Deliverable 2.1 – Overview of research activities in Europe to reduce acrylamide in cereal-based foods for the field of agronomy and plant breeding (M12)

Project Name	ACRYRED
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Working Group	WG2
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ACRYRED

Executive Summary

Abstract of scientific content

The concentration of individual free amino acids in cereal grains varies depending on species and fractions, with asparagine being one of the most abundant. Free asparagine reacts with reducing sugars to form the processing contaminant, acrylamide, in cereal products through the Maillard reaction, promoted by high temperature and low moisture content. The acrylamide monomer is both neurotoxic and carcinogenic. Thus, the European Commission issued Benchmark Levels and codes of practice for the food products in 2017. The presence of acrylamide in food is now a difficult regulatory compliance issue for the food industry.

Cereal breeders could contribute to the reduction of acrylamide level in food by reducing the free asparagine level in cereals. A variation of 0.56–11.82 mmol/kg of free asparagine was measured in different studies on 25–270 wheat varieties grown on 3-6 sites. Variation was also found among different cereal species, with rye having the largest values and the widest variation. Although a high variation was found in asparagine concentration, no genetic resources have so far been identified for breeding purposes.

The most frequent heritability value of free asparagine in wheat was 0.6 (0.13–0.94) in different studies, showing the relevance of breeding for this trait. Nine QTLs were identified for free asparagine concentration, but with 5A chromosome being the most determinant in one study and 7B in another. From the enzymes involved in asparagine metabolism, asparagine synthetase was the most widely studied and genes were identified in different species, but so far, no molecular markers suitable for marker-assisted selection have been developed. Efforts have also been carried out to develop fast, cost-effective methods for measuring free asparagine concentration in cereals but without a realistic solution.

Environmental factors and field management practices can also influence the asparagine level in cereals. Asparagine increased with a delayed harvest, more solar radiation and disease pressure, while the sulphur supply, fungicide treatment or organic farming reduced it in cereal grain. The latter might also be related to the smaller kernel size, harder grain texture and lower protein content of the seed. This may also have contributed to the failure of the selection of the appropriate gene resource.

The working group WG2 „Agronomy and cereal breeding” in COST ACRYRED Action (CA21149) aims to assess diversity of free asparagine in cereal species and cultivars and to identify those having low content. The understanding of the pathways for accumulation of free asparagine and identification of efficient ways for reduction of free asparagine concentrations in cereal grains through plant breeding including genetic tools, cereal production and/or field management practices is an important aspect, and developing fast techniques for screening free asparagine level in cereals is also unsolved. Providing recommendations for farmers and food industry is also of interest.

ACRYRED

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Introduction

Aims of the working group 2 (WG2) „Agronomy and cereal breeding” is to assess diversity of asparagine in cereal species and cultivars and to identify potential sources of low asparagine cereal species and cultivars.
Understanding pathways for the reduction of free asparagine levels in cereal and identification of efficient ways for the reduction of free asparagine levels in cereal through plant breeding (including genetics & genomics tools), cereal production and/or field management practices (*e.g., N application, resistance to biotic and abiotic stress, organic vs. low input vs. conventional systems etc*.) is also an important question to be solved. Furthermore developing/identifying fast techniques for screening free asparagine level in cereals, providing recommendations for the reduction of free asparagine levels in cereal from breeder's and agronomy point of view for farmers and food industry would also be a crucial achivement. In order to achieve the aforementioned aims, our first deliverable in the first year is the overview of previous and current research activities in Europe to reduce acrylamide in cereal-based foods for the field of agronomy and plant breeding. WG2 also had cooperative activities by fulfilling the STSMs, ITC grants, publishing special issues, papers, participating at conferences and organizing meetings.

Objectives of the document

This document aims to summarize the activities of WG2 „Agronomy and cereal breeding” in the first year, (including the publications, conference participations, STSMs, meetings to be organized, special issues, training schools, conference grants etc.) and also to summarize research activities carried out in Europe to reduce acrylamide in cereal-based foods for the field of agronomy and plant breeding.

