (**Atanasijevic *et al.*, 2006: Gupta *et al.*, 2005; Ito *et al.*, 2005**), sensor development (**Katz et al., 2004**), water purification (**Savage *et al.*, 2005**) and environmental remediation (**Liu, 2006; Tratnyek *et al.*, 2006; Zhang, 2003**). Superparamagnetism of magnetic nanoparticles (MNPs) is a size-dependent property that is useful for applications requiring manipulation of MNPs by an external magnetic field. Such particles do not retain any residual magnetism once the magnetic field is removed (**Gupta *et al.*, 2005; Ito *et al.*, 2005**). Using magnetic nanoparticles in biological processes and protein immobilization is considered one of the novel methods of immobilization. MNPs have found many applications in various processes due to their high specific surface area ratio, magnetic properties and special features (**Wang *et al.*, 2012**). The large surface- area-to-volume ratio of a nanoparticle allows it to serve as an efficient carrier of biomolecules. This feature has resulted in the development of many biomolecule-nanoparticle (bio-NP) hybrids for biomedical applications in the diagnosis and localized treatment of disease (**Atanasijevic *et al.*, 2006: Gupta *et al.*, 2005; Ito *et al.*, 2005; Harris *et al.*, 2006**). MNP-enzyme conjugates (MNP-Es) represent a specific class of bio-NP conjugates that are of particular interest for biotechnological applications where high catalytic specificity, prolonged reaction time, and in some cases the ability to recycle an expensive biocatalyst is required (**Alcalde *et al.*, 2006; Swanson, 1999**). The covalent bonding and ionic bonding are two methods of MNP binding to biomolecules. Covalent binding is a method that is widely used in the biopolymers immobilization. Covalent binding is usually carried out by direct biomolecule reactive groups binding or by binding to an intermediate. The purpose of this study is to immobilize OPH enzyme on MNP covalently and immobilization optimization and evaluation of storage stability of immobilized OPH enzyme.

MATERIAL AND METHODS

Bacterial strain

Flavobacterium ATCC 27551 was obtained from Microbial Type Culture Collection (MTCC, Chandigarh, India). OPH enzyme was then isolated and purified from bacteria (**Brown, 1980**).

Magnetic nanoparticles and chemicals

Utilized magnetic nanoparticles with dextran surface cover and NH_2 agent with average size of 130nm (amino-modified magnetic nanoparticles (AMN)), *N*-(3-dimethylaminopropyl)- *N*–ethyl carbodiimide (EDAC) and 2-N-morpholino-ethanesulfonic acid (MES) buffer and parathion were purchased from Micromode, Merck and Sigma Companies respectively. All of the experimental materials in this study were of high purity and were purchased from Fluka and Merck Companies.

Immobilization of OPH enzyme on AMNs

In order to Immobilize OPH enzyme on AMNs surfaces, 20 µl of AMNs was transferred to a 2ml microtube. Ferric nanoparticles were then washed three times

with MES buffer solution and suspended in100 µl of MES buffer solution. After shaking for one minute by putting permanent magnet under the micro tube for 1-2 minute(s) AMNs were separated from the solution. Then, a 100 µl MES solution containing 0.7mg EDAC was added to the nanoparticles that had been suspended in the 100 µl MES solution and mixed with shaker for 10 minutes. A 10 µl stock enzyme solution with 2.2 U/ml activity was added to microtube containing active nanoparticles, then the microtube was incubated for 3 h at 4°C with 250 rpm. After the end of incubation, nanoparticles were washed three times with a 300 µl PBS buffer solution and suspended with a 300 µl PBS buffer solution in pH=8.

Optimization process

The optimum conditions for the covalent binding of OPH enzyme to AMNs surface were obtained using Taguchi experiment design method and Qualitek-4 software. This method allows observation and optimization of variables simultaneously. In the present study, a standard Taguchi L9 orthogonal array due to its ability to operate 4 parameters in 3 levels was chosen. Parameters and their levels for the covalent binding of OPH enzyme to AMNs are shown in Tab 1.

Table 1 Factors and level of parameters used in Taguchi experimental design.

Factor	Level 1	Level 2	Level 3
EDAC/AMN ratio(w/w)	2.45	6.126	15.313
Enzyme/AMN ratio(U/mg)	0.0894	0.1341	0.1788
Time (h)	1.5	3	4.5
pH	6	6.15	6.30

Evaluation of Immobilized enzyme stability

Free and immobilized enzyme stability was determined by using relative activity calculation. The immobilized enzyme and the samples containing free enzyme as the observer were incubated at the same condition of 4, 25, 45°C and enzyme activity in different periods was determined intermittently. The acidity stability of both immobilized enzyme and free enzyme was determined at the same condition at pH range of 4-11. The samples were incubated at 4°C for 3 h. In order to determine activity of samples, first samples were incubated at 25°C and pH=8 for 30 minutes then their activities were determined.

Analytical methods

The phosphotriesterase activity was measured on the basis of Spectrophotometric Assay method. The rate of ethyl-parathion hydrolysis was determined by measuring para-nitrophenol production (**Robatjazi *et al.*, 2010**). For this purpose, 150 µl of solution containing immobilized enzyme and 150 µl of PBS buffer at pH=8 were mixed. Then 10 µl of 50 mM $CoCl_2$ solution was added and the mixture was shaken for 30 minutes at room temperature. Next, 5 µl of 40 mM ethyl-parathion was added and p-nitrophenol production rate was determined by measuring the increase of absorbance rate at 410 nm. One unit of phosphotriesterase activity (U) was defined as the amount of enzyme required to hydrolyze 1 µmol of ethyl-parathion per minute at 30°C (as described previously in detail by **Robatjazi *et al.* (2010)**).

VSM and FTIR

Immobilized and free enzyme Sample spectrum was determined using FTIR spectroscopy. OPH enzyme was immobilized on AMNs by the determined optimized condition. Then, modified magnetic nanoparticles (enzyme-AMNs) and AMNs were freeze-dried for 24 hours at -60°C (model Alpha 2-4 LSC; Martin Christ, Osterode, Germany). The Samples spectrums were measured from 450 cm^{-1} to 4000 cm^{-1}. In order to perform the vibrating sample magnetometer (VSM) test, modified AMNs were washed twice with distilled water and then dried using freeze-drier on aluminum plates for 48 hours. VSM test measurement was carried out on magnetometer device (VSM, Kashan University, Iran) by changing the magnetic field from +8000 to -8000.

RESULTS AND DISCUSSION

Optimization experiment results are shown in Tab 2 on the basis of enzyme specific activity in mg of nanoparticles. Each parameter's effect and its level effect is shown in figure 1, according to the obtained results it can be deduced that pH parameters, enzyme(U)/mg_{AMN} ratio, mg_{EDAC}/mg_{AMN} and incubation time for enzyme covalent binding on the surface of AMNs were levels of 2, 2, 2, 2 respectively.

