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Detection and quantification of *Aspergillus* section *Flavi* spp. in stored peanuts by real-time PCR of *nor*-1 gene, and effects of storage conditions on aflatoxin production

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ABSTRACT

Aspergillus flavus and *A. parasiticus* are the main species from section *Flavi* responsible for aflatoxin accumulation in stored peanuts. A real-time PCR (RT-PCR) system directed against the *nor*-1 gene of the aflatoxin biosynthetic pathway as target sequence was applied to monitor and quantify *Aspergillus* section *Flavi* population in peanuts. Kernels were conditioned at four water activity (a_W) levels and stored during a 4-month period. The quantification of fungal genomic DNA in naturally contaminated peanut samples was performed using TaqMan fluorescent probe technology. Sensitivity tests demonstrated that DNA amounts accounting for a single conidium of *A. parasiticus* RCP08300 can be detected. A standard curve relating *nor*-1 copy numbers to colony forming units (cfu) was constructed. Counts of species of *Aspergillus* section *Flavi* from unknown samples obtained by molecular and conventional count (CC) methodologies were compared. A correlation between cfu data obtained by RT-PCR and CC methods was observed ($r = 0.613$; $p < 0.0001$); and the former always showed values higher by 0.5–1 log units. A decrease of fungal density was observed throughout the storage period, regardless of the quantification methodology applied. Total aflatoxin levels ranging from 1.1 to 200.4 ng/g were registered in peanuts conditioned at the higher a_W values (0.94–0.84 a_W).
The RT-PCR assay developed appears to be a promising tool in the prediction of potential aflatoxigenic risk in stored peanuts, even in case of low-level infections, and suitable for rapid, automated and high throughput analysis.

1. Introduction

Aflatoxins are highly toxic and carcinogenic secondary metabolites of concern in food safety. Aflatoxin B_1 (AFB_1) has been classified by the International Agency for Research on Cancer as a human carcinogen (group 1A) (IARC, 1993). The evidence regarding the potent carcinogenicity of aflatoxins has forced government regulatory agencies to establish very low tolerances in food, including peanuts and related products (Van Egmond, 2003). The European Union upper limit for aflatoxins in peanuts is 2 μg/kg for AFB_1 and 4 μg/kg for total aflatoxins ($B_1 + B_2 + G_1 + G_2$) (Commission of the European Communities, 2006). In addition to health concerns related to aflatoxins, the rejection of contaminated peanuts with aflatoxin values exceeding the minimum acceptable level results in large economic losses. Peanut is an important food commodity in Argentina with an annual production exceeding 6×10^5 tons (SAGPyA, 2008). In commercial plants, peanut seeds are stored for many months before being exported or used locally. Most of the production is exported whereas the remainder is used by local industry for human consumption, oil production and as raw materials for feedstuffs (SAGPyA 2008).

Aflatoxigenic species occur in three sections of *Aspergillus* genus, but section *Flavi* contains the greatest number of potential producers (Cary et al., 2005; Pildain et al., 2008). According to previous mycological surveys, Argentinean peanuts are frequently contaminated by *A. flavus* and *A. parasiticus* and this infection can occur during growth, harvesting, transportation or storage (Barros et al., 2003; Novas and Cabral, 2002; Passone et al., 2008). The level of fungal infection as well as the identification of main species is important, since they could give an indication of the food quality as well as of the future potential due the presence of mycotoxins (Suanthie et al., 2009).

The development of a rapid, sensitive method for detection and differentiation of potential aflatoxigenic species in foods is needed to estimate any potential health risk associated (Valasek and Repa, 2005). Conventional methods for identification and detection of fungi in foods rely on microscopic or culture techniques, which are time consuming and laborious. Information derived from these test would

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allow informed decisions about storage life of the product and the need for specific mycotoxin analysis. In this direction, DNA-based detection methods such as RT-PCR appear more sensitive and specific. Protocols have been already developed and applied for the detection of aflatoxigenic fungi in different food matrices such as maize, pepper, and paprika (Mayer et al., 2003; Shapira et al., 1996).

The applicability of these assays to detect fungi in natural samples, including material from field or storage plants, needs special consideration since food matrices are usually very complex (Hanna et al., 2005) and some of the matrix-associated compounds can inhibit RT-PCR or reduce its efficiency. The high concentration of peanut oils (48%) (Savage and Keenan, 1994) can reduce the purity of DNA extracted, whereas phenolic and polysaccharide compounds, acting as inhibitors, are often associated to peanuts (Savage and Keenan, 1994; Yu et al., 2004). These compounds may cause problems in real-time reactions by binding or denaturing the polymerase (Wilson, 1997). Inhibition can manifest itself as a complete reaction failure leading to false negative results or as a reduced detection sensitivity. The use of a convenient DNA extraction procedure would then, to a great extent, overcome inhibition of nucleic acid amplification (Selma et al., 2008).

