JURNAL KARTIKA KIMIA

J. Kartika Kimia, November 2025, 8, (2), 199-206
Journal homepage: http://jkk.unjani.ac.id/index.php/jkk
P-ISSN: 2655-1322 E-ISSN: 2655-0938

Application of Germination and Combination Germination-*Rhyzopus* sp. treatment for Reduction of Ochratoxin A in Peanuts

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DOI: https://doi.org/10.26874/jkk.v8i2.855

Received: 20 Oct 2025, *Revised*: 17 Dec 2025, *Accepted*: 18 Dec 2025, O*nline*: 23 Dec 2025

Abstract

Peanuts are a major food commodity in Indonesia and an important source of protein, but their safety is compromised due to contamination by mycotoxins, including ochratoxin A (OTA). Several methods for OTA degradation have been explored, including chemical and physical approaches. However, biological methods like germination and a combination of germination with fungal treatment have shown potential for reducing OTA at a low cost. This study aimed to evaluate the effect of germination and a combined germination-fungal treatment using *Rhyzopus* sp. on the OTA content in peanuts. Peanuts spiked with 89.9 ppb OTA were subjected to germination for three days and the combination treatment. OTA degradation was analyzed using UHPLC-ESI-QQQ-MS/MS. The study found that germination and the germination-fungal treatment reduced OTA concentration on peanuts by 89.25% to 96.4%.

Keywords: *Fungal treatment, germination, ochratoxin A, Rhizopus sp.*

1 Introduction

Food safety is fundamental to human health and quality of life, and it has become a serious global issue. Approximately 600 million people, or 1 in 10 worldwide, suffer from foodborne illnesses, with 420,000 deaths reported annually [1]. The European Commission's Knowledge for Policy identifies mycotoxins as one of biological contaminants that can significantly impact food safety at both national and international levels. Factors including temperature and water activity are crucial for the growth of pathogen fungi that produce mycotoxin [2]. Indonesia as a tropical country has high rainfall, temperature as well as high humidity, creates ideal conditions for the proliferation of these fungi. This condition can affect the quality and safety of food commodities in Indonesia, particularly food contaminated by pathogenic fungi that can produced mycotoxin.

Mycotoxins are the group of toxic compounds that produce from pathogenic fungi. *Aspergillus*, a group of fungi, is commonly known to produce mycotoxins that pose serious health risks [3]. Fungal species like *Aspergillus niger*, *Aspergillus ochraceus*, and *Aspergillus carbonarius* are specifically known to produce ochratoxin. Food ingredients can contain high levels of mycotoxin produced by fungi, such as aflatoxin and ochratoxin A [4]. Legumes are one of the main commodities frequently contaminated by these fungi [5]. Peanuts, a type of annual legume that thrives in tropical climates, are particularly vulnerable to fungal contamination [6]. Due to these risks, extensive research has been conducted on mycotoxin in peanuts. Studies have shown that peanuts are heavily contaminated by *Aspergillus* species, with 85.7% of ochratoxin A (OTA) found in contaminated samples [7]. Additionally, 50% of peanut samples were found to contain OTA, with average levels ranging from 5.6 to 130 ng/g [8]. OTA is known to contaminate peanuts before harvesting process; however the recent research has found that OTA can contaminate grain during the storage period [9]. Therefore, it is crucial to explore methods to reduce OTA in in food products, particularly in peanuts.

Various strategies to minimize OTA contamination in peanuts is essential during the initial harvest, storage, and food process stages, some of the method has been reported including chemical, physical, and biological methods. However, physical and chemical methods have limitation such as high costs, inefficiency, nutrient

loss, and the potential formation of toxic by-products [10, 11]. In contrast, biological methods, like germination, have been shown successfully reduce OTA contamination at low cost while enhancing the nutritional value of food, one of the reports shown germination has decreased OTA in wheat by 38% [12]. Additionally, the use of fungi as biotic elicitors, such as *Rhizopus homothallicus*, *Rhizopus oryzae*, and *Rhizopus stolonifera*, has been reported to degrade over 95% of 7.5 mg OTA/L after 16 days of incubation [13]. More recently, a fungus from different family, *Aspergillus oryzae*, was found to detoxify OTA in liquid media with a maximum degradation rate of 94% [14]. These studies demonstrate that biological method including germination and elicitation using several fungal species have the potential to effectively reduce or degrade OTA. However, the impact of germination and the combination of germination with fungal treatment, particularly using *Rhizopus* sp. on OTA levels in the peanut matrix has not yet been explored. In this study was conducted to assess the effect of germination and the combination of germination and fungal treatment using Rhizopus sp. on OTA content in peanuts.

