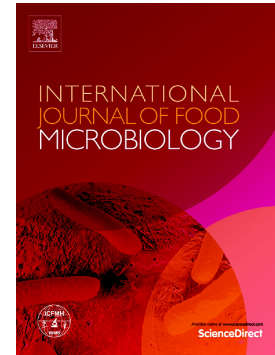


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**The major *Alternaria alternata* allergen, Alt a 1: a reliable and specific marker of
fungal contamination in Citrus fruits**

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Abstract

The ubiquitously present spores of *Alternaria alternata* can spoil a wide variety of foodstuffs, including a variety of fruits belonging to the *Citrus* genus. The major allergenic protein of *A. alternata*, Alt a 1, is a species-specific molecular marker that has been strongly associated with allergenicity and phytopathogenicity of this fungal species. This study aimed to evaluate the potential of the detection of Alt a 1 as a reliable indicator of *A. alternata* contamination in citrus fruits. To accomplish this aim, sixty oranges were artificially infected with a spore suspension of *A. alternata*. Internal fruit material was collected at different incubation times (one, two and three weeks after the fungal inoculation) and used for both total RNA extraction and protein extraction. Alt a 1 detection was then performed by polymerase chain reaction (PCR) amplification using Alt a 1 specific primers and by enzyme-linked immunosorbent assay (ELISA). The experimental model presented in this work was effective to simulate the typical *Alternaria* black rot phenotype and its progression. Although both PCR and ELISA techniques have been successfully carried out for detecting Alt a 1 allergen in *A. alternata* infected oranges, the PCR method was found to be more sensitive than ELISA. Nevertheless, ELISA results were highly valuable to demonstrate that considerable amounts of Alt a 1 are produced during *A. alternata* fruit infection process, corroborating the recently proposed hypothesis that this protein plays a role in the pathogenicity and virulence of *Alternaria* species. Such evidence suggests that the detection of Alt a 1 by PCR-based assay may be used as a specific indicator of the presence of pathogenic and allergenic fungal species, *A. alternata*, in fruits. This knowledge can be employed to control the fungal infection and mitigate agricultural losses as well as human exposure to *A. alternata* allergens and toxins.

Keywords

Alternaria black rot, Alt a 1 detection, ELISA, food biosecurity, polymerase chain reaction

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1. Introduction

Several species belonging to the fungal genus *Alternaria* are well known plant pathogens that may cause damage in the field as well as spoil postharvest fruits, vegetables and cereals resulting in serious agriculture and economic losses (Barkai-Golan and Paster, 2008; Samson et al., 2010). Epidemiological studies have showed that *Alternaria alternata* spores are one of the most frequently identified fungal spore types in the atmosphere (Woudenberg et al., 2015). The ubiquitous presence in nature of *A. alternata* probably justifies why this species is one of the most common contaminating fungi recorded in various agricultural commodities, such as tomatoes, melons, cucumbers, squashes, peppers, citrus fruits, apples and olives, among others (Barkai-Golan, 2001; Barkai-Golan and Paster, 2008; Morris et al., 2000). In particular, *A. alternata* colonization of citrus fruits is recognized to be the major cause of phenotypes of plant diseases, such as *Alternaria* black rot, *Alternaria* leaf spot and *Alternaria* brown spot (Logrieco et al., 2009; Peever et al., 2005). The exact etiology of infection of fruits by molds is still unclear, however, it has been supposed that fungal colonization mainly occurs by taking advantage of the existence of surface lesions as a result of sunburn, bruising or cracking of ripe fruit (Morris et al., 2000).