Early detection of aflatoxin-producing species is critical to prevent this mycotoxin entering the food chain and aflatoxin concentration can be correlated with the density levels of aflatoxigenic species detected on naturally contaminated samples (Shapira et al., 1996). Hence, identification and quantification of species from *Aspergillus* section *Flavi* in raw peanuts could predict potential risk of aflatoxin contamination, during the storage period.

The aim of the present work was to apply a rapid, sensitive and specific assay, not inhibited by matrix effects, to detect and quantify *Aspergillus* section *Flavi* spp. in stored peanuts. The assays were performed in combination with an efficient and economic fungal DNA extraction procedure, tested in peanuts stored and conditioned at different water activities during a 4-month period. The effects of fungal density and storage conditions on aflatoxin produced were also investigated.

2. Materials and methods

2.1. Storage silos and sample collections

One-thousand kilograms of in-pod peanuts destined for human consumption were artificially dried up to 0.92 ± 0.01, 0.88 ± 0.01, 0.84 ± 0.01 and 0.76 ± 0.02 a_W, distributed in four flexible and air-permeable containers (called "big bag") manufactured of polypropylene raffia of high resistance and tenacity and used to carry out the study from July to November 2008. The four experimental units were placed alongside a 60 t stockpiled cell in a storage company located in the south of Córdoba, Argentina. The stockpiled cell had air-extractors that were used to renew the air during the storage period and thermocouples to monitor peanut temperature.

A total of one-hundred samples were monthly collected from July to November 2008. Five points of each big bag were sampled at each collection time by using a compartment-sampling spear, which enabled samples to be taken from different depths. Each 1 kg sample was collected in polyethylene bags (to minimize water loss), sealed, transferred immediately to the laboratory and kept at low temperature (−20 °C). After each storage period, each sample was analyzed for the presence of *Aspergillus* section *Flavi* and aflatoxins in kernels.

2.2. Physical properties of sample determination

Water availability of peanut kernels at each sampling period was determined by measurement a_W with AQUALAB CX2 (Decagon, Devices, USA) according to the operator's Manual. Water activity was determined once per sample ($n = 5$ per big bag at each sampling). The temperature changes of stored peanuts were measured monthly by using distance-reading thermometers.

2.3. Mycological studies

Counts of *Aspergillus* section *Flavi* spp. were determined by culturing all samples on plates containing dichloran rose bengal chloramphenicol agar (DRBC) supplemented with 3% NaCl (Horn, 2005). Quantitative enumeration was done using the surface-spread method. Ten grams of each milled peanut sample was homogenized in 90 mL 0.1% peptone–water solution for 30 min in an orbital shaker. Serial decimal dilutions up to 10^{-6} were made and 0.1 mL aliquots were inoculated in duplicate onto the culture media. The plates were incubated at 25 °C for 5–7 days. Only plates containing 10–100 colony forming units (cfu) were used for counting. The results were expressed as colony forming units per g of peanut kernels (cfu/g). Isolates were identified to section level according to Klich (2002).

2.4. Aflatoxin analyses

Aflatoxin contents from 100 peanut kernels samples were determined following the methodology proposed by Trucksess et al. (1994). For aflatoxin analysis each sample consisted of 500 g of in-pod peanuts that was manually shelled, ground and mixed to obtain peanut paste, and kept at −20 °C until analysis. A representative sub-sample of 50 g of thoroughly homogenized peanuts was taken by quartering method and extracted with acetonitrile:water (90:10, v/v) by shaking the sample with solvent for 30 min on an orbital shaker, filtering the extracts through Whatman No. 4 filter paper. A 3 mL aliquot of each extract was applied to a clean-up column (Mycosep 224 MFC, Romer). A 200 µL aliquot was derivatized with 700 µL of trifluoracetic acid:acetic acid: water (20:10:70, v/v/v). The determination of aflatoxin levels was performed by high-performance liquid chromatography (HPLC). The HPLC system consisted of a Hewlett Packard 1100 pump (Palo Alto, CA, USA) connected to an HP 1046A programmable fluorescence detector, and quantification was done with an HP workstation. Chromatographic separations were performed on a stainless steel C_{18} reversed phase column (150 × 4.6 mm i.d., 5 µm particle size; Luna-Phenomenex, Torrance, CA, USA). Water:methanol:acetonitrile (4:1:1, v/v/v) was used as the mobile phase at a flow rate of 1.5 mL/min. Fluorescence of aflatoxin derivatives was recorded at excitation and emission wavelengths of 360 and 440 nm, respectively. Aflatoxins were quantified on the basis of HPLC fluorometric response compared with aflatoxin standards (Sigma Chemical, St Louis, MO, USA). The mean recovery of the method used was calculated by spiking peanut kernels at different levels ranging from 5 to 100 ng/g of aflatoxins and was estimated at 94.5%. The lowest limit of detection was 1 ng/g.