1. Chapter 1 Deliverable report

The main aim of this grant period was to collect the knowledge on acrylamide and asparagine of cereals that could be important for breeders and to review what is available in the published literature. It was found, that there are only a few groups working on low-asparagine cereals, and no papers have been published until this time on breeding efforts. Thus, it is still an interesting question for breeders, whether it makes sense at all to breed low-asparagine cereals?

The most important questions that may arise are the following:

- What is the main components in cereals for acrylamide formation?
- What is known about the variation of asparagine within and between species? Which cereals species are of greatest interest?
- What is known about the genetic background that determine asparagine level in cereals?
- What are the effects of the environment and agronomy on the asparagine level in cereals?
- What kind of methods are available to measure asparagine level in cereals?

Acrylamide was discovered in food in 2002. The main pathways leading to the formation of acrylamide in food is called the Maillard reaction. There are different models for the proposed mechanism of acrylamide formation. However, the main point in each is, that during cooking and baking processes free asparagine reacts with reducing sugars (such as glucose, fructose or maltose) to form acrylamide as a result of heat and low moisture.

In cereal products, free asparagine concentration was shown to be the major determinant of acrylamide formation as the carbonyl sources are available in high amounts. Consequently, asparagine is the precursor, limiting the formation of acrylamide in cereal products. The products of the Maillard reaction include melanoidin pigments and complex mixtures of compounds that impart flavour and aroma. Acrylamide is formed at the end of the reaction, which has neurotoxic and carcinogenic effects on human health. It is readily absorbed through the skin, by inhalation and from the gastro-intestinal tract.

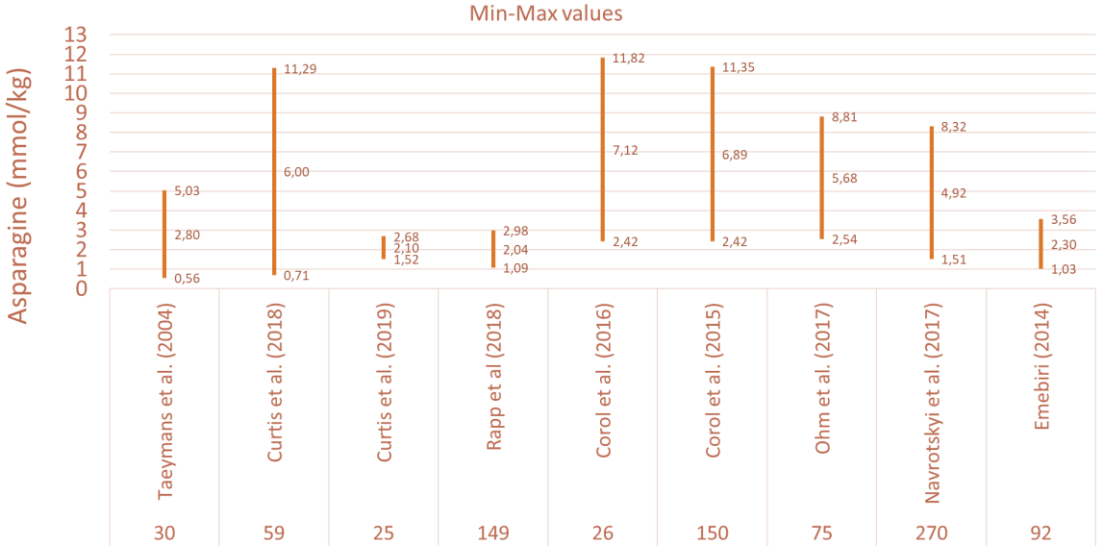
In 2007, the European Commission issued a Recommendation on the monitoring of acrylamide levels in food (2007/331/EC). Later, in 2011, they introduced the concept of Indicative Values for acrylamide levels in food. Indicative Values were not meant to be regulatory limits or safety thresholds, but they were set at levels that food industry ought to be able to achieve, for different food categories. In 2018, Commission Regulations replaced Indicative values with Benchmark Levels, which are lower than the corresponding Indicative Value for almost all product types, but they are performance indicators rather than triggers for investigation.

