P77: BUCKWHEAT ANTIOXIDANT PROFILE MODULATES *ASPERGILLUS FLAVUS* AFB_1 PRODUCTION, *IN VITRO*

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Abstract – Field commodities are exposed to fungal contamination with potential negative impact on human health. Bioactive plant molecules, as for example antioxidant compounds present in common buckwheat (*Fagopyrum esculentum* Moench), can provide natural means of biocontrol.

To this purpose, the ability of flavonols extracted from common buckwheat hull to modulate, in *in vitro* conditions, *Aspergillus flavus* production of aflatoxin B_1 (AFB_1) was tested in comparison to synthetic rutin.

Positive results achieved let infer that the extract could be furtherly tested also *in vivo*.

Keywords: *Fagopyrum esculentum*, biomolecules, biocontrol, *Aspergillus flavus*, AFB_1.

1.INTRODUCTION

Over recent years, in popular thinking, the words food and health are often associated together. Issues like nutritional facts, traceability and health effects, lay hidden in a corner of consumer's minds and unawares guide them through their dietary choices, with the arguable belief that this alone may contribute to the health status. Further food related subjects, as for example fungal contamination and consequent mycotoxin production, should not be overlooked and kept as well in mind in view of their potential harmfulness. Indeed, the insurgence of pathogens may impair the food production chain already at field cultivation stage and cause severe crop damage. Fungal contamination has been traditionally restrained by the use of chemical compounds, whose residues have gradually accumulated determining a toxic effect on the environment. Alternatively, modern innovative techniques take advantage of the biocidal properties of natural biodegradable plant-based molecules, as polyphenols. These molecules show a broad spectrum of action capable to suppress various virulence factors [1] [2] with potential of application within integrated pest control management systems. Buckwheat (*Fagopyrum* spp.) represents a natural rich source of polyphenols, in particular rutin [3]. These can be recovered also from non-edible parts of the plant, such as hulls.

In this study, initially, the antioxidant profile (polyphenols and enzymes) of viable achenes, artificially inoculated with AFB_1 producer-*Aspergillus flavus*, was analysed. Achenes infection determined an increase of polyphenols concentration and a higher activation of antioxidant enzymes. In this context, polyphenols can be considered as markers of tolerance, and their effect on AFB_1 production was investigated *in vitro*, in first instance amending cultural medium with synthetic rutin and in alternative with common buckwheat hull extract.

2.EXPERIMENTAL

2.1Buckwheat achenes and hulls

Fagopyrum esculentum (var. Aelita) achenes originally were kindly supplied by "The Crop Research Institute", Prague (CZ) and subsequently multiplied. Hulls were grinded with a "CYCLOTEC 1093 Sample mill" (Tecator) and sieved with a 1000 µm mesh prior to use.

Fungal strain:AFB_1-producing *Aspergillus flavus* strain - NRRL 3357 - was kindly provided by ATCC.

Analytical procedures: The inoculation procedure was carried out, enzymes and antioxidant activities were evaluated, total polyphenols content, polyphenols analysis and aflatoxin extraction and analysis were performed, as previously described [4].

Evaluation of rutin antifungal activity: Erlenmeyer flasks containing 50 ml of potato dextrose broth (PDB) were added with synthetic rutin to obtain individual concentrations of 0.02; 0.2; 2 mg/mL and

subsequently inoculated with 500 µL of a *A. flavus* spore suspension containing approximately 1.0×10^5 conidia/mL. PDB without addition of rutin was used as negative control. Samples were incubated at 30 °C for 168 hours and measured at 24 hours intervals for rutin, quercetin and aflatoxin content.

Evaluation of hulls' extract antifungal activity: 600 mg of ground hulls were extracted with 4 ml of ethanol for 1 hour. AFB_1 production was evaluated for 168 hours by inoculating PDB amended at three different concentrations (50, 100, 500 ng/mL) of the hulls' ethanolic extract (EE). Not amended inoculated PDB represented the positive control.

Statistics: All data are reported as the mean of three replicates ± standard deviation (SD) and were subjected to statistical analysis through the t test with significance level $p<0.05$.
Principal Components Analysis (PCA) [5], based on Spearman test, was employed for the correlation between AFB_1 production in seeds (variable AFB_1_ACH) and the other variables under investigation, namely rutin content (rutin), quercetin content (quercetin), antioxidant activity (PO) and enzymatic activity (Σ). All the statistical analyses were performed using XLSTAT, 2009.4.06 software (Addinsoft, Paris, France).