Results showed that 3 hours incubation time had the most positive effect on enzyme-AMN reaction. The enzyme to magnetic nanoparticle ratio and mg_{EDAC}/mg_{AMN} and pH for enzyme covalent binding on AMNs surface had 15, 45 and 65 percent effectiveness on immobilization. Immobilization process was carried out in optimized condition. Optimized condition for covalent binding by amino groups for incubation time, U/mg_{MNP} ratio, mg $_{EDAC}$/mg_{MNP} ratio, and pH, was determined 3 h, 0.1341, 6.125 U/mg, and 6.5 respectively.

Table 2 The results of immobilized enzyme activity in L_9 orthogonal array of the Taguchi experimental design.

Trial	Factors	Results
1	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=2.45,U_{enzyme}/mg_{AMN} ratio(U/w)=0.0894	0.038
2	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=2.45, U_{enzyme}/mg_{AMN} ratio(U/w)=0.1341	0.076
3	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=2.45, U_{enzyme}/mg_{AMN} ratio(U/w)=0.1788	0.032
4	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=6.126, U_{enzyme}/mg_{AMN} ratio(U/w)=0.0894	0.061
5	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=6.126, U_{enzyme}/mg_{AMN} ratio(U/w)=0.1341	0.043
6	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=6.126, U_{enzyme}/mg_{AMN} ratio(U/w)=0.1788	0.089
7	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=15.313, U_{enzyme}/mg_{AMN} ratio(U/w)=0.0894	0.027
8	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=15.313, U_{enzyme}/mg_{AMN} ratio(U/w)=0.1341	0.078
9	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=15.313, U_{enzyme}/mg_{AMN} ratio(U/w)=0.1788	0.048

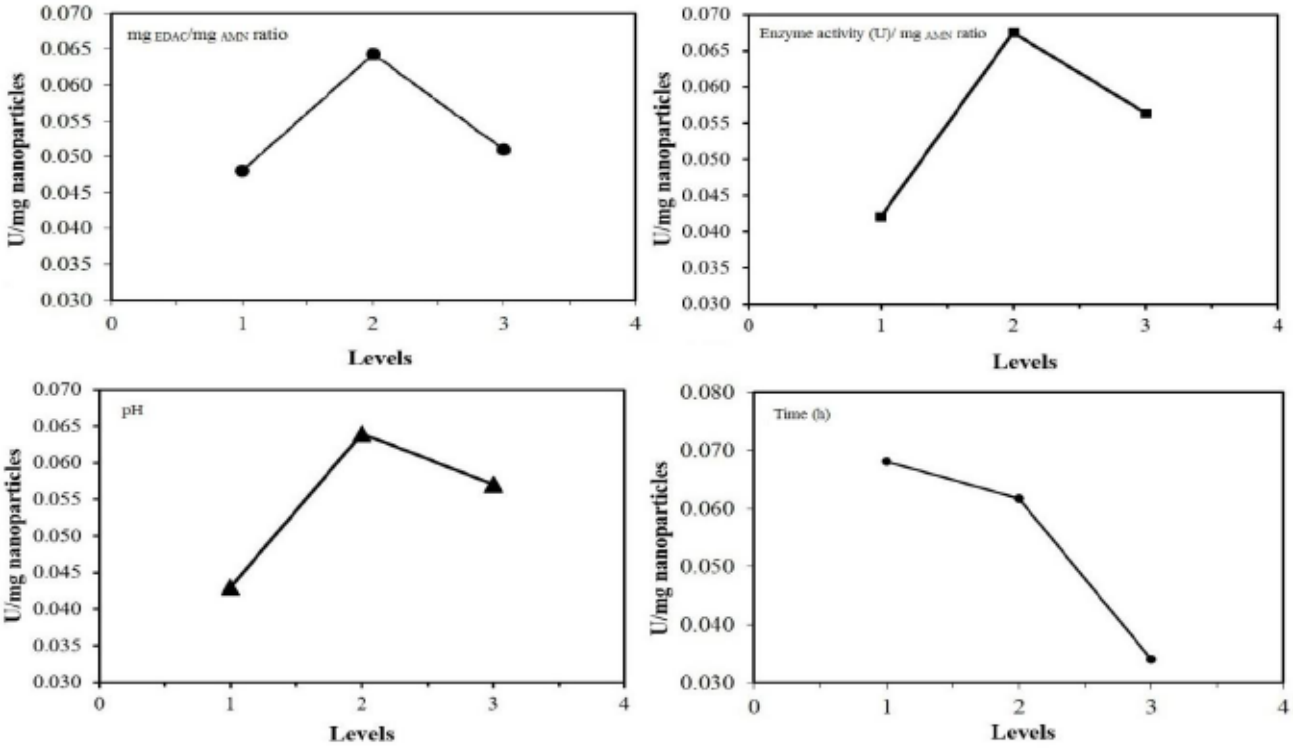

Figure 1 Average response levels for different parameters

Experiments were performed several times using the optimized conditions, and the results were in good agreement with those predicted from Taguchi experiment design. Results showed in optimized condition that amount of immobilized enzyme on nanoparticles was 0.25 U/$mg_{nanoparticles}$ with about 70% efficiency. The obtained results were in good agreement with studies performed by **Jiang *et al.* (2008)** . In a similar study by **Kuo *et al.* (2012)** immobilization efficiency was reported 58.3% for Lipase enzyme immobilization, Highest obtained activity was reported 20 unit per gram and in similar studies by **Sahoo *et al.* (2011)** , urease enzyme immobilization was reported 57% on ferric magnetic nanoparticles.

Figure 2 shows the pH stability of immobilized enzyme. The pH stability increased for the immobilized enzyme compared with the free enzyme. As the pH shifted towards the alkaline or acidic conditions, the enzyme activity of the immobilized enzyme remained higher compared with the free enzyme. This may be due to the protection of the enzyme by MNPs against extreme pH values. The remaining enzyme activity of the immobilized enzyme and the free enzyme after 8 h of incubation at pH 4 were 78% and 20% respectively.

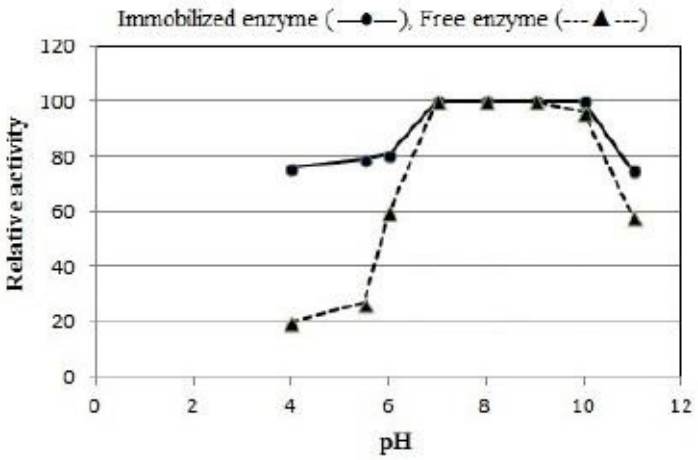

Figure 2 pH stability of immobilized enzyme

Stability studies showed significant increase in immobilized enzyme stability at 4, 25 and 45°C. The results are shown in the figure 3.The results show after 120 h of incubation at 4 °C that the relative activity has been 91% and 43% for the immobilized enzyme and free enzyme, respectively (figure 3-a). Immobilized enzyme stability was determined 6.3-fold increase in comparison to free enzyme at 4°C. A 50% reduction in the initial enzyme activity of the immobilized enzyme determined after 135 and 62 h of incubation at 25 and 45 °C, respectively, (figure 3-b, c). The results demonstrated that coupling of OPH to AMNs enhanced the enzyme thermal stability at 4, 25 and 45 °C. The Immobilized enzyme saved 87.7% of its activity for more than 10 days at 4 °C.

