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Buckwheat achenes antioxidant profile modulates *Aspergillus flavus* growth and aflatoxin production

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ABSTRACT

Buckwheat (*Fagopyrum* spp.) is a "pseudo-cereal" of great interest in the production of healthy foods since its flour, derived from achenes, is enriched with bioactive compounds and, due to the absence of gluten, may be used in composition of celiac diets. Amongst buckwheat species, *F. tataricum* achenes possess a larger amount of the antioxidant flavenol rutin than the common buckwheat *F. esculentum*. Ongoing climate change may favor plant susceptibility to the attack by pathogenic, often mycotoxigenic, fungi with consequent increase of mycotoxins in previously unexploited feeds and foodstuffs. In particular, *Aspergillus flavus*, under suitable environmental conditions such as those currently occurring in Italy, may produce aflatoxin B_1 (AFB_1), the most carcinogenic compound of fungal origin which is classified by IARC as Category 1. In this study, the viable achenes of two buckwheat species, *F. tataricum* (var. Golden) and *F. esculentum* (var. Aelita) were inoculated with an AFB_1-producing *A. flavus* NRRL 3357 to analyze their relative performances against fungal invasion and toxin contamination. Notably, we sought the existence of a correlation between the amount of tocols/flavonols in the achenes of buckwheat, infected and non-infected with *A. flavus*, and to analyze the ability of the pathogen to grow and produce toxin during achene infection. Results suggest that achenes of *F. tataricum*, the best producer of antioxidant compounds in this study, are less susceptible to *A. flavus* infection and consequently, but not proportionally, to mycotoxin contamination compared with *F. esculentum*. Moreover, rutin-derived quercetin appears to be more efficient in inhibiting aflatoxin biosynthesis than the parent compound.

1. Introduction

Over recent years, the close connection between food and health has become increasingly important for consumers, food industry and scientists. Thus, healthy, added-value foods containing bioactive compounds are constantly entering the market. Among grain crops, buckwheat (*Fagopyrum* spp.) is gaining momentum because of its content of such healthy compounds (Christa and Soral-Śmietana, 2008). Furthermore, buckwheat achenes can be used to obtain flours that are gluten-free and thus less harmful to celiacs (Alvarez-Jubete et al., 2010). The high content in buckwheat of phenolic compounds, several of which also exhibit antioxidant activity, can be beneficial for human health (Holasova et al., 2002; Wijngaard and Arendt, 2006). Plants produce phenolic compounds for pigmentation, growth and reproduction (Bhattacharya et al., 2010). Nevertheless, wounding, drought and pathogen attack deeply affect the synthesis and accumulation of phenolics by plants (Kefeli et al., 2003; Zapprometov, 1989). Defense-induced phenolics may act as natural toxicants and pesticides against invading organisms, such as herbivores, nematodes, phytophagous insects, fungal and bacterial pathogens (Dakora and Phillips, 2002; Lattanzio et al., 2006; Ravin et al., 1989).

Rutin and quercetin are the main phenolic antioxidant compounds present in buckwheat (Fabjan et al., 2003; Morishita et al., 2007; Quettier-Deleu et al., 2000). These compounds also have significant therapeutic properties (Polito et al., 2013). Significant variations in rutin content were reported within buckwheat grain varieties and also species (Kitabayashi et al., 1995; Ohsawa and Tsutsumi, 1995; Oomah and Mazza, 1996). Amongst these, *Fagopyrum tataricum* is characterized by a higher content of phenolic compounds (Li et al., 2010) compared to other species used in trade such as *Fagopyrum esculentum.* The amount of rutin in *F. tataricum* can reach values up to 200 times higher than that of *F. esculentum* (Brunori et al., 2008; Brunori and Végvári, 2007). Although antioxidant properties of rutin have been largely investigated, little is known about the impact of this compound on fungal pathogenesis in buckwheat.

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Other than phenolics, plants present a complex profile of antioxidant compounds (e.g. tocopherols, ascorbic acid, glutathione) and enzymes [e.g. superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX)] to combat environmental and intracellular oxidative insults. These antioxidants play an important role not only in scavenging oxidative species, but also in reducing microbial growth and in controlling the development of off-flavors and nutritional changes in foods (Hussain et al., 2013). Among antioxidant compounds, tocopherols are important for controlling non-enzymatic lipid peroxidation during seed dormancy and seedling germination. Tocopherols are lipid soluble antioxidants present in all photosynthetic organisms. They are capable of donating a single electron to form a stabilized tocopherol radical probably responsible for the quenching of reactive oxygen species (ROS) and other lipid by-products of oxidative stress (Brigelius-Flohé and Traber, 1999). A coordinated response to stress of tocopherols toward other antioxidants has also been suggested (Hussain et al., 2013).

Buckwheat has been recently included in the list of cereals and derived products which may be susceptible to invasion by *Aspergillus flavus*, and consequently, aflatoxin contamination (EFSA, Supporting Publications 2013:EN-406). Aflatoxins remain essentially unaltered during food processing and, for this reason, could be present in processed foods (flour, pasta, baked goods) (Krysinska-Traczyk et al., 2007), and can reduce the quality and safety of products obtained from buckwheat.