3.RESULTS AND DISCUSSION

Analysis of antioxidant activities in achenes: Plants respond to pathogens by activating a variety of defense mechanisms, including the rapid production and accumulation of reactive oxygen species (ROS) [6]. The increase of internal ROS levels, induces plant cells to activate enzymatic (i.e. catalases-CAT, superoxide dismutases-SOD) and non-enzymatic (i.e. glutathione system-GPX) antioxidant responses [6] [7]. Taking into account these considerations, CAT, SOD and GPX activities were analysed in infected and non-infected viable common buckwheat achenes.
Results, visible in Fig. 1 A, B, C respectively, speak for a quite evident, infection related, antioxidant defensive response, showing for all three enzymes a peak of the activities, at 72 hours post inoculation (hpi). Non-infected grains did not show significant differences in ROS scavenging enzyme activities.

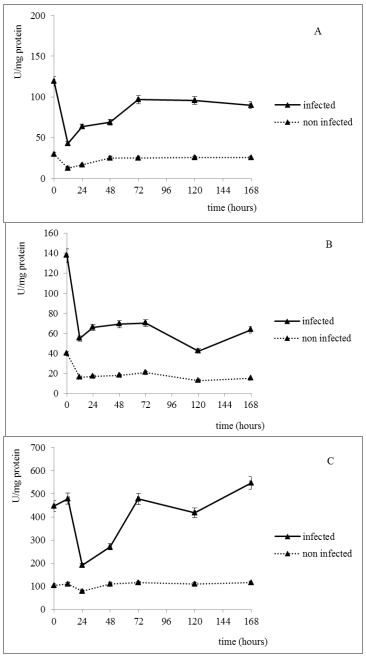

Figure 1. (A) Catalase (CAT), (B) superoxide dismutase (SOD) and (C) glutathione peroxidase (GPX) activity trend in achenes either inoculated or not with *Aspergillus flavus* .

The free radical scavenging capacity of common buckwheat achenes was measured (Fig. 2).

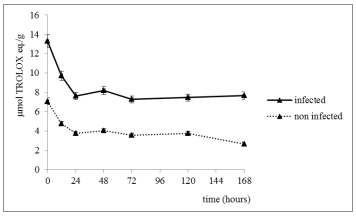

Figure 2. Total antioxidant activity in common buckwheat achenes either inoculated or not with *Aspergillus flavus*.

The results of the Trolox assay showed a significantly higher antioxidant activity associated to infected achenes, twofold compared to those non inoculated.

Phenolic compounds in achenes: Rutin and quercetin contents were measured in *A. flavus* - infected and non-infected achenes (Fig. 3).

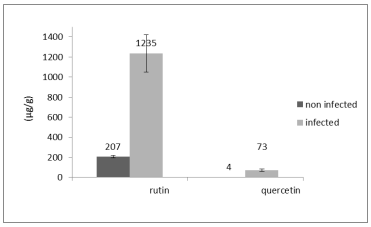

Figure 3. Difference in total rutin and quercetin production in achenes, either inoculated with *A. flavus* (infected) or non-infected at 168 hpi.

Rutin resulted the main flavonoid present in *F. esculentum*, confirming data previously reported [8]. At any time during the experiment, rutin and quercetin amounts were significantly higher in inoculated achenes compared to those who were not.

Aflatoxin occurrence in inoculated achenes: AFB_1 production was monitored in achenes at 24 hours intervals, up to 168 hours after inoculation. Its presence was detected at 72 hpi (167 ng/g) and increase with time: 1300 ng/g at 120 hpi and 3005 ng/g at 168 hpi.

Correlation between aflatoxin biosynthesis and antioxidant profile: PCA plot (Fig. 4) showed a significant positive correlation of AFB_1 biosynthesis (AFB_1_ACH) with the other variables under investigation.

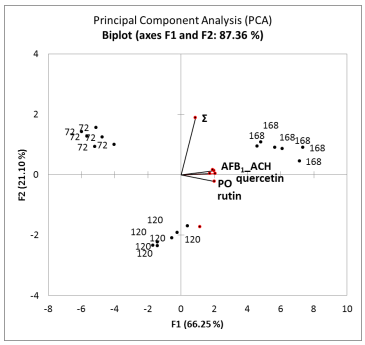

Figure 4. PCA showing correlation between AFB_1 production in infected achenes (AFB_1_ACH), rutin (rutin) and quercetin content (quercetin), antioxidant activity (PO) and antioxidant enzymes (∑) in infected achenes at 72 hpi, 120 hpi and 168 hpi.

Furthermore, it appeared evident how the reaction to fungal infection started at 168 hpi. These significant correlations suggest the existence of an important underlying physiological mechanism that possibly controls achenes reaction to fungal infection.