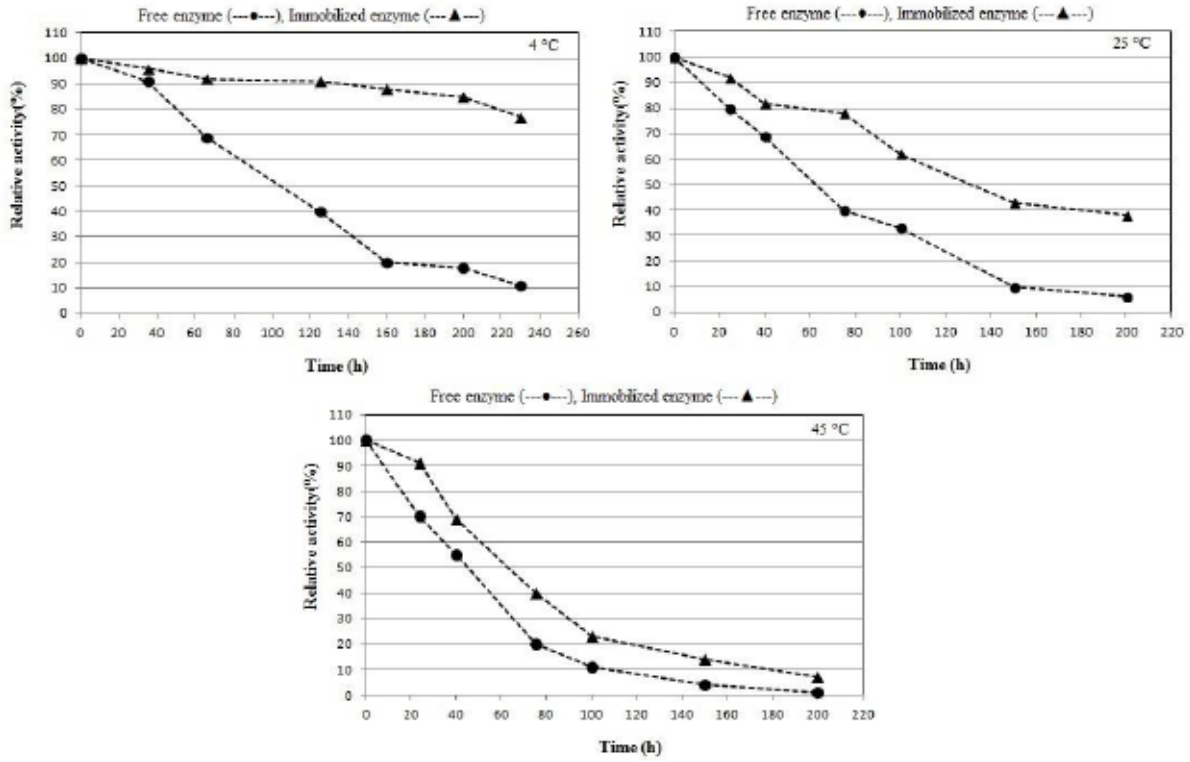

Figure 3 Enzyme thermal stability of immobilized enzyme

The immobilized enzyme was usable and recoverable for about seven cycles. The activity of immobilized enzyme was assayed up to seven cycles. The investigation depicted that 96% enzyme activity was retained after third cycle and 80% after fifth cycle. According these results, the relative enzyme activity was decreased 23% after seven cycles (figure 4).

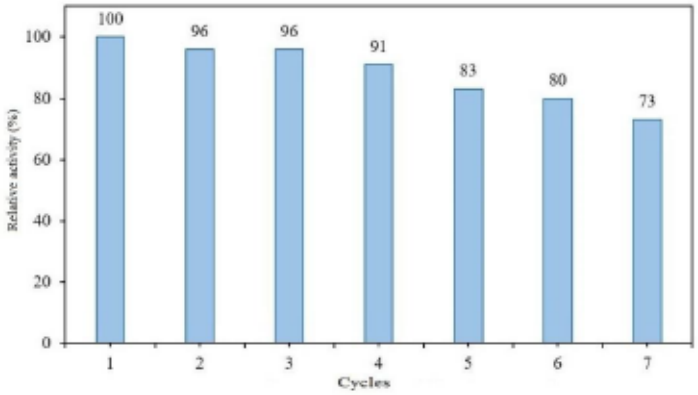

Figure 4 Relative activity of immobilized enzyme after each cycle of use

FTIR test results confirmed the covalent bonding between enzyme and magnetic nanoparticles agent groups (figure 5). On this basis, bonds are visible at 571 cm^{-1} and 1635 cm^{-1} which proves the enzyme bonding to nanoparticles such as reported by **Teste *et al.* (2010)** previously. VSM test results showed that the enzyme coated magnetic nanoparticles retain their superparamagnetic nature during the immobilization process and only about 10% of the magnetic property is decreased (figure 6).

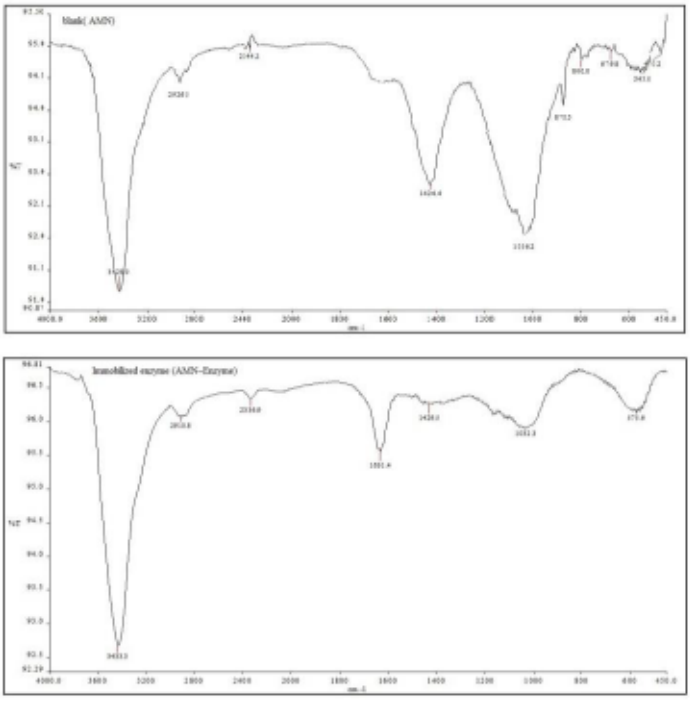

Figure 5 FTIR spectroscopy spectrum of Immobilized enzyme, a: blank (AMN), b: Immobilized enzyme (AMN–Enzyme)