2 Method

Materials

The peanuts were purchased from a local farmer in Sumedang, West Java, Indonesia. Rhizopus sp. was obtained from PT. Aneka Fermentasi Industri in Bandung, Indonesia. The Ochratoxin A (OTA) standard CRM46912, dissolved in benzene/acetic acid solution (99:1, v/v), was purchased from Sigma-Aldrich, USA. Ethanol and acetic acid glacial and were supplied by J.T. Baker, while LC-MS grade water, formic acid, and acetonitrile were purchased from Fulltime, and a 5.25% natrium hypochlorite (NaOCl) solution.

Sterilization, germination, and germination-fungal treatment condition

Ten grams of peanuts were sterilized by soaking them in 50 mL of a 1% NaOCl solution for 2 minutes to eliminate any microbial contamination. The peanuts were then rinsed four times with sterile distilled water. After sterilization, the peanuts were immersed in a 0.1 µg/mL OTA solution at a ratio of 10 mL per 10 grams of peanuts. This step served a both soaking process before germination and the OTA spiking procedure. Instead of water, the soaking solution used for germination consisted of 10% acetonitrile and 4% acetic acid diluted in water containing OTA. The peanuts were soaked in the dark with continuous shaking at room temperature for 24 hours to ensure even distribution and absorption of the OTA. And then the sample will be called the spike peanuts.

Germination was conducted in a dark box to prevent external light exposure. The germination setup including a timer, heating mantle, and fog generator were adjusted to control humidity and temperature while germination process. Prior to use, the germinator was sterilized with UV light for 15 minutes, followed by spraying with a 70% alcohol and 0.07% NaOCl solution (v/v) and left for 15 minutes to minimize microbial contamination during germination process. Peanuts experienced three different treatments during germination: 1) germination in darkness (samples were coded as G), 2) germination with fungal treatment, where Rhizopus sp. was introduced immediately after soaking or on day 1 of germination (samples were coded as G0_F), and 3) germination with fungal treatment, where Rhizopus sp. was added after day 1 of germination (samples were coded as G1_F). The germination process lasted for three days in total. Ungerminated peanuts were used as controls to evaluate the effect of germination and the combination of germination and fungal treatment on OTA reduction (samples were coded as U). A summary of the different sample treatments is provided in the **Table 1**.

Table 1. The summary of the sample treatments

Sample	Step					
	NaOCl 1%	*Spike*	Germination	Day inoculation		
				1	2	3
UG	√	√	-	-	-	-
G	√	√	√	-	-	-
G0_F	√	√	√	√	√	√
G1_F	√	√	√	-	√	√

For the G samples, the sample (peanuts that has been spiked by OTA before) were placed on a tray lined with sterile cheesecloth that had been sterilized by autoclaving at 121 °C for 60 minutes. The optimal conditions for dark germination were established by operating the fog generator and fan for 5 minutes every 3 hours, with the temperature maintained between 25 and 30 °C. For the samples undergoing combines germination and fungal treatment, 1 gram of Rhizopus sp. powder was suspended in 15 mL of sterile water. The spiked peanuts were then inoculated by adding the fungal

culture suspension at a ration of 0.75 mL per 10 grams of peanuts (15 mL per 200 grams) in a petri dish and mixed thoroughly [15]. For the G0_F samples, were inoculated at 30 °C with relative humidity (RH) of 55% - 85%. For the G1_F samples, fungal inoculation occurred 24 hours after germination at 25 °C and 100% RH. Humidity during the germination process of each sample was maintained by using the fog generator and fan every 5 hours for 2 minutes over the total 3-day germination period.

