Comparative Study of Different Cereals as Substrates for T-2 and HT-2 Production by *Fusarium langsethiae*

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***Abstract*—*Fusarium langsethiae* has been isolated from infected oats, wheat and barley, mainly, in Central and Northern Europe. This species has been implicated in the production of high levels of T-2 and HT-2 toxins in cereals, especially in oats. Cereals and their by-products are basic foods in human and animal diet. They are a primary source of components that are beneficial to human and animal health. There are limited data available regarding the host sensitivity and effect of environmental variables such as relative humidity and temperature on the biosynthesis of T-2 and HT-2 by this species. The aim of this work was to know the effect of cereal type and environmental conditions on production of T-2 and HT-2 by different isolates of *F. langsethiae* isolated from cereals in order to determine the effect of host and physical, chemical or biological variables, associated to cereal technology, in the accumulation of T-2 and HT-2 in the grain. Toxins were analyzed by an optimized HPLC method. In general, the highest T-2 and HT-2 levels were found in oat grains, followed by barley, wheat, corn, sorghum, rye and rice grains incubated at 25 ℃ and 0.98 a_w. At 20 ℃ and 0.95 a_w toxin levels were very low in all the assayed cereals. ANOVA showed that T-2 and HT-2 production by *F. langsethiae* were significantly affected (P > 0.001) by temperature, a_w and type of cereal.**

Index Terms*—cereals, T-2 and HT-2 toxins, temperature, relative humidity, *Fusarium langsethiae

I. Introduction

Cereals and their by-products are basic foods of human and animal diet. They have high beta-glucan content, good taste, dietetic properties, and anticarcinogenic effects. Beta-glucan is known as a prebiotic, stimulating the growth of some beneficial residential colon microorganisms such as bifidobacteria [1]. *Fusarium* species occur widely in nature as saprophytes and plant parasites. All cereals are prone to *Fusarium* spp. infection in pre-and post-harvest and this infection, in general, reduces grain yield and/or contaminates the grain with a range of toxic metabolites detrimental to human and animal health.

Trichothecene mycotoxins produced by *Fusarium* species have been subdivided into type-A trichothecenes, which include T-2 toxin (T-2), HT-2 toxin (HT-2) and diacetoxyscirpenol (DAS), and type-B trichothecenes, such as deoxynivalenol (DON) and nivalenol (NIV). This categorisation is based upon the absence (type-A) or presence (type-B) of a keto group at C-8 of the trichothecene skeleton.

Fusarium langsethiae has been isolated from infected oats, wheat and barley in Central and Northern Europe [2], [3]. This toxigenic species is difficult to be detected because, generally, it does not produce visible symptoms on the seeds. *F. langsethiae* is closely related to *Fusarium sporotrichioides*, *F. poae* and *Fusarium sibiricum* [4] and has been involved in the production of high levels of T-2 and HT-2 in cereals, especially in oats in countries of Central and Northern Europe [5]-[11]. In cereals T-2 is readily metabolized to HT-2, which is a deacetylated form of T-2, but both toxins induce adverse effects with similar potency [12], [13]. T-2 and HT-2 show immunosuppressive and cytotoxic effects both *in vivo* and *in vitro* and induces DNA fragmentation characteristic of apoptosis [14], [15].

Distribution of T-2 and HT-2 appears to be largely restricted to Europe and in the last years highest levels of T-2 and HT-2 have been detected in cereals in Nordic countries and the UK, although this may, in part, be due to the lack of analysis in other regions of the world. HT-2 and T-2 are currently being considered for legislation in the EC. A previous recommendation (2013/165/EU) has been published in the Official Journal of the European Union. The aim of this Recommendation is to collect information that will ultimately support the assessment of trends in levels and exposure to T-2 and HT-2, and that may contribute to the understanding of the factors affecting levels. The wide range and frequent presence of

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these toxins found in cereals reveal an increasing need for research on these factors. Among the cereal species, T-2 and HT-2 usually present higher incidence levels and concentrations in oats (*Avena sativa*) [6]. *Fusarium sporotrichioides* has been reported to be the major producer of T-2 and HT-2 in cereal grains in the South of Europe, although *F. langsethiae* has been detected in barley in Northern Spain [16].