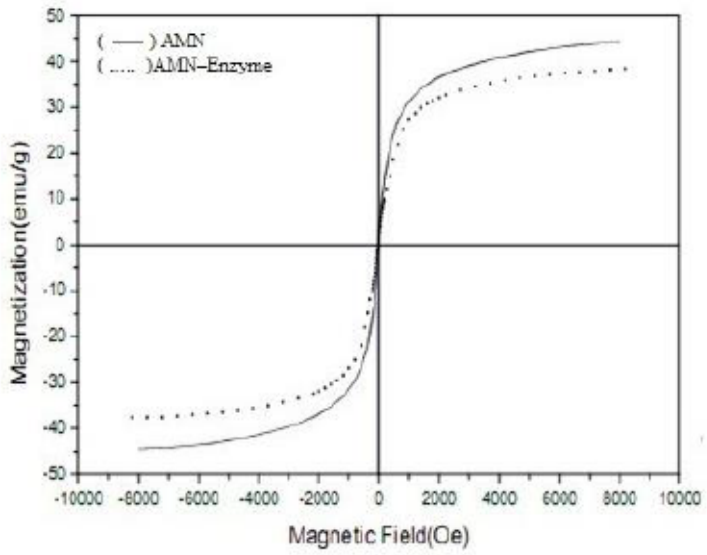

Figure 6 Magnetization curve of AMN–Enzyme

CONCLUSION

In the present study, OPH have been linked to the magnetic nanoparticles's surface by the covalent coupling method. Best conditions were determined for enzyme binding to the magnetic nanoparticle's surface. The thermal stability of immobilized enzyme on the magnetic nanoparticle's surface showed 6.3-fold, 1.4-fold and 1.1-fold increase in comparison to free enzyme after 120 h of incubation at 4, 25 and 45°C respectively. Immobilized enzyme on magnetic nanoparticle's surface can simply be separated with a magnet which leads to reuse and sequential use of enzymes. The enzyme-magnetic nanoparticles can be utilized in applications such as biodegradation, biosensing and they are also appropriate for development of nanoscale smart technologies.

REFRENCES

Alcalde, M., Ferrer, M., Plou, F.J., & Ballesteros, A. (2006). Environmental biocatalysis: from remediation with enzymes to novel green processes. *Trends Biotechnology*, 24, 281-287. http://dx.doi.org/10.1016/j.tibtech.2006.04.002

Atanasijevic, T., Shusteff, M., Fam, P., & Jasanoff, A. (2006). Calcium-sensitive MRI contrast agents based on superparamagnetic iron oxide nanoparticles and calmodulin. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 14707-14712. http://dx.doi.org/10.1073/pnas.0606749103

Brown, K.A., (1980). Phosphotriesterases of *Flavobacterium sp*. Soil Biology and Biochemistry;12:105-12. http://dx.doi.org/10.1016/0038-0717(80)90044-9

Gupta, A.K., & Gupta, M. (2005). Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. *Biomaterials*, 26, 3995-4021. http://dx.doi.org/10.1016/j.biomaterials.2004.10.012

Harper, L.L., McDaniel, C.S., Miller, C.E., & Wild, J.R. (1988). Dissimilar plasmids isolated from *Pseudomonas diminuta MG* and a *Flavobacterium* sp. (ATCC 27551) contain identical opd genes. *Applied and environmental microbiology*, 54, 2586-2589. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC204325

Harris, T.J., von Maltzahn, G., Derfus, A.M., Ruoslahti, E., & Bhatia, S.N. (2006). Proteolytic Actuation of Nanoparticle Self-Assembly. *Angewandte Chemie International Edition*, 45, 3161-3165. http://dx.doi.org/10.1002/anie.200600259

Ito, A., Shinkai, M., Honda, H., & Kobayashi, T. (2005). Medical application of functionalized magnetic nanoparticles. *Journal of Bioscience and Bioengineering*, 100, 1-11. http://dx.doi.org/10.1263/jbb.100.1

Jiang, Y., Guo, C., Xia, H., Mahmood, I., Liu, C., & Liu, H. (2009). Magnetic nanoparticles supported ionic liquids for lipase immobilization: Enzyme activity in catalyzing esterification. *Journal of Molecular Catalysis B: Enzymatic*, 58, 103-109. http://dx.doi.org/10.1016/j.molcatb.2008.12.001

Katz, E., & Willner, I. (2004). Integrated Nanoparticle-Biomolecule Hybrid Systems: Synthesis, Properties, and Applications. *Angewandte Chemie International Edition*, 43, 6042-6108. http://dx.doi.org/10.1002/anie.200400651

Kuo, C., Liu, Y., Chang, C., Chen, J., Chang, C., & Shieh, C. (2012). Optimum conditions for lipase immobilization on chitosan-coated Fe_3O_4 nanoparticles. *Carbohydrate Polymers*, 87, 2538-2545. http://dx.doi.org/10.1016/j.carbpol.2011.11.026

Lei, Y., Mulchandani, A., & Chen, W. (2005). Improved Degradation of Organophosphorus Nerve Agents and p-Nitrophenol by *Pseudomonas putida* JS444 with Surface-Expressed Organophosphorus Hydrolase. *Biotechnology Progress*, 21, 678-681. http://dx.doi.org/10.1021/bp0495901

Liu. W-T. (2006). Nanoparticles and their biological and environmental applications. *Journal of Bioscience and Bioengineering*, 102, 1-7.

Obare, SO, De, C, Guo, W, Haywood , T.L., Samuels, T.A., Adams, C.P., …& Fletcher K. (2010). Fluorescent chemosensors for toxic organophosphorus pesticides: A review. *Sensors*, 10, 7018-7043. http://dx.doi.org/10.1263/jbb.102.1

Robatjazi, S.M., Shojaosadati, S.A., Khalilzadeh, R., & Farahani, E.V. (2010). Optimization of the covalent coupling and ionic adsorption of magnetic nanoparticles on *Flavobacterium* ATCC 27551 using the Taguchi method. *Biocatalysis and Biotransformation*, 28, 304-312. http://dx.doi.org/10.3109/10242422.2010.516390

Sahoo, B., Sahu, S.K., & Pramanik, P. (2011). A novel method for the immobilization of urease on phosphonate grafted iron oxide nanoparticle. *Journal of Molecular Catalysis B: Enzymatic*, 69, 95-102. http://dx.doi.org/10.1016/j.molcatb.2011.01.001

Savage, N., & Diallo, M.S. (2005). Nanomaterials and Water Purification: Opportunities and Challenges. *Journal of Nanoparticle Research*, 7, 331-342. http://dx.doi.org/10.1007/s11051-005-7523-5