Extraction OTA

After completing the germination and germination-fungal treatment process, the samples (U, G, G0_F, and G1_F) were dried at 40 °C for 24 hours. Then, the samples were ground using a spice grinder. The ground samples were then filtered through 80 mesh to standardize the surface area of the samples. OTA extraction was performed according to Soleimany et al., 2012, with solvent ration modification based on X. Zhao et al., 2021 [16,17]. One gram of the powdered samples was diluted in 2 mL of a solution containing acetonitrile, water, and acetic acid in a ration of 84:15:1, and the mixture was shaken at 200 rpm for 60 minutes. Afterward, the mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant was pipetted into 15 mL falcon tube, and the reside was extracted a second time using the same method. The supernatants from both extractions were combined in a falcon tube, vortexed, and the centrifuged again at 3000 rpm for 10 minutes. The 1.5 mL of final extraction product was pipetted into an Eppendorf tube and placed into a micro insert for injection to UHPLC-ESI-QQQ-MS/MS. Each sample in this study was extracted twice.

OTA analysis using UHPC-ESI-QQQ-MS/MS

Sample extracts were analyzed using a UHPLC-ESI-QQQ-MS/MS instrument. The stationary phase used in this study was a Kinetex® 2.6 µm C18 column (50 × 2.1 mm). Mobile phase A consisted of 0.1% formic acid in and 0.1% formic acid in acetonitrile as mobile phase B. Gradient elution conditions (Table 3.2) were used with a flow rate of 400 µL/minute and a sample injection volume of 5 µL at temperature constant column 40°C. A Shimadzu LCMS-8045 triple quadrupole mass spectrometer (Shimadzu, Tokyo, Japan) was equipped with an ESI source in positive ionization mode. Mass spectrometer conditions and parameters adopted from Prakasham et al., 2023 [18]. The heat block temperature at the ion source was set at 300 °C., the DL temperature at 300°C, the interface temperature at 300°C, the nebulizing gas flow rate at 2.7 L min−1, and the heating gas flow rate at 10 L min−1 for the MS/MS analysis.

Qualitative and quantitative analyses of OTA were conducted using Multiple Reaction Monitoring (MRM) scanning mode. In MRM, a precursor ion scan is performed in Q1, followed by the scanning of one or more fragment ions in Q3. MS1 and MS2 are configured to specific m/z values where the selected precursor ion transitions to the chosen product ion. In this study, the mass spectrometry parameters for the OTA compound included a precursor ion at m/z 404.1 [M+H]+. The MRM transition with the highest intensity, 404.1 → 238.85 (-24 eV), was utilized for quantification, while the confirmation fragments were 404.01 → 358 (-15 eV) and 404.01 → 221 (-39 eV).

The percentage reduction and concentration of OTA in peanuts resulting from the treatments were analyzed by comparing the averages of two technical replicates for each treatment. The experiments were conducted using two technical replicates per treatment.

3 Result and Discussion

The effect of germination and combination germination-fungal treatment peanut morphology

Based on the experiment shown that as morphology the peanuts radicle, both in germination only and combination germination-fungal treatment, looks like no difference in term of the length of sprouts (**Fig. 1**). The introducing the OTA to the peanuts known can inhibit the growth of peanut sprout. This phenomena in line with previous study, Singh et al., (2021) reported that mycotoxin can influence the seed germination, seed quality, seed viability, as well as coleoptile and root growth [19]. OTA can affect protein synthesis, thereby indirectly impairing the activity of several cellular enzymes, particularly cytosolic phosphoenolpyruvate carboxykinase, an enzyme involved in the gluconeogenic pathway [20]. Therefore, OTA is thought to indirectly alter carbohydrate metabolism and disrupt germination growth.

A B C

Figure 1. All the germinated peanuts shown in the figure were spiked with OTA. Germinated peanut without fungal treatment (G) had more open cotyledon (A); peanuts with fungal-treatment in the same day or germination (G0_F) exhibit a greenish-yellow surface (B); and germinated peanut with fungal-treatment after 24 hours of germination displayed brown radicle tips (C).