A. alternata is described to be producer of a wide variety of toxic metabolites, including dibenzopyrone, perylene and tetramic acid derivatives, that are potentially harmful for humans and animals (Pose et al., 2010). Apart from being toxin producer, this fungal species is an important source of a broad panel of allergenic proteins that may affect human health, particularly the immune system of atopic individuals, frequently resulting in severe respiratory manifestations (Gabriel et al., 2016; Simon-Nobbe et al., 2008). Among the several allergens described in this mold, the major *A. alternata* allergen, Alt a 1, has been demonstrated to be the most clinically relevant and widely used as a

diagnostic marker of sensitization to Pleosporaceae members (Postigo et al., 2011). Alt a 1 is a species-specific protein, exclusive to a restricted number of *Alternaria* related species, and regarded as an important phylogenetic marker (Hong et al., 2005). Although the function of Alt a 1 in fungal biology remains unclear, some evidence has suggested that the role of Alt a 1 can be related to virulence and fungal infection pathogenicity (Gómez-Casado et al., 2014; Hong et al., 2005).

Fungal contamination of fruits and other foodstuffs probably make them important sources of hazardous mycotoxins and fungal allergens. Thus, the presence of individual fungal products (such as toxins or allergens) in fruits and other foodstuffs might be used as a direct indicator of fungal contamination and thus of food quality and safety for human consumption. With this in mind, in this work, it was aimed to study the value of the *A. alternata* major allergen, Alt a 1, as a molecular, allergenicity and pathogenicity marker with potential to be used as indicator of contamination of citrus fruits by *A. alternata*. The specific detection of Alt a 1 was evaluated by two different methodologies: a recently developed polymerase chain reaction (PCR) method based on the Alt a 1 coding gene and an enzyme-linked immunosorbent assay (ELISA).

2. Materials and Methods

2.1 Strains, spore suspensions preparation and culture conditions

The inclusion criteria for choosing fungal strains were based on the previous work, where the suitability of the Alt a 1 specific PCR assay was tested for several pure fungal species (Gabriel et al., 2015). *A. alternata* strain CBS 104.26 (Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands), which showed ability to express substantial amounts of Alt a 1 was selected to be used in this study. In addition, to confirm the specificity of the detection assays and to ascertain eventual cross-species contamination, another citrus pathogen, which does not produce an Alt a 1-like protein, *Penicillium chrysogenum* PC 4 strain (Municipal Institute for Medical Research, Autonomous University of Barcelona, Spain) was also used as control. Fungal strains were individually grown on Sabouraud dextrose agar plates for 7 days at 25°C and used for *A. alternata* and *P. chrysogenum* spore suspension preparation. Fungal spores were collected as previously described by Otani *et al.* (Otani et al., 1998). The spore suspensions were counted using a hemacytometer and adjusted to a final concentration of 1×10^6 spores/mL sterile distilled water. Ten microliters of the conidial suspension were used for fruit inoculation.

2.2 Fruit infection, collection and processing of samples

A total of 66 sweet oranges "Valencia Late" (*Citrus sinensis*) from Alicante (Spain) were obtained from commercial orchards in the Vitoria area (Spain). Fruits were selected for uniformity in size, appearance and absence of physical defects or lesions. Prior to inoculation, the citrus fruits were surface disinfected by soaking for 1 hour in 1% (w/v) sodium hypochlorite. They were rinsed in sterile distilled water and directly used for inoculation, following the methodology proposed by Isshiki *et al.* (Isshiki et al.,

2003). Briefly, the inoculations were performed under sterile conditions by injecting 10 μ L of spore suspension directly into the central axis (columella) of the surface-sterilized fruits. Sixty oranges were used for inoculation with *A. alternata*. For control of the specificity, 3 oranges were infected with *P. chrysogenum*. The remaining 3 oranges were injected with sterile distilled water and used as non-infected negative controls. To prevent cross-contamination the inoculated oranges were placed in individual sterile plastic boxes sealed with a cotton sterilizing filter in the inlet port and placed in an incubator at 25°C.