Serdar, C.M., & Gibson, D.T. (1985). Enzymatic Hydrolysis of Organophosphates: Cloning and Expression of a Parathion Hydrolase Gene from Pseudomonas diminuta. *Nature Biotechnology*, 3, 567-571. http://dx.doi.org/10.1038/nbt0685-567

Sethunathan, N., & Yoshida, T. (1973). A Flavobacterium sp. that degrades diazinon and parathion. *Canadian Journal of Microbiology*, 19, 873-875. http://dx.doi.org/10.1139/m73-138

Swanson, P.E. (1999). Dehalogenases applied to industrial-scale biocatalysis. *Current Opinion in Biotechnology*, 10, 365-369. http://dx.doi.org/10.1016/S0958-1669(99)80066-4

Teste, B., Vial, J., Descroix, S., Georgelin, T., Siaugue, J.M., Petr, J., …& Hennion, M.C. (2010). A chemometric approach for optimizing protein covalent immobilization on magnetic core–shell nanoparticles in view of an alternative immunoassay. *Talanta*, 81, 1703-1710. http://dx.doi.org/10.1016/j.talanta.2010.03.027

Tratnyek, P.G., & Johnson, R.L. (2006). Nanotechnologies for environmental cleanup. *Nanotoday* 1, 44-48. http://dx.doi.org/10.1016/S1748-0132(06)70048-2

Wang, J., Zhao, G., Li, Y., Liu, X., & Hou, P. (2013). Reversible immobilization of glucoamylase onto magnetic chitosan nanocarriers. *Applied Microbiology and Biotechnology*, 97, 681-692. http://dx.doi.org/10.1007/s00253-012-3979-2

Zhang, W.X. (2003). Nanoscale iron particles for environmental remediation: an overview. *Journal of Nanoparticle Research*, 5, 323-332. http://dx.doi.org/10.1023/A:1025520116015

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THE *IN VITRO* EFFECT OF ELDERBERRY (*SAMBUCUS NIGRA*) EXTRACT ON THE ACTIVITY AND OXIDATIVE PROFILE OF BOVINE SPERMATOZOA

*Abzal Abdramanov[1], Peter Massanyi[2], Nurzhan Sarsembayeva[1], Altay Usenbayev[1], Jahongir Alimov[3], Eva Tvrdá[*2]*

Address(es): MSc. Eva Tvrdá, PhD.,
[1]Kazakh National Agrarian University, Abai street 8, 050010 Almaty city, Kazakhstan.
[2]Slovak University of Agriculture, Tr. A. Hlinku 2, 94976 Nitra, Slovakia.
[3]Gulistan State University, 4th Microrayon, Guliston 120100, Syrdarya, Uzbekistan.

*Corresponding author: evina.tvrda@gmail.com

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ABSTRACT

The paper presents the overall results and experimental details of the *in vitro* assessment of the elderberry (*Sambucus nigra*) extract on the motility, viability and reactive oxygen species (ROS) production of bovine spermatozoa in different time periods (0, 2, 6 and 24 hours). *Sambucus nigra* is often used for medicinal purposes throughout the world. Modern research reveals that *Sambucus nigra* extracts may have anti-inflammatory, antiviral, anticancer and antioxidant properties, because of a high content of biologically active components. Spermatozoa motility was assessed using the Computer-assisted sperm analysis (CASA) system. Cell viability was examined using the metabolic activity MTT assay and ROS generation was quantified using luminometry. The CASA analysis revealed that the motility in the experimental groups supplemented with 100, 50 and 1 µg/mL elderberry extract was lower in comparison with other samples. The experiment showed that the elderberry extract had a considerable *in vitro* effect on the sperm motility, vitality and oxidative profile. The ROS production as well as the CASA assessment proved that the optimal concentration of both extracts was 10 µg/mL in every time with statistically significant results. The MTT test showed a statistically significant increase of mitochondrial at all time periods with 10 µg/mL elderberry extract when compared to the control group. When lower concentrations of the elderberry extract were used (5 and 1 µg/mL), the mitochondrial activity was higher than in the control group but lower than in the group supplemented with 10 µg/mL of the extract. In these groups this indicator increased maximally after 24 h. The findings of the present study indicate that *Sambucus nigra* extract possesses activity promoting properties on bovine spermatozoa at 10 and 5 µg/mL.

Keywords: Elderberry, *Sambucus nigra*, spermatozoa, bull, motility, mitochondrial activity, reactive oxygen species

INTRODUCTION

Several commonly used plants have been reported to affect male reproductive functions in wildlife and humans. The effects observed with most of the plant and plant-based products have been attributed to a wide variety of properties of one or more active compounds present in ethnopharmacologically important medicinal herbs (**D'Cruz *et al.*, 2010**). Evaluation of herbs has been in progress worldwide for several decades to identify effective and safe substances for fertility regulation. This approach proved to be a good alternative to synthetic drugs as the chemicals of plant origin have limited side effects. Various medicinal plants extracts were investigated for their fertility-related activity both in male and female animal models (**Sharma *et al.*, 2013**).

Sambucus nigra Linn, frequently known as 'Sweet elder' belongs to family *Caprifoliaceae*. *Sambucus* species are being investigated for their potential health benefits. It is one of the most attractive trees being put to some useful purpose in Ayurveda, homeopathic medicine and has become a cynosure to modern treatment options. The plant is highly used traditionally in curing diverse disorders. Commonly it is used as an astringent, antiviral and diuretic. The antioxidant activity of elderberry extracts has been evaluated before, and it is estimated to be similar to that of black raspberries, blackberries, and other dark-fleshed small fruit Elderberries contain flavonoids (flavone, flavonone, isoflavone derivatives and anthocyanins), which are reported to possess antioxidant activity and to protect against oxidative stressors, such as hydrogen peroxide, 2-amidinopropane, dihydrochloride (AAPH), ferrous sulfate, and ascorbic acid (Kaur *et al.*, 2014).

Sambucus nigra L. has been found to be effective against some important pathogenic microorganisms involved in wounds, bums, skin infections, enteritis, typhoid and candidiasis. Thus, the extract of *Sambucus nigra* L. berries can be used in order to treat these ailments. Elderberry extracts proved to be active against *Staphylococcus aureus* and *Bacillus subtilis* at low concentration and against *C. albicans* at very low concentrations. It is however, more effective against *Pseudomonas aeruginosa, Escherichia coli* and *Salmonella typhi*. Elderberry can be used in treating diseases caused by selected test organisms. It also contains sterols, tannins, essential oils and can readily be considered as a healthy food. It can also be used as a strong food preservative (**Mohammadsadeghi *et al.*, 2013**). *Sambucus nigra* fruit extract powder has a very high *in vitro* antioxidant activity and no mutagenic effects at low concentrations (**Bratu *et al.*, 2012**).

This study investigated the *in vitro* effects of the *Sambucus nigra* extract on the motility, viability and oxidative profile of bovine spermatozoa.