There are limited data available regarding the host sensitivity and effect of environmental variables linked to weather and agro-climatic regions, such as humidity and temperature, on the biosynthesis of T-2 and HT-2 by *F. langsethiae*. The aim of this work was to know the effect of cereal species and environmental conditions on production of T-2 and HT-2 by different isolates of *F. langsethiae* isolated from cereals in order to determine the effect of host and variables associated to cereal technology in the accumulation of T-2 and HT-2 in the grain. The obtained results can be very useful to predict risk of toxin presence in cereals in pre- and post-harvest and to apply prevention and control measurements.

II. Materials and Methods

A. Fungal Strains and Growth Conditions

Three strains of *F. langsethiae*, Fl22, Fl05 and Fl26 isolated from barley, were used. These strains are held in the Mycology and Mycotoxins Group Culture Collection (Valencia University, Spain). Strains were preserved in 15% glycerol at -20 ℃. Before carrying out the study on the influence of cereal type and environmental factors on mycotoxin accumulation, the strains were grown on YES medium. The culture medium was autoclaved for 30 min at 115 ℃. The medium was poured into 9-cm diameter Petri dishes. The three strains of *F. langsethiae* were inoculated and incubated for 7 days at 25 ℃. These fresh cultures were used to prepare inocula for further experiments on toxin production in cereal grains.

B. Effect of Cereal Type and Environmental Conditions on T-2 and HT-2 Production

Oat, barley, wheat, corn, sorghum, rye and rice grains (15g), previously analysed to ensure they had undetectable levels of T-2 and HT-2, were placed in Erlenmeyer flasks and autoclaved for 20 min at 121 ℃. Then, water activity (a_w) was adjusted to 0.95 and 0.98 by addition of sterile distilled water using a moisture adsorption curve for each cereal (a_w-values were checked with a Novasina RTD 502 equipment (Novasina GmbH, Pfäffikon, Switzerland). The hydrated cereal seeds were placed in sterile 9-cm Petri dishes to form a layer of grains. All treatments were inoculated centrally with a 3-mm diameter agar disk taken from the margin of a 7-day-old growing colony. Inoculated Petri plates of the same a_w were enclosed in sealed plastic containers together with beakers of a glycerol-water solution matching the same a_w as the treatments to maintain a constant equilibrium relative humidity inside the boxes. The experiments were carried out in triplicate. Cultures were incubated at 20 ℃ and 25 ℃ for 15 days.

C. Chemical Analysis

Preparation of standard solutions: Standards of T-2 and HT-2 were supplied by Sigma (Sigma-Aldrich, Alcobendas, Spain). Each standard of T-2 and HT-2 was dissolved in acetonitrile at a concentration of 1.0 mg/mL and stored at -20 ℃ in a sealed vial until use. Working standards were prepared by appropriate dilution of known values of the stock solution with acetonitrile and used to obtain the calibration curves.

Extraction and clean-up of T-2 and HT-2 from oat grains: To determine toxin levels in the cultures, all the cereal culture distributed as a homogenous layer into the Petri dish was used, regardless of the colony diameter reached after 14 incubation days. Grains were milled and homogenized. Two g of each milled cereal grain were placed into a 50-mL screw-capped tube for trichothecene extraction. Fifteen mL of solvent mixture (acetonitrile-water, 84:16, v/v) was added and the tube was shaken in an orbital shaker (Infors-HT Aerotron, Bottminghen, Switzerland) for 90 min. The extract was filtered through Whatman No. 4 filter paper. The filtrate was kept at -20 ℃ until used. Solid-phase extraction cartridges containing alumina, charcoal and C18 were prepared following the methodology described by Valle-Algarra *et al.* [17]. Three mL of filtrate sample extract was passed through the cartridge using a manifold and collected into a vial. The cartridge was washed with 2 mL of the same extraction solvent. The eluate was collected in the same vial and the extract was dried at 45 ℃ under a gentle stream of nitrogen. The dried extract was redissolved in 250 µL of Milli-Q pure water with help of a vortex mixer. In the case of heavily contaminated cultures further dilution (up to 1:15 v/v) was accomplished. Fifty µL of the final extract was injected into the chromatographic system.