2.5. Isolation of DNA from peanut samples

For DNA isolation from peanut samples, 10 g of peanut kernels was milled and homogenized in 90 mL of 0.1% peptone–water solution for 30 min in an orbital shaker. An aliquot of 0.1 mL was centrifuged at 13,000 rpm for 10 min. The pellet was vortexed for 2 min in the presence of 500 µL of extraction buffer (100 mM Tris HCl, 2% CTAB; 1.4 mM NaCl) and glass beads (425–600 µm diameters, Sigma) in order to favor the disruption of fungal material. After incubation at 65 °C for 60 min, 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) was added to the sample, homogenized and centrifuged for 10 min at 13,000 rpm. The aqueous phase was recovered and 1 volume of chloroform:isoamyl alcohol (24:1, v/v) was added. The sample was homogenized and centrifuged again for 10 min at 13,000 rpm. The aqueous phase was recovered and precipitated with 10 µL NaCl (5 M) in the presence of 2 volumes of 100% ethanol. The sample was homogenized by inversion 4 times, centrifuged for 10 min at 13,000 rpm and the aqueous phase was

discarded. Finally the DNA pellet was washed with 70% ethanol and suspended in 25 µL of nuclease-free H_2O.

To analyze the mean recovery percentage of the DNA isolation procedure, peanut samples were contaminated with different defined amounts of *A. parasiticus* RCP08300. DNA was extracted by applying the method previously described and concentrations were determined by UV–visible spectrophotometery (DU 800, Beckman Coulter, USA). The quantity of nucleic acid was determined at 260 nm wavelength. The quality of the DNA solution was accepted at an A_{260}/A_{280} ratio of 2.0 ± 0.15. The additional DNA extracted from peanut samples was compared with a control without fungal DNA.

2.6. Real-time PCR

The real-time PCR reactions were performed in a Mx3000P Stratagene (USA). The primers and the internal probe used in the reaction were those proposed by Mayer et al. (2003). The used primer/probe set had the following nucleotide sequence: nortaq-1, 5′-GTCCAAGCAACAGGCCAAGT-3′; nortaq-2, 5′-TCGTGCATGTTGGTGATGGT-3′; norprobe, 5′-TGTCTTGATCGGCGCCCG-3′ enclosing an amplicon of 66 bp from nucleotide 514 to 580 according to the published sequence of *nor*-1 for the isolate AF36 of *A. flavus* (AY510455) (Geisen, 1996). The sequence was checked by BLAST (www.ncbi.nlm.nih.gov/BLAST) nucleotide database search.

The method sensitivity was tested by amplification of serial dilutions of total DNA extracted from 5000 conidia of *A. parasiticus* RCP08300, estimated under optical microscopy using a Newbauer camera, and quantified by spectrophotometer (DU 800, Beckman Coulter, USA). For PCR reaction 1 µL of the DNA sample solution was mixed with 24 µL of PCR stock solution containing 2.5 µL of 10× PCR buffer (5 Prime, GmbH Hamburg Deutschland), 2 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTP mixture, 1 µL of each primer (10 µM), 0.5 µL probe (10 µM), 0.2 µL of 5 U/µL enzyme (5 Prime, GmbH Hamburg Deutschland) and 16.3 µL sterile deionized H_2O. The amplification thermal profile was: 4 min at 95 °C following of 40 amplification cycles at 95 °C for 30 s, 53 °C for 30 s and 72 °C for 20 s.

For the standard curve generation, 10 g of milled peanut sample was suspended in 90 mL 0.1% peptone–water solution and inoculated with a concentration of *A. parasiticus* RCP08300 spores from which serial decimal dilutions were done. A 100 µL aliquot was inoculated in duplicates onto Petri dishes containing malt extract agar, from which the cfu/g was estimated. At the same time, a 100 µL aliquot of each dilution was taken for extracting the DNA according to the methodology described before. An aliquot of each dilution was used in standard reactions during each setup of the RT-PCR reaction. Initial DNA concentrations of each standard were related with the cfu values of each dilution. Colony forming units of unknown samples were calculated by the MxP-3000 system according to the generated standard curve.