The relationship between the level of asparagine and the formation of acrylamide was studied on wheat and rye heated flour. A linear regression/relationship and a positive correlation was found between the two components.

The contents of the individual amino acids varies, depending on the type of cereal and its fraction. In wheat, Asn, Asp, Glu, Ala, and Ser, are the major amino acids, with Asn being the most abundant.

The variation of the asparagine content in wheat was determined in several studies. Figure 1 shows the results from nine papers, and these seemed to be the most significant as they included the highest number of studied samples (25-270). The concentration is given in mmol/kg in each study and the minimum, maximum and average values are indicated on the figure as well. The lowest value was found to be 0.56, while the highest was 11.82 mmol/kg, which represents a 20 times higher value than the minimum, demonstrating a high variation. However, because of the strong environmental and methodological effects, this variation does not seem to be enough to identify potential candidates for breeding.

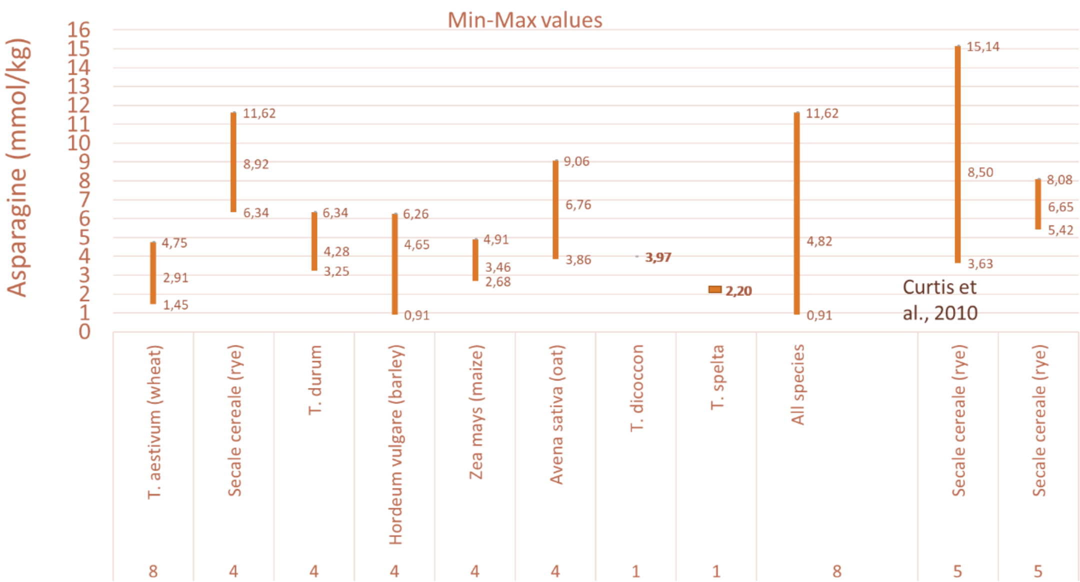
Figure 1. Variation of asparagine in wheat based on different studies



Variation of the asparagine content was also studied in different wheat quality groups, and a high variation was found in each of them. Quality groups were not significantly different from each other in their asparagine content. However, lower values are expected in soft wheat, as they usually having lower protein content (Curtis, Powers et al., 2018).

The asparagine content of the different cereal species was also compared in the work of Zilic et al. (2017). They found the lowest values in wheat, barley and spelt, although this study included low number of samples for each species. The highest values and highest variation of asparagine content was found in rye (Figure 2.).