Chromatographic analysis: The LC system consisted of a Waters 600E system controller, a Waters 717 Plus autosampler and a Waters 996 UV diode array detector (DAD) (Waters, Milford, MA, USA). T-2 and HT-2 were separated using a C18 Zorbax Eclipse Plus (150 x 4.6 mm, 3.5 µm) (Agilent Technologies, Waldbronn, Germany), with a guard column of the same material. Analysis was performed in the gradient mode using water (A) and acetonitrile (B) solvents. Gradient conditions were initiated by holding the mobile phase composition for 3 min with 30% B. After that, it was linearly changed to 55% B during 18 min. The composition was then changed to 99% B in 1 min and maintained for 5 min as a cleaning step to improve results. After cleaning, the eluent composition was returned to the initial 30% B. The flow-rate of the mobile phase was 1 mL/min [18].

Validation of the analytical method: The analytical method used for T-2 and HT-2 was assessed for selectivity, linearity, and precision according to Mateo *et al.* [18]. Selectivity was checked by injecting three times 50 µL of T-2 and HT-2 standard solutions before injecting extracted samples and comparing the peak retention times and the UV spectra of the substances that produce these peaks. Linearity was assessed by performing triplicate injections of standard solutions whose concentrations

were in the 0.5-10 mg/L range for both mycotoxins. Standard curves were generated by linear regression of peak areas against concentrations. Precision and recovery were established by determination of T-2 and HT-2 in each grain, covering the range of the method. Limit of detections (LOD) were considered as the T-2 and HT-2 concentrations that provide a signal equal to b + $3s_b$, where b is the intercept of the respective calibration curves and s_b is the standard error of the estimate assuming to be the blank. The limit of quantification (LOQ) was considered equal to 3 x LOD [19].

Statistical analysis: The data were treated by multifactor ANOVA and Duncan's test of multiple comparisons using Statgraphics Centurion XV.2.11 software (Statpoint Inc., VA, USA). A 95% confidence level was used to assess influence of individual and interacting treatments.

III. Results and Discussion

T-2 and HT-2 toxins were determined in cereal grain cultures of the three *F. langsethiae* strains under all the assayed conditions. Fig. 1, Fig. 2, Fig. 3 and Fig. 4 show the effect of temperature, a_w, and class of cereal on T-2 and HT-2 production.

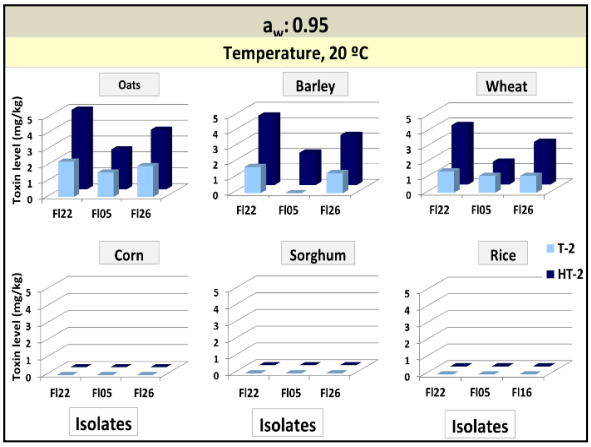

Figure 1. T-2 and HT-2 toxin production by three isolates of *F. langsethiae* (Fl22, Fl05 and Fl26) in cereal grains at 0.95 a_w and 20 ℃. Toxin levels are averages of three assays.