The collection and processing of the oranges was performed according to Katoh et al, (Katoh et al., 2007). The citrus fruits were examined and processed at different times post inoculation (one, two and three weeks after the fungal inoculation). Twenty *A. alternata*- inoculated, one *P. chrysogenum*-infected and one sterile water-injected citrus fruits were recovered and processed at each analysis time point (Week 1, 2 and 3). First, the fruits were longitudinally cut using sterile surgical blades. Then, they were examined to assess internal changes related to mold growth. For each fruit, material from the central area including the central axis and adjacent pulp was recovered. A slice of recovered citrus material was placed in the center of DRBC agar plates for analysis of mold viability and plates were incubated for 21 days at 25°C. The remaining recovered fruit material was incubated at -40°C overnight and freeze-dried. Freeze-dried samples were then pulverized with mortar and pestle and 100 mg and 300 mg were used for later RNA and protein extraction, respectively.

2.3 RNA Extraction

Total RNA was extracted from all the samples collected in 2.2. (100 mg) using the RNeasy® Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol

for fungi. For PCR positive control, total RNA was obtained from 100 mg from pure fungal mat of 5-day-old *A. alternata* culture. The final elution step was carried out with 30 μ L of sterile distilled water and from this volume, 11 μ L was used for reverse transcription. The RNA quality was analyzed with 1.2% agarose gel electrophoresis.

2.4 cDNA synthesis, primer design and PCR amplification

Reverse transcription reactions were carried out with the RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany), employing the Oligo(dT)18 Primer and following the manufacturer's instructions. Afterwards, PCR reactions were performed with Expand High Fidelity System (Roche, Mannheim, Germany). Ten μ L of cDNA as the template and an *A. alternata* specific primer set A21F (5'-GCGGATCCATGTCCTGCCTGTCACCAC-3') and A21R (5'-GCGGATCCTTAAGAGCTCTTGGGGAGA-3') (Gabriel et al., 2015) were employed in the preparation of PCR reactions. The detection system sensitivity was found to be 2×10^3 *A. alternata* spores (Gabriel et al., 2015). The PCR products were purified with electrophoresis on an agarose gel, sub-cloned into the pJET1.2/blunt vector and transformed into *Escherichia coli* DH5 alpha competent cells. The plasmid DNA was purified, and the nucleotide sequence of the cDNA insert was determined. Sequencing reactions were performed using the BigDye Terminator v.3 sequencing kit (Applied Biosystems) according to the manufacturer's specifications and were run in an ABI 3130XL sequencer (Servei de Genòmica, Universitat Autònoma de Barcelona). The amplicon sequence identity was confirmed with NCBI BLAST.

2.5 Protein extraction

Three milliliters of ammonium bicarbonate (pH 7.2) were added to the 300 mg of the lyophilized powder of each sample. The resulting suspensions were homogenized by

vortex and incubated overnight at 4°C under agitation. After centrifugation at 3000 rpm for 10 minutes, the respective supernatants were collected and filtered through a 0.45 µm syringe filter (Sartorius Stedim Biotech, Germany). Precipitated proteins were resuspended in 300 µL of PBS with 1% BSA and 0.05% Tween 20 (PBS-B-T) and frozen at -20°C until use.

2.6 Enzyme-linked immunosorbent assay (ELISA)

Quantification of Alt a 1 in the samples was carried out using 1D6 monoclonal antibody anti-Alt a 1 in solid phase and biotinilated polyclonal anti-Alt a 1 antibodies as a second antibody reagent (Bial Laboratorios, Bilbao, Spain), as previously described by Asturias *et al.* (Asturias *et al.*, 2003). A standard curve ranging from 0.25 to 100 ng/mL of native Alt a 1 (nAlt a 1, Bial Laboratorios, Bilbao, Spain) was performed. The absorbance was measured at a wavelength of 492 nm in a plate reader Multiskan® Bichromatic (Labsystems, Finland). According to data presented by Asturias *et al.* the detection limit of the assay is lower than 0.05 ng Alt a 1/mL. Statistical analysis and graphical representation of the results were carried out using the SPSS Software. Means of Alt a 1 concentration were analyzed by t-test.