Figure 2. Variation of asparagine in different cereal species



Further question is that how much of this trait is genetically determined? Heritability of asparagine has already been studied in wheat and rye, with minimum values being 0.13 and the maximum being 0.94. The most frequent values were between 0.4 and 0.6 (Table 1.).

SNP array analysis and QTL studies were also carried out to identify markers in relation to low asparagine content. In one study, 9 SNPs were identified explaining 14 to 24% of the genetic variance of asparagine. In another study, 8 QTLs were identified, which explained 78.5% of the genetic variance. From the 8QTLs, the one on chromosome 7B explained 18.4% of the total genetic variance.

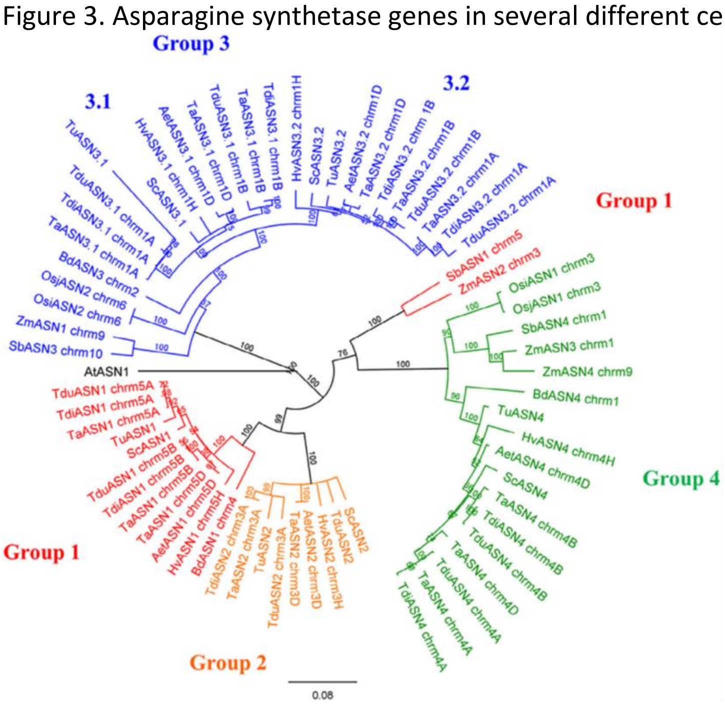
Table 1. Genotype and Environmental effects and heritability of asparagine

Specie	G	GxE	Heritability	Reference
T. aestivum, wheat			0.32	Emebiri, 2014
	26%	41%	0.56	Ohm et al., 2017
			0.13	Corol et al. 2016
			0.41	Poudel et al., 2021
	44.5%	3.7%	0.94	Navrotskyi et al., 2017
	***	***	0.65	Rapp et al., 2018
Secale cereale, rye	23%	24%	0.62	Curtis et al., 2010

The core enzymes involved in the asparagine metabolism has already been identified (asparagine synthetase, asparaginase, glutamine synthetase, glutamate dehydrogenase, ferredoxin-dependent glutamate synthase, NADH-dependent glutamate synthase, aspartate amino transferase, glutamate decarboxylase, aspartate kinase, nitrate reductase and nitrite reductase), from which asparagine synthetase seems to be the most determinant and the most studied. It should be noted that asparagine synthases are known to play important roles in germination in other species.

Asparagine synthetase genes have already been identified in several different cereal species, such as wheat, maize, barley, durum, rice, rye etc.

Figure 3. Asparagine synthetase genes in several different cereal species



With this background knowledge, direct and indirect methods are suggested as strategies for breeding low-asparagine wheat. The direct method uses the natural variation found within the species. Identification of genotypes with deletion in TaSN-B2 gene and identification of markers for this purpose could help in the efficient selection. Indirect methods include using induced variation achieved by gene editing of the genes of enzymes involved in asparagine metabolism. The other option is to carry out selection for disease resistance or drought tolerance or for those varieties, which have sprouting resistance. Researchers found that these resistance properties may be related to low asparagine content, but the effect of other environmental factors and field management practices can be contributary factors in such studies.