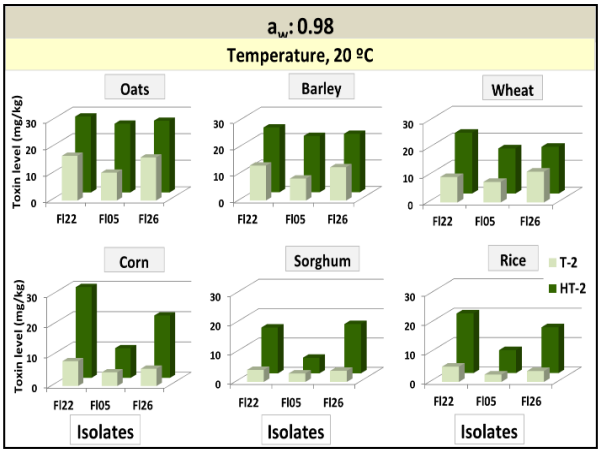

Figure 2. T-2 and HT-2 toxin production by three isolates of *F. langsethiae* (Fl22, Fl05 and Fl26) in cereal grains at 0.98 a_w and 20 ℃. Toxin levels are averages of three assays.

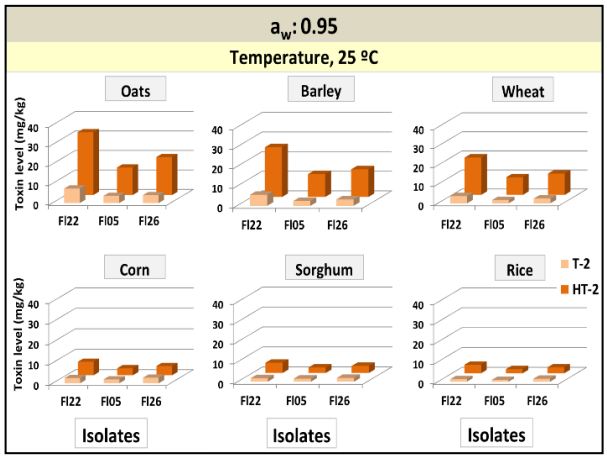

Figure 3. T-2 and HT-2 toxin production by three isolates of *F. langsethiae* (Fl22, Fl05 and Fl26) in cereal grains at 0.95 a_w and 25 ℃. Toxin levels are averages of three assays.

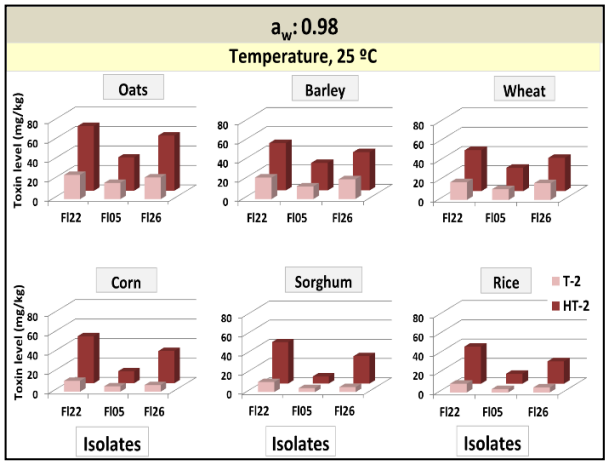

Figure 4. T-2 and HT-2 toxin production by three isolates of *F. langsethiae* (Fl22, Fl05 and Fl26) in cereal grains at 0.98 a_w and 25 ℃. Toxin levels are averages of three assays.

In general, concentrations of HT-2 were higher than those of T-2, regardless of environmental conditions and type of cereal. HT-2 concentration was always higher at 25 ℃ than at 20 ℃ and higher at 0.98 a_w than at 0.95 a_w and in the order oats > barley > wheat > corn > sorghum > rice. ANOVA showed that T-2 and HT-2 levels were significantly affected (P > 0.001) by temperature, a_w and cereal type. The highest HT-2 level (74.6 mg/kg) and T-2 level (28.9 mg/kg) were detected in oat seeds colonized by isolate Fl22 at 25 ℃ and 0.98 a_w.