MATERIAL AND METHODS

Plant Material

Sambucus nigra berries were obtained from the Botanical Garden at the Slovak University of Agriculture in Nitra. After drying, the plant tissues were crushed, weighed and soaked in ethanol p.a. (96%, Centralchem, Bratislava, Slovak Republic) during two weeks at room temperature in the dark. Exposure to sunlight was avoided to prevent the degradation of active components. The ethanolic plant extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove any residual ethanol (Stuart RE300DB rotary evaporator, Bibby Scientific Limited, UK, and vacuum pump KNF N838.1.2KT.45.18, KNF, Germany). Crude plant extracts were dissolved in DMSO (Dimethyl sulfoxide; Sigma-Aldrich, St. Louis, USA) to equal 100.4 mg/mL as a stock solution.

Sample Collection and Processing

Bovine semen samples were obtained from 10 adult Holstein breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). The animals were of similar age and were kept under uniform feeding and housing conditions. Two

samples were obtained from each bull on a regular collection schedule with the help of an artificial vagina. Subsequently, sperm concentration and motility was evaluated using phase contrast microscopy (200 x). Only semen samples with a minimum 70% progressive motility were used for the experiments.
Each sample was diluted in physiological saline solution (PS; sodium chloride 0.9% w/v; Bieffe Medital, Italia) containing different concentrations of the elderberry extract (1, 5, 10, 50 and 100 µg/mL) using a dilution ratio of 1:40. The samples were cultured at laboratory temperature (22-25°C). The control (Ctrl) group (medium without *Sambucus nigra* extract supplementation, containing 0.5% DMSO) was compared with the experimental groups.

Spermatozoa Motility Analysis

Spermatozoa motility (%; MOT) was assessed by using the computer-aided sperm analysis (CASA, Version 14.0 TOX IVOS II.; Hamilton-Thome Biosciences, Beverly, MA, USA). Ten µL of each sample were placed into the Makler counting chamber (depth 10 µm, 37 °C; Sefi Medical Instruments. Haifa, Israel) and immediately assessed. Ten microscopic fields were subjected to each analysis in order to include at least 300 cells.

Mitochondrial Activity (MTT Test)

Viability of the cells exposed to *Sambucus nigra* was evaluated by the metabolic activity (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT test. This colorimetric assay measures the conversion of a yellow tetrazolium salt (MTT) to blue formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria within living cells. Formazan can be measured spectrophotometrically.
The MTT tetrazolium salt (Sigma-Aldrich, St. Louis, USA) was dissolved in phosphate-buffered saline (Dulbecco's PBS; Sigma-Aldrich) at 5 mg/mL. A 10 µL of the solution was added to the cells (in 100 µL medium per well). After 2h of incubation (shaker, 37 °C, 95 % air atmosphere, 5% C02), the cells and the formazan crystals were dissolved in 150 µL of acidified (0.08 M HC1; Centralchem, Bratislava, Slovak Republic) isopropanol (Centralchem). The optical density was determined at a wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Anthos MultiRead 400, Austria). The data were expressed as percentage of the control, set to 100% (**Knazicka *et al.*, 2012**).

ROS Generation

ROS levels in samples were assessed by the chemiluminescence assay using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma-Aldrich) as the probe. The test samples consisted of luminol (10 uL, 5 mM) and 400 uL of control or experimental sample. Negative controls were prepared by using 400 uL Dulbecco's PBS (Sigma-Aldrich). Positive control included 400 uL Dulbecco's PBS and 50 uL of hydrogen peroxide (30%; 8.8 M; Sigma-Aldrich) in triplicates. Chemiluminescence was measured on a 48-well plate for 15 min by using the Glomax MultiPlus Combined Spectro-Fluoro-Luminometer (Promega, Madison, WI, USA). The results were expressed as relative light units (RLU)/sec/10^6 sperm (**Kashou *et al.*, 2013**).

Statistical Analysis

Statistical analysis was carried out by using the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). Descriptive statistical characteristics (mean, standard error) were evaluated at first. As we focused to study the impact of different elderberry concentrations on the spermatozoa activity (experimental groups) in comparison to the control at a specific time frame, thus taking one factor into consideration, one-way ANOVA was used for specific statistical evaluations. Dunnett test was used as a follow-up test to ANOVA, based on a comparison of every mean to a control mean, and computing a confidence interval for the difference between the two means. The level of significance was set at *** (P<0 .001); ** (P<0.01); * (P<0.05).

RESULTS AND DISCUSSION

Over the past years, natural compounds isolated from plants have emerged exhibiting a complex biological activity. Due to their broad range of effects, particularly with respect to antibacterial, anti-inflammatory protection and antioxidant mechanisms, plant extracts have attracted a widespread scientific and consumer interest (**Putheti and Okigbo, 2008; Omogbadegun *et al.*, 2011; Tohamy *et al.*, 2012; Hamidpour *et al.*, 2014**).
The CASA assessment showed a continuous decrease of spermatozoa motility in all groups over the course of a 24h *in vitro* culture (Table 1). The initial (Time 0h) MOT was higher in the experimental groups supplemented with 100 and 1 µg/mL elderberry extract and lower in other groups with extract when compared to the control group, although without any statistical significance (P>0.05). No significant differences among the control and experimental groups were recorded at Time 2h: the groups supplemented with the extract at 100 and 50 µg/mL the MOT was lower, as at the 10, 5 and 1 µg/mL – higher. A statistically significant motion-activating effect became visible after 6h in the group supplemented with 10 µg/mL of the *Sambucus nigra* extract whereas in other groups no significant effects of the extract were observed. At the end of the experiment (Time 24h), the motility observed in the experimental groups supplemented with 1-100 µg/mL elderberry extract was higher in comparison with the control, but a significantly highest MOT was established at the 10 µg/mL concentration. *Sambucus nigra* concentrations ranging between 1-100 µg/mL had an impact of activation on the sperm MOT when compared to the control. Nevertheless a significant influence was shown with respect to the extract at a concentration of 10 µg/mL at all assessment periods (Table 1).

Table 1 Bovine spermatozoa motility (MOT, %) in the absence (Ctrl) or presence of elderberry extract during different time periods (Mean±SEM; n=10)

Time	Ctrl	Concentration of *Sambucus nigra*, µg/mL				
		100	50	10	5	1
0h	74.75±7.21	78.25±5.80	69.00±1.91	74.00±3.12	71.00±8.18	77.50±2.17
2h	63.75±1.88	58.25±1.73	58.00±5.97	68.75±4.87	67.75±1.49	64.75±3.80
6h	51.25±2.25	48.75±1.49	50.75±3.80	60.25±2.31*	50.00±5.34	49.50±1.30
24h	20.25±6.80	20.50±4.87	24.75±1.23	31.00±1.62*	27.00±2.31	25.25±3.90

*P<0.05; **P<0.01; ***P<0.001

Progressive motility of control and experimental groups at 0 hours of experimental period, with different concentrations of the elderberry extract, did not exhibit significant differences. At 2h of experimental period the higher concentration of *Sambucus nigra* extract with 100 and 50 µg/mL decreased spermatozoa progressive motility (PRG, %). After 6 and 24 hours of experimental periods showed, that the progressive motility of the control and experimental samples with the 100, 50 and 1 µg/mL of the elderberry extract was lower, compared to 10 and 5 µg/mL (Table 2).