3. Results

3.1 Pathogenicity assays

Spore suspensions were injected directly into the central axis of sterilized citrus fruits and symptoms of fungal infection were examined after different incubation times. From this examination, it was observed that citrus fruits artificially inoculated with *A. alternata* exhibited the typical decay symptoms of *Alternaria* black rot (Fig. 1). Macroscopically, rot was observed as the internal fruit area had turned dark as a consequence of fungal sporulation. Fungal growth was restricted to the internal area in both the central axis and adjacent juice sacs, whereas the peel remained apparently unaffected. As verified in figure 1, lesions were increasing in size through the time of incubation after inoculation. Fruits that were incubated for one week after contamination with *A. alternata* presented black rot limited to the small area of injection (Fig. 1A), approximately half the columella was found to be rotted two weeks after inoculation (Fig. 1B), and in the third week, the entire columella and adjacent juice sacs were visibly affected by *Alternaria* black rot (Fig. 1C). A similar phenotype of infection was obtained for *P. chrysogenum*-contaminated oranges, with difference on the color of rot, which presented a characteristic green tone. No visible fungal rot symptoms were noticed in the fruits that were injected with sterile distilled water and used as negative control. Fruit material from columella and adjacent juice sacs area was recovered and used for protein and RNA extraction. To confirm that Alt a 1 was expressed in *A. alternata* infected oranges, specific detection by PCR and ELISA were then performed.

3.2 PCR amplification Assays

Analysis of the presence of Alt a 1 encoding gene by PCR was performed using a primer set A21F and A21R, which could specifically detect Alt a 1 of *A. alternata* and closely related species (Gabriel et al., 2015).

As showed in the Fig. 2, the detection of Alt a 1 encoding gene by PCR in citrus fruits that were artificially contaminated with *A. alternata* was successfully accomplished. Only two samples recovered from *A. alternata* infected oranges that had been processed one week after inoculation gave negative PCR results (sample/lines 11 and 15, Fig. 2A). All positive PCR reactions showed similar electrophoretic profiles, where a single amplified DNA band of 390 bp was observed. Nonetheless, the intensity of amplified bands visualized in the agarose gel was very variable. The sequencing results and BLAST analysis indicated that all the amplified fragments indeed matched the Alt a 1 DNA sequences existing in the GenBank database under accession number AF288160.1.

It is known that the presence of inhibitory substances in food samples may often compromise DNA amplification and lead to the failure of the PCR reaction. To prevent false-negative results, positive controls using cDNA obtained from mycelia of an *A. alternata* pure strain were also included (Fig. 2, lines C+). Sequence analysis of the obtained PCR products allowed for the confirmation of the identity of all amplified Alt a 1 DNA fragments. No amplification products were detected in the PCR reactions using cDNA obtained from both water-inoculated and *P. chrysogenum*-contaminated oranges (Fig. 2). These results indicate that no cross-species amplification was obtained.

3.3 Quantification of Alt a 1 by ELISA

Figure 3 shows the mean values of Alt a 1 levels measured by ELISA in the soluble protein extracts obtained from citrus fruits for each incubation point after inoculation.

Alt a 1 values measured by ELISA varied from 0 to 7.80 ng/mL, 0 to 28.08 ng/mL and 0 to 30.50 ng/mL for samples from the first, second and third weeks after *A. alternata* artificial infection, respectively. Statistically significant differences were only obtained between Alt a 1 concentrations that were produced after 1 week and 2 weeks ($p < 0.05$), where a substantial increase expression of Alt a 1 was detected. A decrease in the Alt a 1 protein levels between samples from second and third weeks after inoculation with *A. alternata* was observed, however such difference was not statistically significant (Fig. 3). None of the negative or specificity controls gave a positive ELISA result.