The isolate Fl22 was the largest producer of T-2 and HT-2 in all the assayed cereals. The concentrations of T-2 in oat, barley, wheat, corn, sorghum and rice seed cultures of isolate Fl22 were 28.9, 26.4, 24.9, 22.4, 18.3 and 17.3 mg/kg, respectively, whereas the levels of HT-2 were 74.6, 63.2, 56.6, 49.3, 42.5, and 32.5 mg/kg, respectively.

The lowest levels of T-2 were registered in the cultures at 0.95 a_w and 20 ℃ of the isolate Fl05. T-2 concentrations were 1.9, 1.6, 0.9, 0.56, 0.35 and 0.22 mg/kg in oat, barley, wheat, corn, sorghum and rice cultures, respectively, and HT-2 concentrations were 3.4, 2.7, 2.1, 1.2, 0.9 and 0.62 mg/kg, respectively.

Although comparative studies about cereal susceptibility to T-2 and HT-2 production by *F. langsethiae* have not yet been reported, some studies have shown the effects of environmental conditions on the growth of *F. langsethiae* and T-2 and HT-2 production by strains of this species from England, Finland, Norway and Sweden on synthetic or semisynthetic media [20]-[22]. In these studies, the optimal levels of a_w and temperature for T-2 and HT-2 production by *F. langsethiae* strains, in general, agree with those obtained in the present study, regardless of the geographical origin of the strains.

Another relevant aspect to highlight in the present study is that the levels of T-2 in the six cereal cultures were lower than those of HT-2, regardless of the cereal type and environmental conditions. In previous studies [18], [23], the interaction between fungicides, environmental conditions, growth, and T-2 and HT-2 production in cultures of *F. langsethiae* in 3% milled oat media and in oat grains have been shown [18], [23]. In these reports it was demonstrated that there is a change in the T-2/HT-2 ratio depending on the substrates.

In semisynthetic medium, T-2 levels were higher than those of HT-2 whereas in oat grains, the opposite was found. Studies on the metabolism of T-2 have suggested that in animals the liver is the major organ for this transformation in HT-2 [24], [25].

Hepatic carboxylesterases have been shown to be responsible for the deacetylation of T-2 giving HT-2 as the major metabolite. The ability of plant carboxylesterases to control secondary metabolites has been reviewed.

In cereals, detailed studies have been carried out leading to the identification of esterases, which are active in pesticide metabolism [26].

Lattanzio, Solfrizzo, and Visconti [27] studied carboxylesterase activities in oats and other cereals. An enriched protein fraction with esterase activity toward T-2 was used to increase the activity of the carboxylesterases naturally present in wheat and oats. These authors found that in maize, the initial T-2 content was completely converted into HT-2 after 90-min incubation. Lower conversion rates (89%, 42% and 35%) were observed in wheat, oats and barley, respectively, after 120 min. Although T-2 can be readily metabolised to HT-2, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has evaluated the safety of different mycotoxins in food and has concluded that the toxic effects of T-2 and its metabolite HT-2 could not be differentiated, and that research into the factors involved in the presence of T-2 and HT-2 in cereals and cereal products, in particular oats and oat products, is necessary and of a high priority. In the present study in cultures at 0.98 and 0.95 a_w the ANOVA shows that all factors and their interactions were significant to both T-2 and HT-2 production.

In summary, no previous studies have examined the impact that interacting environmental conditions and type of cereal have on T-2 and HT-2 production by *F. langsethiae* on cereals. *Fusarium* mycotoxins are produced within and on the grains and it is important to understand how pre- and post-harvest environmental conditions can affect mycotoxin contamination of cereal. In the present study, we have looked at conditions that can be interesting within guidelines on "Good Storage Practice" to minimize contamination of different cereals with T-2 and HT-2 toxins produced by *F. langsethiae*. The results have shown, for the first time, data on the impact of cereal type under the influence of interacting ecological factors on T-2 and HT-2 accumulation in oat, barley, wheat, corn, sorghum, and rice seeds.