For example, it was established in different studies that the delayed harvest and thus, more solar radiation, the disease pressure and the larger TKW (thousand kernel weight) can increase the asparagine content of cereals. On the other hand, the doubled haploidization, the sulphur supply, the fungicide treatment, the use of organic production practices and the softer kernels contribute to the reduction of the asparagine content (Navrotskyi et al., 2017, Curtis et al., 2018, Stockmann et al., 2019).
A study evaluated the complex effects of the field management system, the species, the year and the interaction of these three factors. Almost all of those factors significantly affected the free asparagine content (Stockmann et al., 2019).
When the plants were treated with nitrogen and sulphur fertilizer, it was found that the addition of sulphur significantly reduced the quantity of asparagine in wheat (Curtis, Powers, et al. 2018).
Fungicide treatment also reduced the level of asparagine in wheat as free asparagine is known to be accumulated in many plant species in response to infection by pathogens (Curtis et al., 2016).
The year of wheat variety registration did not affect the asparagine content of the seed. That could be the results of its independence from the traits used for selection in the last 60 years (Rapp et al., 2018).

The relationship between the asparagine content and different quality and agronomical traits was studied in different papers. A correlation was found with the protein content and composition, the preharvest sprouting or the baking quality traits. From agronomical properties, the nitrogen use efficiency, the stress tolerance and senescence, the soil nitrogen:sulphur ratio and the fertilizer use had the greatest influence on asparagine content.

In relation to the coefficients of correlation found in different studies for protein content and composition, gluten content, gluten index and Zeleny sedimentation, the results are often contradictory or show low significance. The highest correlation was found with nitrogen and sulphur content and their ratios. Sulphur application was found to decrease free asparagine content and to improve protein composition (Table 2.) (Oddy et al., 2022).

Table 2. Correlation of asparagine with other compositional and quality traits of wheat

Properties	**Asparagine**	**R^2**	**Reference**
Protein content UPP%,MW	Untransformed Log_e transformation	0.27-0.93 -0.37-0.43 -0.03—- 0.37	Martinek et al., 2009; Corol et al., 2016; Navrotskyi et al., 2018; Malunga et al.,2019; Weber et al., 2008; Stockmann et al., 2018; Simsek et al., 2014; Ohm et al., 2018.
Nitrogen content, N/S ratio, Sulphur content	Log_e transformation Log_{10} back-transformed	0.62, 0.73, 0.14	Liu et al., 2011; Rapp et al., 2018

Gluten content	untransformed	0.44	Corol et al., 2016
Starch content	untransformed	-0.32	Corol et al., 2016
Gluten index	untransformed	-0.36	Malunga et al., 2019
Farinograph water absorption	untransformed Log_e transformation	0.35, 0.94, -0.03,	Corol et al., 2016; Liu et al., 2011; Ohm et al., 2017
Zeleny sedimentation	Untransformed Log_{10} back-transformed	0.37 -0.29	Corol et al., 2016; Rapp et al., 2018
Yield	per unit protein untransformed	0.74, -0.56 0.75, -0.32	Xia et al., 2021; Malunga et al., 2021; Navrotskyi et al., 2018
Sprouting score, Endoprotease activity sprouted and ΔD, Falling number	Log_e transformation Log_{10} back-transformed	0.68, 0.69, 0.6, 0.03	Simsek et al., 2014; Rapp et al., 2018

The correlation of free asparagine with the yield, the physical properties of the seed and some agronomical traits, such as flowering time, days of harvest or sprouting score was also studied.
The most definitely positive correlation was found for sprouting score. However, the relationship between grain asparagine content and the germination exists when the asparagine concentration is very low or very high. The low-grain asparagine may inhibit germination and could affect preharvest sprouting, although correlation with the Hagberg falling number was not found (Oddy et al., 2022).