The percentages of positive results for the presence of Alt a 1 assayed by PCR and ELISA are presented in Table 1. The PCR assays revealed the presence of the fragment corresponding to the Alt a 1 gene of 390 bp in 90%, 100% and 100% of the samples collected at one, two and three weeks after the *A. alternata* contamination, respectively; while Alt a 1 quantification by ELISA in the same samples yielded a slightly lower sensitivity (75%, 85% and 85%).

4. Discussion

Species in the genus *Alternaria* are well-known destructive pathogens of a variety of agricultural commodities and food-stuffs (Chowdappa et al., 2013; Thomma, 2003). The colonization of fruits by these molds often results in the qualitative deterioration of the product by *Alternaria* black rot (Chung, 2014; Soylu and Kose, 2015). Here, results from pathogenicity assays showed that citrus fruits are very susceptible to contamination with both *A. alternata* and *P. chrysogenum* spores. In particular, the artificial contamination of oranges with *A. alternata* spore suspensions resulted in fungal invasion, colonization of the columella and adjacent juice sacs leading to the typical phenotype of *Alternaria* black rot. As described in the literature, *Alternaria* black rot occurs mostly as a core rot of citrus fruits with often little or no external evidence of infection, so consumers cannot avoid buying contaminated fruits (Peever et al., 2005).

Because *A. alternata* is a well-recognized producer of harmful toxins and potent allergenic proteins, exposure to *A. alternata* contaminated foodstuffs is likely to represent an inherent and relevant risk for human health condition (Gabriel et al., 2016; Simon-Nobbe et al., 2008). This issue has been underestimated and, to date, there are no specific regulations for any *Alternaria* toxin or mold allergens in food. The correct and early detection of *A. alternata* contamination is necessary to establish and prevent health risks and economic losses related to fungal colonization of foods such as fruits and vegetables (Logrieco et al., 2009). In recent years, Alt a 1 has been presented as a valuable molecular, allergenicity and pathogenicity marker of the presence of the fungus *A. alternata*. Accordingly, it is expected that the use of approaches targeting the Alt a 1 gene and protein to unequivocally identify and detect pathogen *A. alternata* will yield privileged and powerful information regarding quality and biosecurity control of

foodstuffs. With this in mind, our research group have recently developed a polymerase chain reaction (PCR)-based method targeting the gene coding for Alt a 1 using species-specific primer sets (Gabriel et al., 2015). The suitability of the proposed method was here tested and successfully confirmed in citrus fruits exhibiting *Alternaria* black rot disease. Results reported in this work suggest that the PCR-based assay targeting the species-specific *A. alternata* molecular marker Alt a 1 constitutes a faster, simpler and sensitive valuable tool in assessing Alt a 1 occurrence and fruit contamination/colonization by *A. alternata*, alternative to the laborious traditional species-level identification (Guarro et al., 1999). Further work should be conducted to take advantage of the potential of this early detection method to reduce and control postharvest losses during storage and transportation of fresh fruits. In particular, campaigns of field work to collect representative samples of fruits need to be planned. In a real field scenario, by way of example, for a storage lot comprising 20000 fruits, a sample size of 377 fruits should be analysed to obtain the true percentage of *A. alternata* contaminated fruits with an confidence level of 95% and a confidence interval of 5.

Quantitative assessment of the presence of Alt a 1 by ELISA was also successfully employed in most of samples, but a moderately lower sensitivity was observed. ELISA analysis demonstrated that in *A. alternata* contaminated oranges, Alt a 1 concentration presented a peak in the second week after inoculation. The production of high amounts of Alt a 1 in the first two weeks after inoculation might probably corroborate the reports that suggest that Alt a 1 plays an important role in the virulence and fungal infection pathogenicity of *Alternaria* species (Cramer and Lawrence, 2004; Gómez-Casado et al., 2014).

