SUMMARY

Work-package 1

In order to elucidate the role of lipoxygenases (LOXs) in plant defense mechanisms, in particular for what concerns fungal infections producing mycotoxins, the Unit of Research Biochemistry and Molecular Biology, University of Teramo, analyzed the catalytic activity of LOX in wheat flour (*Triticum aestivum*). Therefore, in this work the specific LOX catalytic activity was analyzed in different cultivars in order to identify a possible correlation between the activity of endogenous LOX and the presence of fungal mycotoxins. The seeds were sampled from CREA and milled by the IZS Lazio-Toscana. The primary sequence comparison between the soybean LOX-1 and wheat LOX yielded a sequence homology of about 52.24% between the two enzymes. Therefore, we were able to develop for the first time a Western blot method of identification of LOX in wheat by using an anti-soybean-LOX monoclonal antibody. Thus, using this approach LOX was detected in all analyzed samples. In addition, the specific LOX catalytic activity was also evaluated by spectrophotometric analysis. The specific activity of LOX resulted significantly different among the analyzed samples. To verify if these changes could be due to seed chemical treatment or to any generalized alterations due to physicochemical alterations of the seeds, the specific activity of a constitutive enzyme was also analyzed. In particular, the specific activity of glucose 6-phosphate dehydrogenase (G6PDH), of the pentose phosphate pathway was investigated. The results showed that the observed differences in LOX specific activity are not due to physical-chemical alterations of the seeds nor to extraction protocols of the enzymes. Indeed, the G6PDH specific activity resulted comparable in all analyzed samples. In addition, the presence of a specific LOX activity in wheat samples was further demonstrated using a specific irreversible LOX inhibitor, the eicosatetraenoic acid (ETYA). Taken together, these results demonstrated for the first time the presence of an intrinsic variability of the LOX enzymatic activity in different wheat cultivars, both before and after tanning treatment. However, the comparison between the specific LOX activity in treated and untreated samples suggests that the tanning procedure of seeds could reduce the activity of the enzyme, thus modulating the LOX pathway. Taken together, these data suggest the possible presence of a greater fungal contamination in those samples with a lower LOX activity. The different specific activity found in the various cultivars of wheat could be due either to genetic polymorphisms leading to specific biochemical and metabolic characteristics or to the presence of epigenetic modifications modulating 1-*lox* gene expression.

In conclusion, these results suggest a possible use of LOX both as a useful biomarker for assessing fungal contamination but also for the selection of more resistant cultivars against micotoxins producing fungi.

Work-package 2

Common wheat is one of the major crops in the world in terms of food and agronomic interest. Belonging to *Triticum* genus, common wheat is one of the most widespread staple cereal in food industry. It consists of several cold-resistent cultivars allowing its cultivation also in frigid climate areas. Durum wheat (*Triticum durum)* is a minor worldwide distribution and it is mainly use for pasta and bread products.

In the nineties Monsanto, one of the major industries operating in the biotech sector, was authorised in the USA to field test genetically modified common wheat tolerant to glyphosate herbicide. However, after completion of the experimental phase, this GM wheat never stepped into commercialisation. Nevertheless, some years ago in Oregon, the presence of glyphosate tolerant wheat was noticed by a researcher of the Oregon State University and than confirmed by United States Department of Agriculture (USDA) several years later (2013). At a later time the Animal and Plant Health Inspection Service (APHIS) notified the event to the European Commission and the member states of the European Union. In order to evaluate the extent of the problem in imported food materials, if any, the Commission decided border inspection posts to start random sampling on consignments of common wheat at import from the United States of America.

In lack of event-specific methods for the identification of such unauthorised GM wheat, the

European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) developed a strategy to detect GM wheat in wheat samples by using both taxon-specific and screening tests. This strategy was then described in a guidance document published on the EURL-GMFF website, together with a comprehensive review on all available relevant literature on taxon-specific real time PCR systems for the identification of *Triticum aestivum*. The results of the studysuggest that the method described by Matsuoka et al 2012, targeting SSII-D gene coding for starch synthase, and the one described by Iida et al 2005, targeting waxy-D1 gene, coding for granule-bound starch synthase, may represent good candidates to uniquely identify common wheat in complex food samples.
In our project, unit 5 (OU5 Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria-CREA), after having conducted a census of *Triticum* cultivars of food and feed interest, in particular *Triticum aestivum*, started collecting seed samples from its seed testing activity. In order to evaluate possible within species copy number variation of the two above mentioned target sequences (waxy-D1 and SSII-D), all wheat cultivar samples collected were analysed in parallel by real time PCR and digital droplet PCR platform, being the latter one of the most recent and promising technique in the field of quantitative PCR, allowing absolute quantification of the nucleic acid target in the sample. Data analysis showed that the copy number of both target PCR sequences seem to be stable among all wheat cultivars under investigation.
Concerning real time PCR, the gold standard for GMO analysis, the two system waxy and SSII-D were compared for several validation parameters such as specificity, PCR efficiency (slope and R2), limit of quantification and precision, in accordance with "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" document.
Specificity was evaluated on potatoes, maize, rice, soybean, canola and cotton DNA and both systems showed to be 100% specific for common wheat. Table 1 shows slope and R_2 of regression curves for all cultivars tested with the two PCR systems: sixteen calibration curves were obtained by matching real time PCR Ct values to copy number observed by ddPCR. Regarding precision, Real time PCR waxy and SSII-D methods showed the same standard deviation trend all over the dynamic range (five dilutions 1:4 from 300 ng of DNA). Finally, sequencing of PCR target regions of waxy and SSII-D didn't show single nucleotide polymorfism (SNP) for any of the 16 cultivars tested.
The two methods seem to be equivalent in terms of validation parameters and they could be employed, as taxon specific module, for quantiying GM common wheat in food and feed samples. Nevertheless waxy system shows a more complete literature that highlights target stability in terms of copies number and sequence conservation among cultivars also at international level.