PHYSICAL CHEMISTRY 2018

14th International Conference
on Fundamental and Applied Aspects of
Physical Chemistry

Proceedings
Volume I

September 24-28, 2018
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ISBN 978-86-82475-36-1
Title: Physical Chemistry 2018 (Proceedings)
Editors: Željko Čupić and Slobodan Anić
Published by: Society of Physical Chemists of Serbia, Studentski Trg 12-16, 11158, Belgrade, Serbia
Publisher: Society of Physical Chemists of Serbia
For Publisher: S. Anić, President of Society of Physical Chemists of Serbia
Printed by: ″Jovan″, <Printing and Publishing Company, 200 Copies
Number og pages: 550+6, Format B5, printing finished in September 2018

Text and Layout: ″Jovan″

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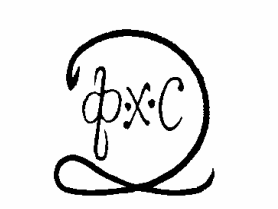

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14th International Conference on
Fundamental and Applied Aspects of
Physical Chemistry

Organized by

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in co-operation with

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and

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NON-INVASIVE CHARACTERIZATIONOF AFLATOXIN-STRESSED WHEAT SEEDSUSING 2D EPR IMAGING AND EEM FLUORESCENCE SPECTROSCOPY

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ABSTRACT

In this work, two non-invasive and independent methods for the characterization of aflatoxin-stressed wheat seeds were applied.Aflatoxins (AFs) are secondary toxic fungi metabolites, which exhibit hazardous effects on plants (phytotoxic), as well as on humans and animals. First, for 2D EPR imaging, we used 3-CP spin probe for the estimation of changes in the redox state of the aflatoxin-stressed seed. On the other side, an excitation-emission fluorescence approach was used to evaluate the spectral profiles of the key biological molecules. We determined their areas and the ratio of the two emission peaks at 355 nm (Ex 290) and 430 nm (Ex 350), corresponding to protein and phenol emission, respectively. Our results showed that the ratio of the two peaks (p/f) decreased in the presence of AFs. Both methods revealed to be useful for the rapid, non-invasive assessment of the seeds' stress state induced by AFs.

INTRODUCTION

Aflatoxins are mycotoxins, which are toxic secondary metabolites produced by fungi that colonize crops and are commonly found as contaminants in cereal food. Chemically, they are difuranocoumarin derivatives produced in a polyketide pathway; among the known aflatoxins, Aflatoxin B1 (AFB1) is the most toxic, mutagenic and carcinogenic [1, 2]. It has been shown that AFB1 can be harmful to both consumer health and plants (phytotoxic effects) [2]. Several analytical methods have been employed to analyze aflatoxins in agricultural food crops and feed [1]. Non-invasive methods, such are EPR imaging and fluorescence spectroscopy provide useful information about normal and physiological as well as pathophysiological processes of the biological systems [3, 4, 5]. These methods were used to analyse AFB1 contaminated wheat seeds. The changes in redox state of the stressed seeds comparing with unstressed ones were correlated with the structural changes observed by fluorescence spectroscopy.

EXPERIMENTAL

Wheat (*Triticum aestivum* L.) seeds, contaminated - and uncontaminated with aflatoxin B1 (AFB1), were used in this study. EPR experiments were carried outwith a Bruker ELEXSYSII spectrometer, operating in the X-band frequency domain (9.8 GHz). The following conditions were set for the measurement: Sweep width, 284.85 G; microwave power, 10.02 mW. All measurements were performed at ambient temperature. 2D imaging was done in the YX plane with respect to the main fields; the magnetic field gradient was 30 G/cm. We used 3-carbamoyl-2, 2, 5, 5- tetramethyl-1-pyrrolidonyl-N-oxyl (3-carbamoyl PROXYL or 3-CP) as spin probe. The seeds were imbibed one hour before the start of the measurement. The program software The Bruker Xepr, was used to process the EPR data. Also, we study the fluorescence characteristics of seed samples via their excitation-emission matrix (EEM) spectrum. The samples' fluorescence spectra were recorded using an Fl3-221 P spectrofluorimeter (JobinYvon, Horiba, France), equipped with a 450 W Xe lamp and a photomultiplier tube. The emission spectra ranging from 300 to 600 nm were recorded with an excitation wavelength ranging from 280 to 350 nm. Integration time was 0.1 s. A spectral bandwidth of 2 nm was for both the excitation and emission slits.

RESULTS AND DISCUSSION

Figure 1 (A) shows the 2D image of the unstressed (-AFB1) and AFB1-stressed (+AFB1) wheat seeds. The seeds were positioned in a quartz capillary, one above another, as shown in Figure 1 A. The EPR imaging was applied for the mapping of redox state of the seeds through detection and localization of the 3-CP spin probe. The levels of 3-CP redox-sensitive spin active probe and the rate at which it is reduced, depends partly on the tissue redox status [4]. We observed the higher signal intensity of the 3-CP spin probe in the unstressed seed compared with AFB1-stressed seeds (Figure 3 A). The 2D images demonstrated that the spin probe appears to be concentrated in a particular region of the seed coat.