Table 2 Bovine spermatozoa progressive motility (PRG, %) in the absence (Ctrl) or presence of *Sambucus nigra* extract during different time periods (Mean±SEM; n=10)

Time	Ctrl	Concentration of *Sambucus nigra*, µg/mL				
		100	50	10	5	1
0h	46.75±4.79	44.00±1.87	48.50±3.86	48.00±5.80	48.00±3.10	44.25±4.90
2h	45.75±1.76	29.00±5.76*	34.25±3.87	48.00±6.80	46.00±3.43	44.50±1.88
6h	23.00±1.46	17.25±1.39*	23.00±1.37	31.50±1.74*	28.50±2.35	21.75±3.60
24h	3.50±0.46	1.75±0.11*	1.25±0.34	6.00±0.33*	6.20±0.87*	2.25±0.46

*P<0.05; **P<0.01; ***P<0.001

According to the MTT assay, an instant *Sambucus nigra* supplementation (Time 0 h) had different effects on the sperm mitochondrial activity in any of the experimental groups (Fig. 1). It was established that 100 µg/mL extract had no specific impact on the mitochondrial activity: at 0 and 2 h it was lower and through 6 and 24 h it was almost equal to the control group. Impact of the 50 µg/mL concentration to the sperm was analogical with a higher activation at 2 and 6 h (Fig. 1). A statistically significant increase of mitochondrial activity was observed at all time periods with 10 µg/mL elderberry extract when compared to the control group. When lower concentrations of the elderberry extract were used (5 and 1 µg/mL) the mitochondrial activity was higher than in the control group but lower than in the group supplemented with 10 µg/mL of the plant extract (Fig. 1). In these groups this indicator increased maximally after 24 h (Fig. 1).

From these results we may hypothesize that the 10 µg/mL of the elderberry extract have a direct activating effect on the mitochondrial energy metabolism, a crucial factor supporting key spermatozoa motion. Activation of the mitochondrial function can increase sperm motility, and subsequently male fertilizing capacity.

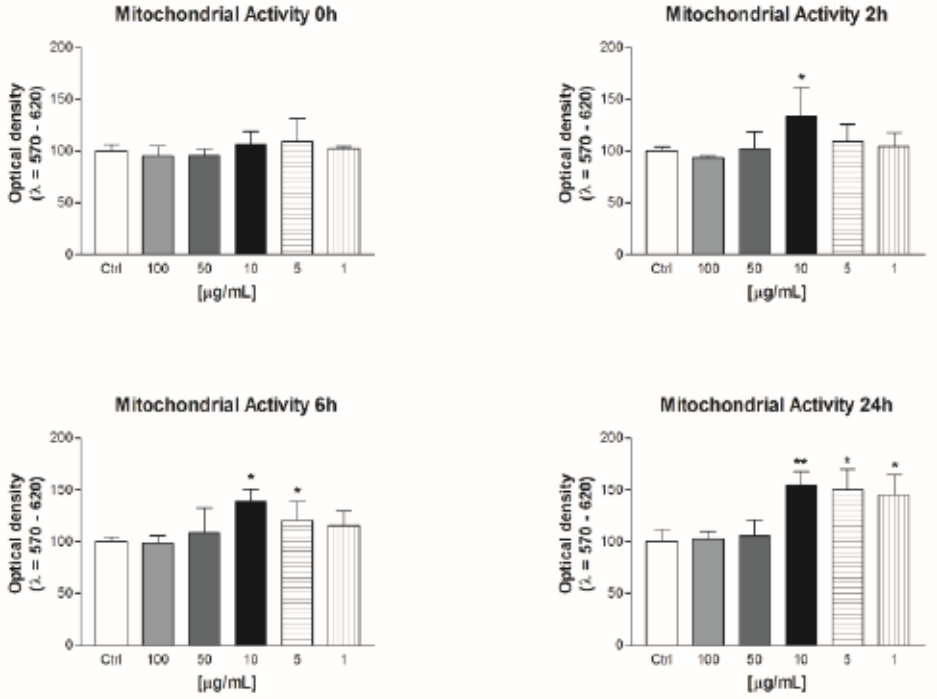

Figure 1 Mitochondrial activity of bovine spermatozoa (%) in the absence (Ctrl) or presence of the *Sambucus nigra* extract in different time periods. MEAN±SEM; ** (P<0.01); *(P<0.05)

Oxidative stress (OS) has become one of the leading causes related to the loss of viable spermatozoa during cryopreservation. ROS over generation is nowadays accepted as a notable side effect of in vitro processing and handling protocols of semen, leading to major disruptions in the cellular oxidative metabolism. The resulting OS may subsequently lead to irreversible alterations of membrane structures via LPO, as well as oxidative degradation of proteins or DNA, followed by apoptotic activation (**Ball, 2008**).

Our investigation revealed that the lowest ROS production was observed in the group with 10 µg/mL extract, where after 2 hours cultivation with extract the ROS production was almost half of the control group (P<0.01). After 6 and 24 hours we could definitely confirm a significant positive antioxidant effect in this group compared with control and other experimental groups (P<0.05 in case of 50, 10 and 5 µg/mL).

Table 3 Reactive oxygen species production (ROS) production by bovine spermatozoa (RLU/sec/10^6 sperm) in the absence (Ctrl) or presence of the *Sambucus nigra* extract in different time periods.

Time	Ctrl	Concentration of *Sambucus nigra*, µg/mL				
		100	50	10	5	1
0h	0.92±0.03	1.86±0.05	1.49±0.06	0.70±0.03	0.61±0.02	0.81±0.04
2h	1.49±0.13	2.95±0.44*	2.61±0.38	0.89±0.08*	1.11±0.09	1.26±0.07
6h	1.77±0.03	3.21±0.11**	3.01±0.70*	1.22±0.17*	1.36±0.14	1.58±0.09
24h	3.59±0.09	5.13±0.13**	4.33±0.38*	2.26±0.52*	2.33±0.08*	2.42±0.17

*P<0.05; **P<0.01; ***P<0.001

(OS) oxidative stress is considered as a crucial factor among causes of male infertility's pathogenesis (**Lanzafame *et al.*, 2008; Tvrdá *et al.*, 2011**). Spermatozoa was the first type of cells to be reported as susceptible to OS. The inability to restore the damage induced by OS coupled with cell membranes rich in polyunsaturated fatty acids (PUFAs), render spermatozoa to be highly susceptible to ROS-induced damage. Subsequently, a rapid loss of intracellular ATP causes mitochondrial and axonemal damage, decreased sperm viability, and increased mid-piece sperm morphological defects, all of which contribute to a decreased sperm motility (**Bansal and Bilaspuri, 2011; Gharagozloo and Aitken, 2011**). OS has become a great concern for clinicians and scientists as this programmed deterioration may lead to poor fertilization and embryonic development, pregnancy loss and birth defects (**Butler *et al.*, 2002; De Iuliis *et al.*, 2006; Tremellen, 2008; Aitken *et al.*, 2010**).