Overall, data obtained from this study confirmed that the spoilage of citrus fruits by *A. alternata* is accompanied by the production of high amounts of the *A. alternata* major allergen Alt a 1, thus suggesting that Alt a 1 is a reliable detection marker of infection by pathogenic and allergenic *A. alternata*. Considering that sensitization to Alt a 1 is considered a risk factor for development and severity of asthma, a potentially relevant contribution of mold-infected food in increasing the spore and airborne allergen exposure should not be disregarded. Furthermore, even though exposure to *A. alternata* allergens by ingestion of contaminated fruits is probably low, contribution of *A. alternata* infected products to the human diet should not be excluded and probably deserves further investigations. In a recent case report, we found that a severe food reaction to mushrooms was associated to a prior sensitization to *A. alternata* allergens (Gabriel et al., 2015). More in-depth knowledge of the role of *A. alternata* allergens as causal agents of severe food reactions is clearly necessary to improve diagnosis and to allow effective avoidance and prevention of further systemic reactions. Likewise, monitoring the occurrence of *Alternaria* species in food using effective methods, such as the Alt a 1 specific PCR-based assay presented here, will provide information of food quality for consumption and etiology of contamination in agricultural commodities. This knowledge will be useful to elaborate strategies to predict and control fungal infection. Furthermore, this will also allow for the better understanding and avoidance of human exposure to *A. alternata* allergens and toxins, providing information on the implementation of appropriate personal/public protective health measures.

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Figure legends

Fig. 1. Longitudinal section through *Citrus sinensis* fruits, one (A) two (B) and three (C) weeks after the artificial inoculation with *Alternaria alternata*.

Fig. 2. Electrophoretic analysis of the PCR products obtained using Alt a 1 specific primers and cDNA from samples collected after one (A) two (B) and three weeks (C) from the fungal infection. Lines 1-60: samples collected from citrus fruits inoculated with *A. alternata*; C-: negative control (non-infected oranges); Pc: *P. chrysogenum* infected oranges; C+: positive control. M: GeneRuler™ DNA Ladder Mix.

Fig. 3. Mean values of Alt a 1 concentrations determined by ELISA in the citrus fruits samples inoculated with *A. alternata* in the first, second and third weeks of incubation. Asterisk (*) indicates statistically significant difference resulting from t-test ($p < 0,05$)

Table 1. Percentage of positive results obtained for the detection of Alt a 1 by PCR and ELISA in citrus fruits, one, two and three weeks after their contamination with an *A. alternata* spore suspension.

Time after inoculation (weeks)	PCR % of positive results	ELISA
		% of positive results
		Mean ± SD
1 (n=20)	90	75
		2.7 ± 0.8
2 (n=20)	100	85
		6.8 ± 2
3 (n=20)	100	85
		4.1 ± 1,8
SD, standard deviation		