For the determination of asparagine in cereals, the most frequently used methods are the amino acid analyzer or GC method (Rapp et al., 2018, Mustafa et al. 2007 Ohm et al., 2018). As a correlation was found between asparagine and the composition of the proteins, Ohm et al. (2018) indirectly tried to estimate the level of asparagine from the ratio of the unextractable polymeric proteins (UPP%). A spectrophotometric method (Novrotskyi et al., 2017) was also applied in one study and there was an attempt to develop NIR calibration but without much success (Rapp et al., 2018). However, there is a company who measures amino acids by NIR as a service (Evonik, AminoNIR).

The take-home messages from the papers for breeders, the questions to be solved and the future prospects are the following:

- 64% reduction could be achieved in asparagine level by good variety selection
- Selection for low asparagine in later generations in breeding programmes is recommended
- Development of markers (MAS) is necessary for selection in early generations
- Pyramiding of QTL alleles for low asparagine can contribute substantially to reduce acrylamide by breeding
- Unravelling the strong GxE effect requires multi-site testing
- NIR calibrations should be developed with larger set of samples (>150)
- Further studies of reducing sugars are recommended
- Innovative approaches for potato already exist: biotechnologically developed varieties have already been marketed in the USA and Canada (Innate, Innate Generation 2).

Based on this background knowledge, the objectives of the Working Group 2 with the title „Agronomy and cereal breeding" within COST Action Acryred are the following:

- Assessing diversity of asparagine levels in cereal species and cultivars and identification of genetic resources for breeding purposes;
- Studying the genetic inheritance of free asparagine level
- Understanding pathways for the reduction of free asparagine levels in cereal and identification of efficient ways of reducing free asparagine levels in cereals through plant breeding (including genetics and genomics tools), cereal production and/or field management practices (e.g., N application, resistance to biotic and abiotic stress, organic vs. low input vs. conventional systems, etc.)
- Developing/identifying fast techniques for screening free asparagine level in cereals;
- Providing knowledge-based guidances for the reduction of free asparagine levels in cereals and their products for farmers and food industry.

These recommended approaches give the basis for fomulating the research agenda of the next 5 years in agronomy and plant breeding. (D2.2 Draft agenda for the next 5 years for agronomy and plant breeding research (M20), D2.3 Agenda for the next 5 years for agronomy and plant breeding research.

2. Chapter 2 Activities

1. WG2 Meetings

12.05.2023, at Centre for Agricultural Research, Martonvásár, Hungary, hybrid meeting

2. STSMs

Barbora Karnikova, Mendel University, Brno, Czech Republic, at 17 July 2023 – 4 August 2023, 3 weeks at Centre for Agricultural Research, Hungary, project title: 'Selection of barley genotypes for food utilization while learning breeding techniques'

3. Conference participation

1. Rakszegi Marianna, Korzun Viktor, Halford Nigel, 2023. Does it make sense to breed low-asparagine cereals? In: Mihály-Langó, Bernadett; Bona, Lajos; Tóth, Beáta; Börner, Andreas Cereal Breeding - Challenges and Opportunities for Global Improvement Book of Abstracts of the Eucarpia Cereals Section Conference Szeged, Hungary, pp. 28.
2. Nigel Halford, Naveet Kaur, 2023, Acrylamide in food: regulatory developments, progress in producing low acrylamide wheat, and the aims of the ACRYRED cost action (ca21149). In: Mihály-Langó, Bernadett; Bona, Lajos; Tóth, Beáta; Börner, Andreas Cereal Breeding - Challenges and Opportunities for Global Improvement Book of Abstracts of the Eucarpia Cereals Section Conference Szeged, Hungary, pp. 26.
3. Navneet Kaur, Martin Urban, Kim E. Hammond-Kosack, Nigel G. Halford, 2023, Uncoupling the signalling network linking biotic stress and free asparagine accumulation in wheat and the aims of the ACRYRED cost action (ca21149). In: Mihály-Langó, Bernadett; Bona, Lajos; Tóth, Beáta; Börner, Andreas Cereal Breeding - Challenges and Opportunities for Global Improvement Book of Abstracts of the Eucarpia Cereals Section Conference Szeged, Hungary, pp. 27.
4. Sarić Beka, Gürsul Aktağ Işıl, Žilić Slađana, Simić Marijana, Nikolić Valentina, Gökmen Vural (2023). Effect of dry-heat treatment on acrylamide and HMF formation in maize flour. XXII EuroFoodChem Congress, 14-16. June, Belgrade, Serbia. Book of abstracts. pp. 276.
5. Žilić Slađana, Gürsul Aktağ Işıl, Gökmen Vural (2023). Relation of free asparagine content in small-grain cereals and the generation of acrylamide in the cookies. XXII EuroFoodChem Congress, 14-16. June, Belgrade, Serbia. Book of abstracts. pp. 277.