All factors appear to be very important in determining accumulation levels of these toxins in the cereal. In cereal technology, specially, in oats, barley and wheat, where the highest levels of toxins were found, a suitable control of a_w (< 0.95 a_w) and temperature (< 20 ℃) could prevent T-2 and HT-2 accumulation in cereal products ready for consumption. These measures are applicable to all stages of cereal technology. In pre-harvest, especially in humid areas or in unfavourable weather conditions, if necessary, approved and registered fungicides could be used as recommended by the manufacturers to control toxigenic *Fusarium* spp. In harvest and post-harvest, adequate temperature and a_w control should be sufficient to prevent *F. langsethiae* growth and toxin production.

New research is needed to control T-2 and HT-2 in cereal and derivatives food and feed. The Scientific Panel on Contaminants in the Food Chain (CONTAM panel) of the European Food Safety Authority (EFSA) [28] has indicated that "taking into account the conclusions of the scientific opinion, together with the large year to year variation in occurrence of T-2 and HT-2 toxin, it is appropriate to collect more data on T-2 and HT-2 in cereals and cereal products and more information on the effects of food processing (i.e. cooking) and agronomic factors on the presence of T-2 and HT-2 toxins. Furthermore, it is necessary to obtain more information on the different factors which lead to relative high levels of T-2 and HT-2 toxin in cereals and cereal products in order to be able to identify the measures to be taken to avoid or to reduce the presence of T-2 and HT-2 toxin in cereals and cereal products. Investigations have to be undertaken in order to collect information on the factors resulting in relative high levels of T-2 and HT-2 toxin in cereals and cereal products and on the effects of feed and food processing. Based on the available data, T-2 and HT-2 do not occur or only in very low levels in rice and rice products and therefore it is appropriate to exclude these products from the scope of this Recommendation". In the present report rice was the cereal less vulnerable, as a substrate, to T-2 and HT-2 production by *F. langsethiae*. This finding shows that rice appears not to be a probable source of T-2 and HT-2 in the diet, which agrees with the Commission Recommendation.

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Mr. Jose V. Gómez graduated in Biology in 2012 from University of Valencia, and obtained his M.Sc. in 'Research in Molecular and

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Dr. David Romera graduated in both Chemistry in 1997 and Food Science and Technology in 2007 from University of Valencia. Ms.C. in Optical Technologies Applied to Industry in 2008. He earned his Ph.D. in Chemistry in 2016 from University of Valencia. He worked as a Specialist Technician in Food Safety at the 'Generalitat Valenciana' (Valencian Autonomous Government, Spain), Chemist at the Agro-Food Laboratory (EU Reference Laboratory of Residues in Food and Vegetables, DG SANCO, European Commission-'Generalitat Valenciana'). Currently, he is Research Chemist at the 'Conselleria de Sanitat' ('Generalitat Valenciana'). He is author of research papers presented at national and international Conferences.

Ms. Andrea Tarazona graduated in Pharmacy in 2012 from University of Valencia. She has joined the 'Mycology and Mycotoxins' group in 2015 as a postgraduate student and is doing her Ph.D. studies at the Department of Microbiology and Ecology (University of Valencia). Her research is focused on a) the study of host susceptibility related to T-2 and HT-2 toxin production by *Fusarium langsethiae*, b) determination of both toxins in food destined to human consumption by high-resolution chromatographic techniques, c) Identification of ochratoxigenic fungi in food by microbiological techniques and species-specific PCR protocols, and d) study of the occurrence of *Aspergillus niger*, *A. ochraceus* and *A. steynii* in cereals produced in ecological and conventional agriculture, use of microbiological techniques and PCR protocols, physiological study and influence of environmental factors associated to climate change in their development and chemotyping of mycotoxin-producing strains. She also worked as pharmacist and has participated in many courses addressed to pharmacology, pharmaceutical technology and pharmaceutical/health assistance (University Clinic Hospital of Valencia).