Identification and Quantification of Ochratoxin A (OTA) in samples

Ochratoxin A (OTA) in each extracted sample was successfully identified by confirming that the peaks for both OTA-specific MRM transitions matched between the sample and the standard. The quantification transition (Q, quantifier) for OTA was confirmed at m/z 404.1 → 238.85, which exhibit the highest intensity, as illustrated in **Fig. 2**. Additionally, the qualifying peaks (q, qualifier) were observed at m/z 404.1 → 358 and m/z 404.01 → 221. According to Fernandes et al., 2013, the OTA fragmentation pattern at m/z 238.85 indicates the structure of OTA after the loss of phenylalanine $[M+H - Phe]^+$, while the peak used for confirmation at m/z 358 represents the OTA compound after the loss of -COOH, and the m/z value of 221 corresponds to the fragment $[M+H - Phe - H_2O]^+$ [21]. In addition to the specific MRM transition, OTA can also be confirmed by comparing its retention time in the sample with that of standard OTA solutions. The OTA standard eluted at a retention time of 2.54 – 2.55 minutes, while the OTA detected in the sample eluted at 2.47 – 2.57 minutes, confirming its presence based on the standard retention time.

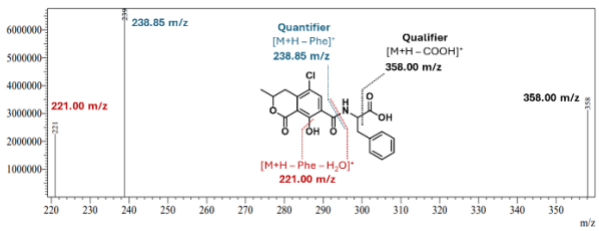

Figure 2. Mass spectrum and fragmentation of OTA in MRM mode in positive ion mode

Quantitative analysis of OTA is conducted using a calibration curve that illustrates the relationship between the response from the UHPLC-ESI-QQQ-MS/MS instrument-measured as peak area-and the standard concentration of the target analyte, OTA. the response is based on a standard series of OTA with known concentrations of 15 ppb, 40 ppb, 90 ppb, and 115 ppb. According to the calibration curve (**Figure 3**), the relationship between peak area and standard concentration can be described by the linear regression with a coefficient of determination (R2) value of 0.9811. This equation is utilized to determine the OTA concentration in the samples, with results from each treatment in two replicates presented in Table 2. The initial concentration of OTA in the peanut sample was 89.8751 ppb shown by the UG sample as a control sample that was not treated.

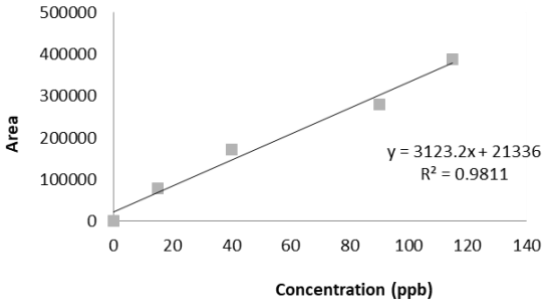

Figure 3. OTA standard calibration curve

Table 2. The results of the OTA concentration

Sample	Area	Conc. (ppb)	Mean (ppb)
UG	282363	83,5768	89,8751
	321705	96,1735	
G	54294	10,5526	9,6604
	48721	8,7682	
G0_F	57057	11,4373	10,9119
	53775	10,3865	
G1_F	32341	3,5236	2,2398
	30568	2,9559	

Effect germination and combination germination-fungal treatment with Rhizopus sp of ochratoxin A reduction in Peanuts

The reduction in OTA concentration was assessed in the extracts of germinated peanuts (G) and peanuts that underwent a combination of fungal treatment at two different inoculation times (G0_F and G1_F), compared to ungerminated peanuts (UG) as a control. The results indicate that

all three treatments effectively reduced OTA concentration in the peanut samples (**Fig. 4**). The germinated peanuts sample (G) showed a decrease in concentration from 89.8751 ppb to 9.6604 ppb, representing a reduction of 89.25%. The most significant reduction in OTA concentration observed in peanut samples germinated with additional of fungal treatment after day 1 of germination (G1_F) sample, resulting in a final concentration of 2.23898 ppb, which reflects a decrease of 96.4%. Conversely, the germination with fungal treatment applied on day 0 of germination (G0_F) resulted in a reduced OTA concentration of 10.9119 ppb, corresponding to reduction of 87.9%.