6. Tafuri A., Pirona R., Fricano A., Mazzucotelli E., Cagliani L. R., Gasser M., Giordano M., Zuccaro M., Ravaglia S., Consonni R., Thomas A., Gilardi F., Ceriotti A., Baldoni E. "Metabolite characterization of durum wheat grain for association studies: exploring the natural variation of free asparagine content" Oral Communications. Proceedings of the LXVI SIGA Annual Congress Bari, 5/8 September, 2023.
7. Sarić Beka, Marijana Simić, Valentina Nikolić, Danka Milovanović, Slađana Žilić (2023). Potential of commercial corn hybrids for acrylamide formation. X Symposium on Plant Breeders and Seed Producers Society of the Republic of Serbia and VII Symposium on the Breeding of Organisms Section of the Serbian Genetic Society, 16-18. October, Vrnjačka Banja, Serbia. Book of abstracts. pp.

4. Special issues

Title: „**Nutrition and Sustainable Development Goal 9: Industry, Innovation, and Infrastructure**"
Guest Editors: João Miguel Rocha, Gheorghe Adrian MartauDan Cristian VodnarDimas Rahadian Aji Muhammad, Calinoiu Florina Lavinia, Marianna Rakszegi, Sanja Kostadinovic Velickovska, Maria D. Papageorgiou
Paper Invitation: IF=5.753, Q1
Deadline: 20 December 2023
Link: https://www.frontiersin.org/research-topics/40804/nutrition-and-sustainable-development-goal-9-industry-innovation-and-infrastructure

Title: "**New Insights into Cereals and Cereal-Based Foods III.** "
Guest Editors: Dr Maria Papageorgiou
Paper Invitation: IF=5.561, Q1
Deadline: 15 November 2023
Link: https://www.mdpi.com/journal/foods/special_issues/9M8B6PC8BZ

Title: **"Strategies to Improve the Security and Nutritional Quality of Crop Species"**
Guest Editors: Prof. Dr Grażyna Podolska, dr Anna Szafrańska
Paper Invitation: IF 3.6
Deadline: 25 November 2023
Link: https://www.mdpi.com/journal/agriculture/special_issues/04DW5K15VU

Title: **"Advances in Cereal and Cereal Product Chemistry, Nutrition and Technology"**
Guest Editors: Dr Žilić Slađana, Dr Jinshui Wang, Dr Ying Liang
Paper Invitation: IF=5.20 Q1
Deadline: 5 February 2024
Link: https://www.mdpi.com/journal/foods/special_issues/9Y8M74ETKL

5. ITC and DCG Conference Grants

ITC Grant:

Type of presentation: Poster
Title: Effects of Fertilizer and Herbicide Applications on Acrylamide Formation Potential Of Corn Genotypes
Presenters: Burçe Ataç Mogol*, Slađana Žilić, Aytül Hamzalıoğlu, Neslihan Taş, Tolgahan Kocadağlı, Vural Gökmen, Marijana Simić, Natalija Kravić
Conference: 9th International conference CHEMICAL REACTIONS IN FOODS IX, 13-15 September 2023