Prof. Jose V. Gimeno-Adelantado graduated in Chemistry from University of Valencia. He joined the Department of Analytical Chemistry and completed his Ph.D. in Chemistry in 1981 from University of Valencia. He was Lecturer from 1973 to 1989, Associate Professor in 1989 and Full Professor since 2009 at the Department of Analytical Chemistry (University of Valencia). His research since the 80s has focused on the development of analytical methods for samples of mineral and organic origin using electron microscopy, spectroscopic and chromatographic techniques. In the last two decades he has dedicated his research to the analytical study of mycotoxins in food, as well as the study of materials of artistic and archaeological interest, also including electrochemical techniques. This research is carried out in collaboration with the group 'Mycology and Mycotoxins' of the Department. of Microbiology and Ecology (University of Valencia) and the Institute of Heritage Restoration from Polytechnic University of Valencia. He has been senior researcher in some national projects. He has published 150 articles in high-ranking journals, numerous book chapters and 300+ contributions to conferences and symposia.

Prof. Rufino Mateo-Castro graduated in both Chemistry and Pharmacy from University of Valencia. He joined the Department of Analytical Chemistry and completed his Ph.D. in Chemistry in 1989 from University of Valencia. He was head of the departments of instrumental analysis at both the Laboratory of Animal Health and the Laboratory of Pesticide Residues in Vegetal Products (Ministry of Agriculture, Fisheries and Food, Spanish Government) at Valencia from 1976 to 2012. Since 1990 he shared his activities as officer in those centers with docent duties as part-time professor at the Department of Analytical Chemistry. Since 2012 he is Associate Professor at the Department of Analytical Chemistry (University of Valencia). He has been research collaborator in many competitive Projects sharing activities between the group 'Mycology and Mycotoxins' headed by Prof. M. Jiménez and the artwork analytical team headed by Prof. J.V. Gimeno-Adelantado and Prof. A. Doménech-Carbó (University of Valencia). He was member of the COST Action 835 (European Commission, 1999-2003). He has published more than 80 articles in JCR journals and 14 book chapters. He has also presented 130+ research papers at national and international symposia and conferences. At present, his main research interest includes mycotoxin analytical methods and biology of toxigenic fungi infecting food commodities.

Prof. Misericordia Jiménez graduated in Biology and Ms.C. in Microbiology from University of Valencia (Spain), she obtained her Ph.D. degree in Microbiology in 1987 from University of Valencia. The same year she created the 'Mycology and Mycotoxins' research group at the Department of Microbiology and Ecology (University of Valencia), where she is head of this group. This group is internationally recognized for research on ecophysiology and characterization (morphological, physiological and molecular) of toxigenic fungi in crops (cereals, citrus fruits, nuts, grapes…) and derivatives, design and optimization of analytical methods (GC, LC, GC-MS/MS, UHPLC-MS/MS, UHPLC-QTOF-MS) for determination of mycotoxins in food and feed, modelling of toxigenic fungi growth and toxin production (with especial attention to new scenarios associated to climate change), effects of food processing and agronomic factors on toxigenic fungi and mycotoxins, discovery of new strategies and bioactive compounds exhibiting antifungal activity… She has lead numerous research projects about these topics and was member of the Management Committee of COST action-835 (European Commission, 1999-2003). Her group has published 250+ research papers (JCR), numerous book chapters and has been considered a group of excellence by the 'Generalitat Valenciana' (Spain). Dr. Jiménez is full professor at Valencia University in different degrees: Biotechnology, Biochemistry and Biomedicine, Biology and Environmental Sciences and has supervised numerous Ph.D. Theses and 150+ Training Projects (graduated student) in companies, hospitals and research institutes