Intracellular and extracellular oxidative stress is a key factor in stimulating biosynthesis of aflatoxin and other mycotoxins (Reverberi et al., 2010, 2012a, 2013). H_2O_2 and other promoters of oxidation (Fanelli et al., 1985; Jayashree and Subramanyam, 2000; Narasaiah et al. 2006) may trigger aflatoxin synthesis in *Aspergillus* sect. *Flavi* (Reverberi et al., 2008). Further, ROS production at the plant–pathogen interface is also an important factor that contributes to *Aspergillus* virulence and secondary metabolite production (Reverberi et al., 2012b, 2013). At the early stage of the interaction, an early oxidative burst is triggered by the pathogen (Kachroo et al., 2003) to successfully infect the seed. Concomitantly, a burst of H_2O_2 in the seeds contaminated with *Aspergillus* is achieved within a few hours post infection (Reverberi et al., 2007). It is emerging that oxidative stress in the fungus plays an important role not only as inducer of mycotoxin biosynthesis but also as an early factor in the establishment of pathogen/plant interaction. In relation to this, a host cell bases an important part of its defense toolkit on antioxidant content and activity triggered soon after pathogen infection.

The aim of this study was to search for a relationship between the antioxidant profile of the viable achenes of two buckwheat species and their ability to counteract *A. flavus* growth and AFB_1 production during achenes infection.

2. Material and methods

2.1. Fungal strain

AFB_1-producing *A. flavus* strain NRRL 3357 was provided by ATCC. The fungal strain was maintained on Czapek Dox Agar (CDA) (Difco), amended with $ZnSO_4$ (5 mg/L) and $NaMoO_4$ (1 mg/L), for 7 days at 30 °C.

2.2. Buckwheat achenes

F. esculentum (var. Aelita) was supplied by the Research Institute of Crop Production, Praga; *F. tataricum* (acc. Golden), was supplied by the ENEA germplasm collection.

2.3. Inoculation of buckwheat achenes with A. flavus

F. esculentum and *F. tataricum* achenes were harvested in experimental fields located at Camigliatello Silano on the high plain of Sila (Region of Calabria) and in Pollino (Region of Basilicata), respectively, both in the South of Italy. All the achenes were harvested from a randomized block design of parcels 1 x 1.5 m each. These seeds were combined into one batch for each cultivar and from these the amount of seed needed for the experiments was taken. Each sample of buckwheat achenes (35 g) was surface sterilized (2% v/v HClO solution), rinsed threefold with sterilized distilled water, moistened up to 0.90 a_w and then inoculated with 250 μL of *A. flavus* conidia suspension (10^4 conidia/mL) and incubated at 30 °C in the dark. Each biological replica comprised a non-inoculated control and *A. flavus* inoculated achenes of Golden and Aelita, sampled at 7 different time intervals from 0 up to 168 h post inoculation (hpi). The whole set of experiments were performed three times, always following this scheme; thus there were two biological replicas of the experiments, each performed in triplicate ($n = 6$).

2.4. Activities of superoxide dismutase, catalase and glutathione peroxidase

The activities of superoxide dismutase (SOD) pH 7.8, catalase (CAT) and glutathione peroxidase (GPX) were analyzed in the inoculated and uninoculated lyophilized buckwheat seeds (50 mg) as previously described (Nobili et al., 2014). The antioxidant activities were also reported as the average of absolute values relative to SOD, CAT or GPX activity per each time interval divided for the respective values at time 0 (i.e. values normalized to those of day 0) in PCA analyses and in Spearman correlation studies.

2.5. Measurement of antioxidant activity of buckwheat grain

The stable 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) radical was used to measure the free radical scavenging capacity of the sample extracts according to Morishita et al. (2007), with some modifications. The reaction mixture consisted of 100 μL of diluted sample (serial dilutions of the grain extracts were prepared prior analysis) and 2.9 mL of a freshly made DPPH methanol solution (60 μM) prepared in 15 ml tubes. The absorbance of the freshly prepared DPPH solution was measured prior to analysis. After vortexing, the tubes were left in the dark for 15 min at room temperature. The absorbance was then measured against methanol (blank) at 515 nm in 1 mL cuvettes using a spectrophotometer. A reaction was conducted simultaneously using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylicacid, Sigma Aldrich, USA) instead of the buckwheat achenes extract. A 10 mM Trolox solution was prepared by dissolving in 80% ethanol. The DPPH radical-scavenging activity was determined from the decrease of absorbance and expressed as equivalents per g of dry matter (micromol Trolox EQ/g) using a calibration line of Trolox.

2.6. Analysis of polyphenols by HPLC

Samples were freeze-dried, powdered in liquid nitrogen, extracted with 80:20 ethanol:water solution (Morishita et al., 2007) and filtered through a 0.20 μm PTFE filter. Rutin, quercetin and hyperoside were analysed by HPLC according to Vogrinčič et al. (2010). The chromatographic separation was carried out with an HPLC instrument equipped with a ternary pump (ProStar 230 Varian, USA), a Supelco Ascentis C18 RP-Amide (25 cm × 4.6 mm, 5 μm) column, a diode array detector (DAD ProStar 330 Varian, USA) and an autosampler (Infinity 1260 Agilent, USA). Chromatograms were acquired by Galaxie (Agilent, USA) software. The eluent mixture was composed of acetonitrile (solution A) and water (solution B) both acidified with 0.1% HCOOH. The flow rate was set to 1 mL/min, the column was thermostated at 30 °C and the detection wavelength was 362 nm. The separations were performed by gradient elution according to the following program: 3 min of isocratic elution with 60% of B, 5 min of linear gradient from 60 to 95% of B followed by 2 min of isocratic elution with 95% of B. Quantification of individual compounds was performed by the external