Influence of rutin and its aglycone quercetin on aflatoxin synthesis: Results achieved put in evidence the influence that rutin, and possibly its aglycone quercetin, play on the biosynthesis of aflatoxin in *A. flavus* infected common buckwheat achenes.

To further confirm the crucial role played by this natural antioxidant in inhibiting mycotoxin production, *A. flavus* was inoculated in PDB amended with rutin at three different orders of magnitude (Fig. 5).

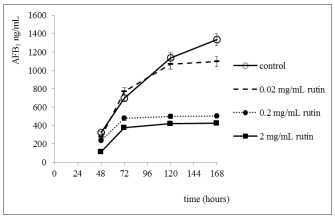

Figure 5. AFB_1 quantification in PDB, containing rutin at 3 different concentrations (0.02; 0.2; 2 mg/mL) inoculated with *Aspergillus flavus* isolate and incubated at 30°C observed at different times (24-168h).

Effects of rutin treatment at 0.02 mg/mL on mycotoxin production were noticed only 168 h after inoculation with a significant reduction of approximately 15%. Samples treated with higher rutin concentrations showed significant levels of inhibition (75%) already after 72 hpi.

Additionally the behaviour of flavonoids in the culture media was analysed. In all cases, a rapid degradation of rutin to quercetin, a powerful antioxidant compound [9] [10], took place starting at 72 hpi, appearing particularly evident in PBD containing higher rutin concentration (Fig. 6).

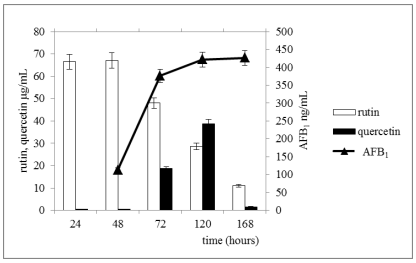

Figure 6. Rutin, quercetin and AFB_1 trend in medium containing rutin at 2 mg/mL rutin.

This can be explained by natural oxidation and additionally by the possible presence of rutin-degrading enzymes (rutinase) produced by *A. flavus* [11], capable to hydrolyse rutin into quercetin and rutinose [12].

The effect of Added RUtin (control; 2; 0.2 and 0.02 mg/ mL-ARU) on the behaviour of the other variables detected, namely aflatoxin production (AFB_1), quercetin (QUE) and rutin (DRU), was evaluated during incubation (Tab. 1).

Table 1: Spearman correlation between aflatoxin B_1 concentration (AFB_1) and other detected variables, at different

incubation times. Values in bold are different from 0 with a significance level alpha=0.05

	48	72	120	168
	AFB_1			
ARU	**-0.775**	**-0.969**	**-0.808**	**-0.775**
DRU	-0.355	0.348	-0.263	-0.375
QUE	-0.199	**-0.942**	**-0.799**	**-0.741**

In this contest, at all times, AFB_1 was negatively correlated in respect to the amounts of rutin added (ARU), proving that a higher concentration of rutin may determine a decreased production of aflatoxin. Moreover, at 72 hpi there was no correlation between DRU and aflatoxin production, while there was a very high significant correlation between AFB_1 and quercetin, thus suggesting the involvement of this flavonol in the mechanism of aflatoxin biosynthesis inhibition.

Influence of common buckwheat hull extracts on AFB_1 synthesis: Having evinced that *F. esculentum* polyphenols play a key role in the plant pathogen interaction, the hull of this species was extracted and the solution obtained (EE) was characterized for the antioxidant activity (7.41 µmol Trolox EQ/g), and the total polyphenols (4.89 mg/g Dry Weight-DW), rutin (306 µg/g DW) and quercetin (25 µg/g DW) content, content prior to testing as antifungal agent (Fig 7).

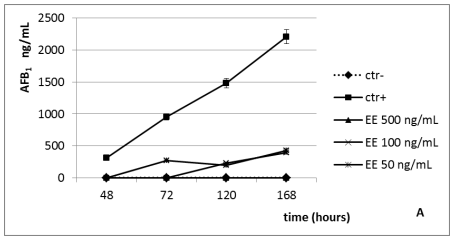

Figure 7. AFB_1 production trend in PDB medium amended with common buckwheat hulls' extract (EE).

Notably, ethanolic extract, provided at 500 ng/mL, totally inhibited AFB_1 synthesis; while at 100 and 50 ng/mL the effect was only partial (75%) and limited in time, thus suggesting that the inhibition process may be concentration-dependent.

Positive results achieved let infer that the extract could be furtherly tested also in *in vivo* conditions.

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