By comparing the percentage of OTA reduction among the UG, G, G0_F, and G1_F treatments, the germination (G) process showed a substantial effect in reducing OTA in peanuts compared to untreated samples (UG). In addition, introducing fungal treatment after 24 hours of germination resulted in greater OTA reduction compared to adding the fungal treatment on the same day as germination. However, these comparisons need to be confirmed through further statistical analysis with an increased number of data points to obtain more quantitative conclusions.

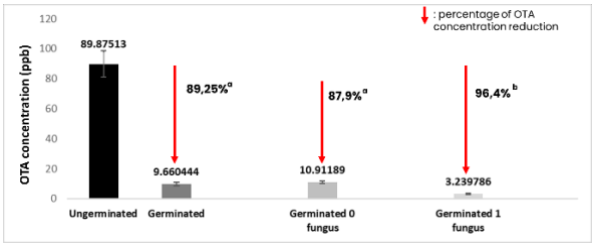

Figure 4. Decreasing OTA concentration

The results indicate that germination plays a crucial role in reducing OTA concentration in peanut samples. The presence of toxin as stressors can biochemically and physiologically affect the cellular mechanisms of peanuts. Study has shown that the interaction between peanuts and toxins can adjust the activity of enzymes in defense-related tissues, such as esterase, peroxidase, and catalase [22]. Additionally, other studies suggest that the germination process can enhance the activity of various enzymes, including amylase, lipase, protease, and β-glucanase [23,24]. Dobritzsch et al., 2014 reported that OTA hydrolysis can be facilitated by lipase, protease, and amidase enzymes [25]. Given its structure, which includes an amide bond, it is likely that protease and amidase enzymes contributed to the hydrolysis of OTA. Furthermore, lipase has been shown to cleave amide bonds and is utilized to deprotect peptides. The lipase enzyme catalyzes both hydrolysis and esterification reactions [26].

In addition to the effect of germination, the introduction of fungi is believed to contributed to the reduction of OTA concentrations in peanuts. Rhizopus sp. possesses several key properties, including its enzymatic activity, particularly the production of enzymes from the protease group [27]. The detoxification of OTA by fungal strains occurs through the action of the carboxypeptidase enzyme, which hydrolyses the amide bond [14]. Fungal isolate Rhizopus sp. has been reported to exhibit carboxypeptidase activity, which may facilitate the degradation of OTA [13]. The carboxypeptidase enzyme is responsible for hydrolyzing peptide bonds in terminal amino acids, resulting in the production of ochratoxin α and L-phenylalanine **(Fig. 5)** [28]. The cleavage of amide bonds is likely to reduce the quantity or concentration of OTA in the peanut samples.

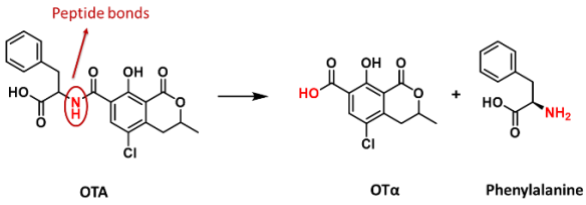

Figure 5. The mechanism of degradation of OTA compounds due to the activity of the carboxypeptidase enzyme [28]

Based on the results obtained, demonstrated that samples germinated for one day and subsequently treated with Rhizopus sp. fungi (G1_F) achieved the greatest reduction in OTA concentration. Therefore, it can be concluded that the combined strategy of germination and fungi addition is more effective in lowering OTA concentration than germination alone. This approach has the potential to make peanuts a safer food option. However, it is essential to conduct further analysis on the product, especially regarding the toxicity of the samples, to ensure the safety of the peanuts for consumption.

4 Conclusion

Germination and the combination of germination-fungal treatment were the best way in reducing OTA concentrations in peanut samples. Peanut samples germinated with a combination of fungal treatment after 1 day of germination

(G1_F) showed the highest reduction in OTA concentration of 96.4%.

Acknowledgement

This research was financially supported by the Institute for Research and Community Service (LPPM), Universitas Pendidikan Indonesia, through the scheme of Penelitian Pembinaan dan Afirmasi Dosen Muda 2024, grant number 392/UN40/PT.01.02/2024.

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