6. Publications

1. Žilić Slađana (2023). Acrylamide in soybean products, roasted nuts and dried fruits. Chapter 11. In: Ed. Vural Gökmen and Burçe Ataç Mogol. Acrylamide in Food: Analysis, Content and Potential Health Effects. Second edition. Elsevier. pp. 644. ISBN: 9780323991193

2. Andrea Tafuri, Melania Zuccaro, Stefano Ravaglia, Raul Pirona, Stefania Masci, Francesco Sestili, Domenico Lafiandra, Aldo Ceriotti, Elena Baldoni (2023) Exploring Variability of Free Asparagine Content in the Grain of Bread Wheat (*Triticum aestivum* L.) Varieties Cultivated in Italy to Reduce Acrylamide-Forming Potential. Plants 2023, 12, 1349. https://doi.org/10.3390/plants12061349

7. Training school

Title: **„Approach to assessment of acrylamide mitigation measures in cereal-based food processing"**
Trainer: dr Slađana Žilić
Title of lecture: Aspects of acrylamide mitigation measures: impact on nutritional profile
Trainees: Beka Sarić, PhD student
Data: September 07 – 08, 2023
Place: National Agricultural and Food Centre, Food Research Institute (NPPC, VUP), Bratislava, Slovakia

8. Database on asparagine content of cereals

1. From STSM of Barbora Karnikova, asparagine of 15 barley genotypes from two years
2. From research of Maize Research Institute, free asparagine of 20 maize genotypes from two years
3. Bilateral project of Republic of Serbia and Republic of Turkey, free asparagine of 4 maize genotipes from two years after application of sulfur-based fertilizers and sulfur-based herbicides (36 combinations)
4. Identification of bread wheat cultivars with low content of free asparagine in the seeds. further activities related to the reduction of free asparagine in this cultivars using technological processes (*e.g.*, fermentaion with lactic bacteria) are in progress (Elena Baldoni)
5. Identification of genomic regions associated to free asparagine accumulation in durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.)) seeds through GWAS. the activity is carried out using a panel of landraces from the Global Durum Panel (https://doi.org/10.3389/fpls.2020.569905) (Elena Baldoni)

9. Deliverable report

D2.1 Overview of research activities in Europe to reduce acrylamide in cereal-based foods for the field of agronomy and plant breeding (M12)

10. Dissemination

Polish Milling Magazine, Poland. 1st Acryred Meeting 11-12 May 2023. Przegląd Zbożowo-Młynarski 2023 (3), 15 (in Polish).
website: https://www.ibprs.pl/en/reducing-acrylamide-exposure-of-consumers-by-a-cereals-supply-chain-approach-targeting-asparagine/ (in English)

11. PhD student progress

- Completed the first year of field expert of the doctoral dissertation on the topic - the effect of sulfur fertilizers on the content of free asparagine and the nutritional profile of wheat, rye and spelt and their phenotypic characteristics and the consequences on the content of acrylamide in biscuits. PhD student: Beka Sarić, Mentor: Dr. Slađana Žilić

- Completed the Doctorate at University of Tuscia, Department of Agriculture and Forest Sciences (DAFNE; Italy) - PhD Programme in Plant and Animal Science - XXXV Cycle. Defence of the PhD Thesis on 19th July 2023. Thesis title: “Exploring variability in free asparagine content to reduce the acrylamide-forming potential of wheat grain”. PhD student: Andrea Tafuri. Supervisor: Dr. Aldo Ceriotti. Co-Supervisors: Dr. Elena Baldoni and Dr. Stefano Ravaglia

3. Conclusion

Members of the Working Group 2 organized the annual meeting of the management committee and the working groups. They published two scientific papers and participated at conferences with five studies. They are editing four special issues, hosted one STSM student and won one ITC grant. There was a dissemination activity and also progress made in PhD projects, which could be useful in building a database on asparagine content of cereals.

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