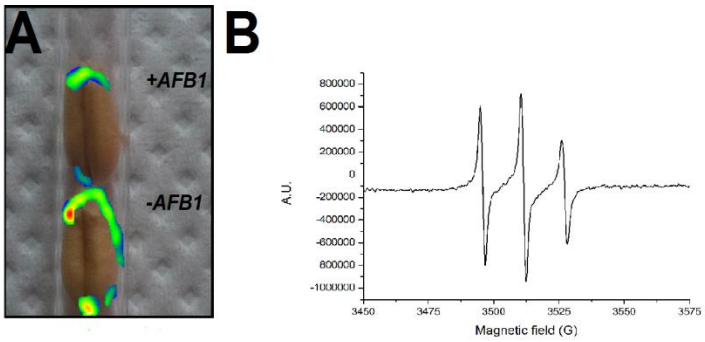

Figure 1. 2D EPR imaging of clearance of the 3-CP spin probe in aflatoxin stressed (+ AFB1) and unstressed (-AFB1) seeds (**A**); and typical EPR spectrum of 3-carbamoyl-PROXYL spin probe in seeds (**B**).

EEM fluorescence spectroscopy was employed to analyze the unstressed (A) and AFB1-stressed (B) seeds of wheat. In Figure 2, the representative contour maps are depicted. The comparison of the contour maps shows that both samples have fluorescence peaks which are characteristic for the protein and phenol regions, located at 355 nm (Ex 290 nm) and 430 nm (Ex 350 nm), respectively. The areas of the analyzed peaks were determined using the equation shown in (**3**).

$$\Phi_i = \int_{\lambda_{em}} I(\lambda_{em}) d\lambda_{em} \quad (3)$$

Φ_i–Area of *i*-th excitation;$I(\lambda_{em})$ - Intensity at emission wavelength atλ_{em};λ_{em}-Emission wavelength.

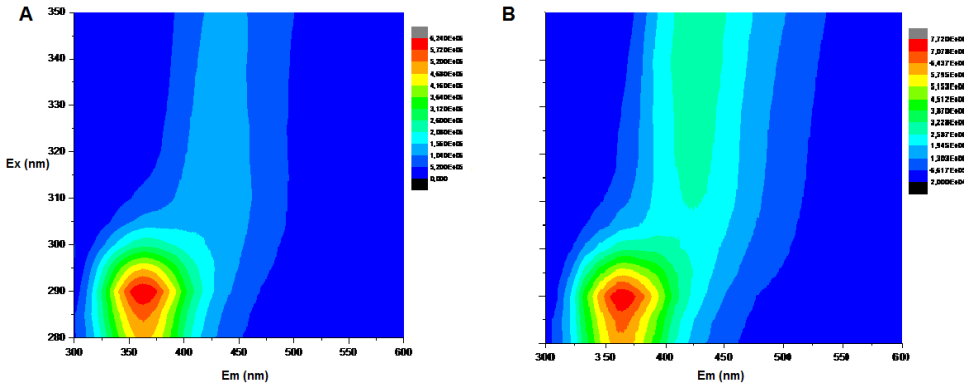

Figure 2. The representative EEMs of (A) unstressed (-AFB1) and (B) AFB1stressed (+AFB1) seeds

The fluorescence areas and ratios of the analyzed peaks are shown in Table 1. The results showed that the areas of the protein peaks were similar, whereas

the areas of the phenol peaks differed. The ratio of the AFB1-stressed seeds was about half of the ratio of the unstressed seed. In the phenol region of the spectrum, aflatoxins exhibit natural fluorescence. It has been proposed that the decrease in the ratio is due to an increase in the concentration of the fluorescence component(s).

Table 1. The fluorescence areas and their ratio for the aflatoxin-unstressed (-AFB1) and AFB1-stressed seeds.

Samples	Fluorescence Area (A.U.)		
	Em 355nm (Ex 290)	Em 430 nm (Ex 350)	**Ratio p/f**
(-AFB1)	85,05	20,814	**4,09**
(+AFB1)	82,36	47,64	**1,73**

CONCLUSION

2D EPR imaging was successfully applied for the redox characterization of aflatoxin-stressed seeds. The spin probe appears to be concentrated mostly in a particular region of the seed coat. To our understanding, the aflatoxin contamination leads to a change in the seeds' redox status, providing important information about its impact on seed metabolism. Also, the investigation of the EEM fluorescence and peak area ratios can contribute to a better understanding of fluorescence species in aflatoxin-stressed seeds.

Acknowledgements

This work was supported by the grants 173017, III41005 and 173040 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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