Fig. 1

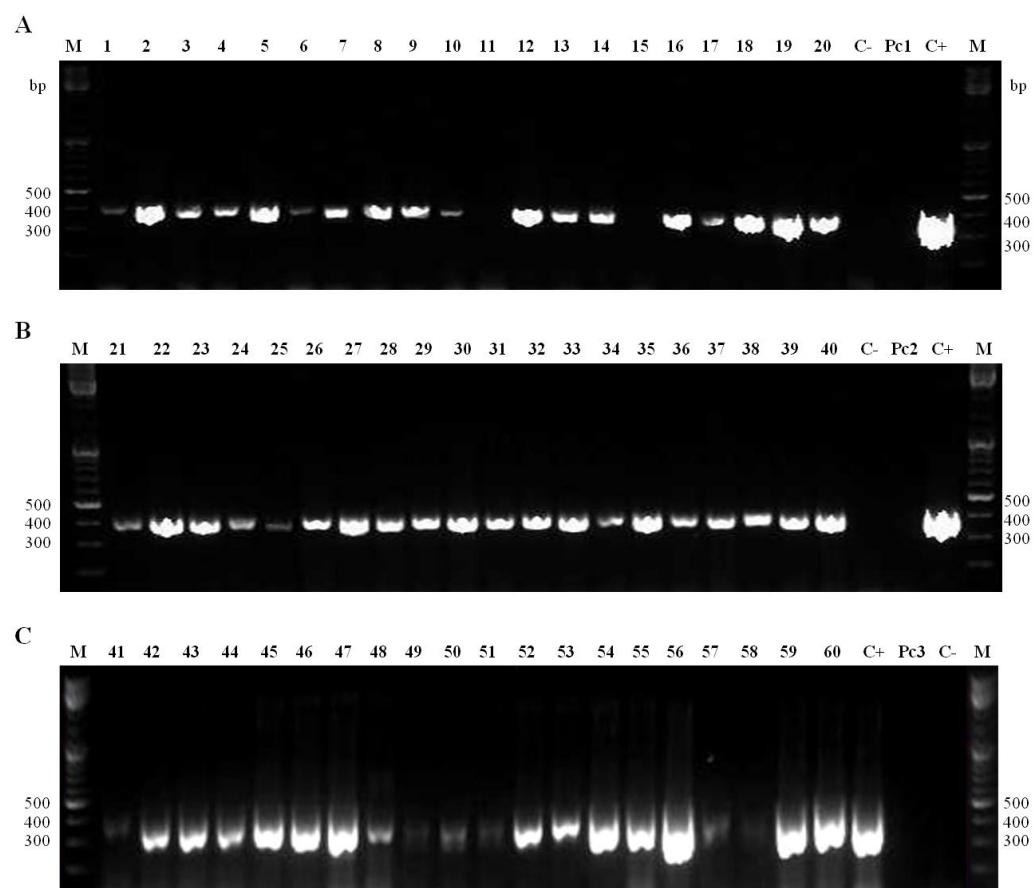


Fig. 2

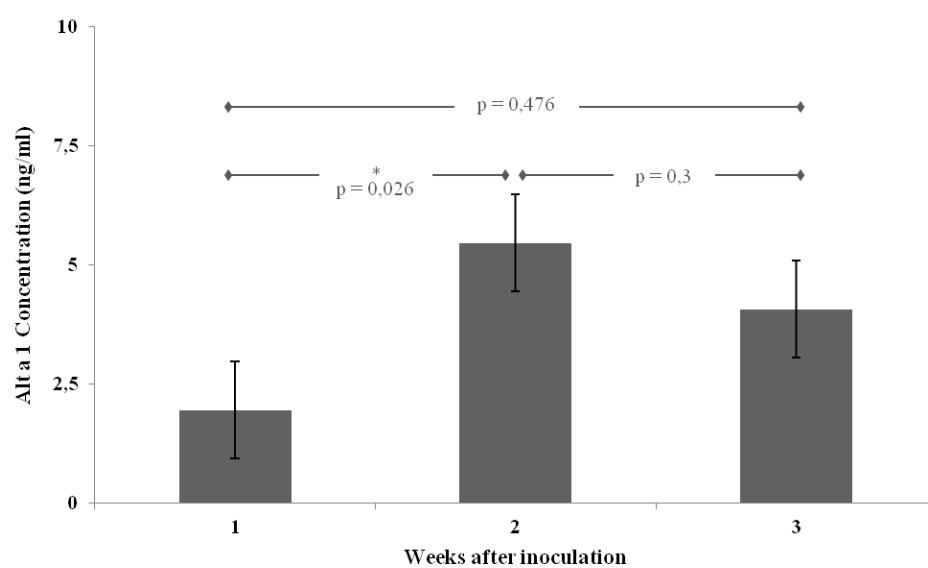


Fig. 3

Highlights

- *Alternaria* black rot was experimentally induced in citrus fruits by *A. alternata* inoculation.
- During the *A. alternata* fruit infection high amounts of Alt a 1 are produced.
- ELISA and PCR are valuable tools for detecting Alt a 1 in moldy fruits.
- Alt a 1 may be used as a specific biosecurity indicator of contamination by *A. alternata*.