The ROS production as well as the CASA assessment proved that the optimal concentration of both extracts was 10 µg/mL in every time with statistically significant results (Table 3). The studies of **Miraj (2016)** defined the beneficial activity as an antioxidant stress protector with the high antioxidant capacity, observed at very low concentrations of the elderberry extract, would be easily fulfilled without any dangers or side effects when used in small amounts. *Sambucus nigra* is known for its medicinal use and contains anthocyanins, flavonoids and other polyphenolics. Our studies point out that maybe because of the antioxidant activity the CASA analyzes was better, while ROS decreased. Also, we can notice that antioxidants mostly reduce oxidative stress and improve sperm motility, protect spermatozoa in the seminal plasma or in spermatozoa itself to prevent oxidative damage. According to the experiment data we may suggest that low concentrations of the *Sambucus nigra* extract decreased activity of the mitochondrial respiratory chain of complex II, thus significantly decreasing the risk of ROS overproduction.

CONCLUSION

The results of the present study indicate that the elderberry extracts have a considerable effect on the functional activity of bovine spermatozoa. The findings of the present study clearly indicate that *Sambucus nigra* extract possesses motility promoting properties on bovine spermatozoa at 10 and 5 µg/mL concentration. However, this study is the first laboratory based experiment. In near future more studies need to be undertaken in a similar direction to prove all the data obtained from this report.

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REFERENCES

Aitken, R.J., Baker, M.A., De Iuliis, G.N. & Nixon, B. (2010). New insights into sperm physiology and pathology. Handbook of Experimental Pharmacology, 1989, 99-115. http://dx.doi.org/10.1007/978-3-642-02062-9_7

Ball, B.A. (2008). Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. Animal Reproduction Science, 107, 257-267. http://dx.doi.org/10.1016/j.anireprosci.2008.04.014

Bansal, A.K. & Bilaspuri, G.S. (2011). Impacts of oxidative stress and antioxidants on semen functions. Veterinary Medicine International. http://dx.doi.org/10.4061/2011/686137

Bratu, M.M, Doroftei, E., Negreanu-Pirjol, T., Hostina, C. & Porta, S. (2012). Determination of Antioxidant Activity and Toxicity of *Sambucus nigra* Fruit Extract Using Alternative Methods. *Food Technology and Biotechnology*, 50(2), 177-182.

Butler, A., He, X., Gordon, R.E., Wu, H.S., Gatt, S. & Schuchman, E.H. (2002). Reproductive pathology and sperm physiology in acid sphingomyelinase-deficient mice, *American Journal of Pathology*, 161, 1061-1075.

D'Cruz, S.C., Vaithinathan, S., Jubendradass, R. & Mathur, P.P. (2010). Effects of plants and plant products on the testis. Asian Journal of Andrology, 12(4), 468-479. http://dx.doi.org/10.1038/aja.2010.43

De Iuliis, G.N., Wingate, J.K., Koppers, A.J., McLaughlin, E.A. & Aitken, R.J. (2006). Definitive evidence for the nonmitochondrial production of superoxide anion by human spermatozoa. *Journal of Clinical Endocrinology & Metabolism*, 91, 1968-1975.

Gharagozloo, P. & Aitken, R.J. (2011). The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. Human Reproduction, 26, 1628-1640. http://dx.doi.org/10.1093/humrep/der132

Hamidpour, M., Hamidpour, R., Hamidpour, S. & Shahlari, M. (2014). Chemistry, pharmacology, and medicinal property of sage (*Salvia*) to prevent and cure illnesses such as obesity, diabetes, depression, dementia, lupus, autism, heart disease, and cancer. *Journal of Traditional and Complementary Medicine*, 4, 82-88. http://dx.doi.org/10.4103/2225-4110.130373

Kashou, A.H., Sharma, R. & Agarwal, A. (2013). Assessment of oxidative stress in sperm and semen. *Methods in Molecular Biology*, 927, 351-361. http://dx.doi.org/10.1007/978-1-62703-038-0_30

Kaur, K., Kaur, R., Kaur, H. & Kaur, S. (2014). A comprehencive review: *Sambucus nigra, Linn. Biolife Journal*, 2(3), 941-948.

Knazicka, Z., Tvrda, E., Bardos, L. & Lukac N. (2012). Dose- and time-dependent effect of copper ions on the viability of bull spermatozoa in different media. Journal of Environmental Science and Health, Part A, 47(9), 1294-1300. http://dx.doi.org/10.1080/10934529.2012.672135

Lanzafame, F.M., La Vignera, S., Vicari, E. & Calogero, A.E. (2009). Oxidative stress and medical antioxidant treatment in male infertility. *Reproductive Biomedicine Online*, 19, 638-659. http://dx.doi.org/10.1016/j.rbmo.2009.09.014

Miraj, S. (2016). Chemical composition and pharmacological effects of Sambucus nigra. *Der Pharma Chemica*, 8(13), 231-234.

Mohammadsadeghi, Sh., Malekpour, A., Zahedi, S. & Eskandari, F. (2013). The Antimicrobial Activity of Elderberry (*Sambucus nigra L.*) Extract Against Gram Positive Bacteria, Gram Negative Bacteria and Yeast. *Research Journal of Applied Sciences*, 8(4), 240-243.

Omogbadegun, Z., Uwadia, C., Ayo, C., Mbanika, V., Omoregbe, N., Otofia, E. and Chieze, F. (2011). Multimedia-based medicinal plants sustainability management system. *International Journal of Computer Science*, 8, 492-503.

Putheti, R. & Okigbo, R.N. (2008). Effects of plants and medicinal plant combinations as antiinfective. *African Journal of Pharmacy and Pharmacology*, 2, 130-135.

Sharma, R.K., Goyal, A.K. & Bhat, R.A. (2013). Antifertility activity of plants extracts on female reproduction: a review. *International Journal of Pharmacy and Biological Sciences*, 3, 493-514.

Tohamy, A.A., Ibrahim, S.R. & Abdel Moneim, A.E. (2012). Studies on the effect of *Salvia aegyptiaca* and *Trigonella foenum graecum* extracts on adult male mice. *Journal of Applied Pharmaceutical Science*, 2, 36-43.

Tremellen, K. (2008). Oxidative stress and male infertility - a clinical perspective, Human Reproduction Update, 14, 243-258. http://dx.doi.org/10.1093/humupd/dmn004

Tvrdá, E., Kňažická, Z., Bárdos, L., Massányi, P. & Lukáč, N. (2011). Impact of oxidative stress on male fertility - a review. *Acta Veterinaria Hungarica*, 59, 465-484. http://dx.doi.org/10.1556/AVet.2011.034