2.7. Statistical analysis

Statistical analyses were performed using SigmaStat program Version 3.10 (Systat Software, USA). Analysis of variance (ANOVA) was performed on total aflatoxin accumulation and Fisher's LSD test ($p < 0.05$) was applied to compare significant differences between the different samplings.

3. Results

3.1. Calibration of DNA isolation, specificity and sensitivity of the RT-PCR assay

According with the observed data, the mean recovery percentage for DNA of *A. parasiticus* RCP08300 extracted from peanut kernels was $94.7 + 16.4\%$ ($n = 5$).

The primers and probe specificity was already tested by Mayer et al. (2003) on genomic DNA of three strains of different *Aspergillus* spp., and on two *Penicillium* spp., four *Fusarium* spp., and one species each of *Mucor*, *Paecilomyces*, *Cladosporium*, and *Alternaria*.

Analysis of total DNA extracted from 5000 conidia of *A. parasiticus* RCP08300 showed that one conidium contains approximately 96 ± 30 pg of DNA. The amplification limit of RT-PCR using nortaq-1, nortaq-2 primers and norprobe was 125 pg, indicating that through the described methodology the detection of a single conidium of *A. parasiticus* RCP08300 was possible.

3.2. Standard curve of the molecular method

Amplification curves obtained with known template concentrations showed a clear relationship between initial DNA concentrations and changes in fluorescence. The DNA extracted from calibrated conidial suspension of *A. parasiticus* RCP08300 in peanut broth, in a range from 2.5×10^3 to 2.5×10^7 cfu/g per reaction, showed progressively lower Ct values (Fig. 1). Data showed an inverse linear correlation between cfu/g and Ct with slope and R^2 values similar in two independent assays (means = −2.8 and 0.99), indicating that the RT-PCR system was highly linear. According to these results the primer/probe system used (nortaq-1, nortaq-2, norprobe) appeared sensitive and accurate for detection of the *nor*-1 fragments extracted from peanut samples.

3.3. Quantification of the nor-1 gene in natural peanut samples and correlation to cfu

A. flavus was predominant in all peanut samples showing isolation percentages of 88.9, 96.4, 100 and 89.2% for big bags 1, 2, 3 and 4, respectively. *A. parasiticus* was isolated in low percentage in big bags 1, 2 and 4 ranging from 3.7 to 13.0%. Only two *A. caelatus* isolates from big bag 1 and at the third sampling were identified. Peanut kernels showed natural infections by members of *Aspergillus* from section *Flavi* during the 4-month storage period, at the different a_W initial values (big bag $1 = 0.92 \pm 0.01$, big bag $2 = 0.88 \pm 0.01$, big bag $3 = 0.84 \pm 0.01$ and big bag $4 = 0.76 \pm 0.02$). Peanut samples taken at different storage times to determine the density of *Aspergillus* section *Flavi* spp. by conventional and molecular methods, showed correlation between the data obtained ($r = 0.613$; $p < 0.0001$) (Fig. 2a–d). However, the cfu values obtained by RT-PCR were usually higher (0.5–1 log units) than those obtained by conventional counts (CC). Both count methods showed that the highest cfu values were in peanut kernels conditioned at the two highest a_W and that this counts were relatively stable during the first three months of storage. The mean counts obtained between the first and fourth samplings were 4.9×10^6 cfu/g (CC) and 2.6×10^7 cfu/g (RT-PCR), and

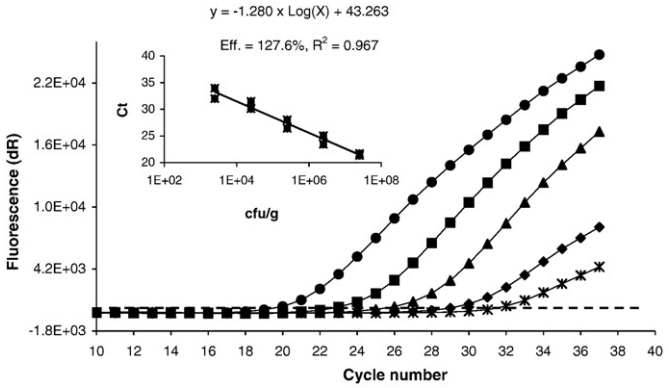

Fig. 1. Amplification plot of standard curves obtained from decimal dilutions of *A. parasiticus* RCP08300 conidial suspensions. Insets show representative standard curve generated from the amplification data: Ct values are plotted against the propagule concentration as cfu/g per reaction. Plate counts are: ●, 2.5×10^7; ♦, 2.5×10^6; ▲, 2.5×10^5; ■, 2.5×10^4; **x**, 2.5×10^3 cfu/g per reaction.

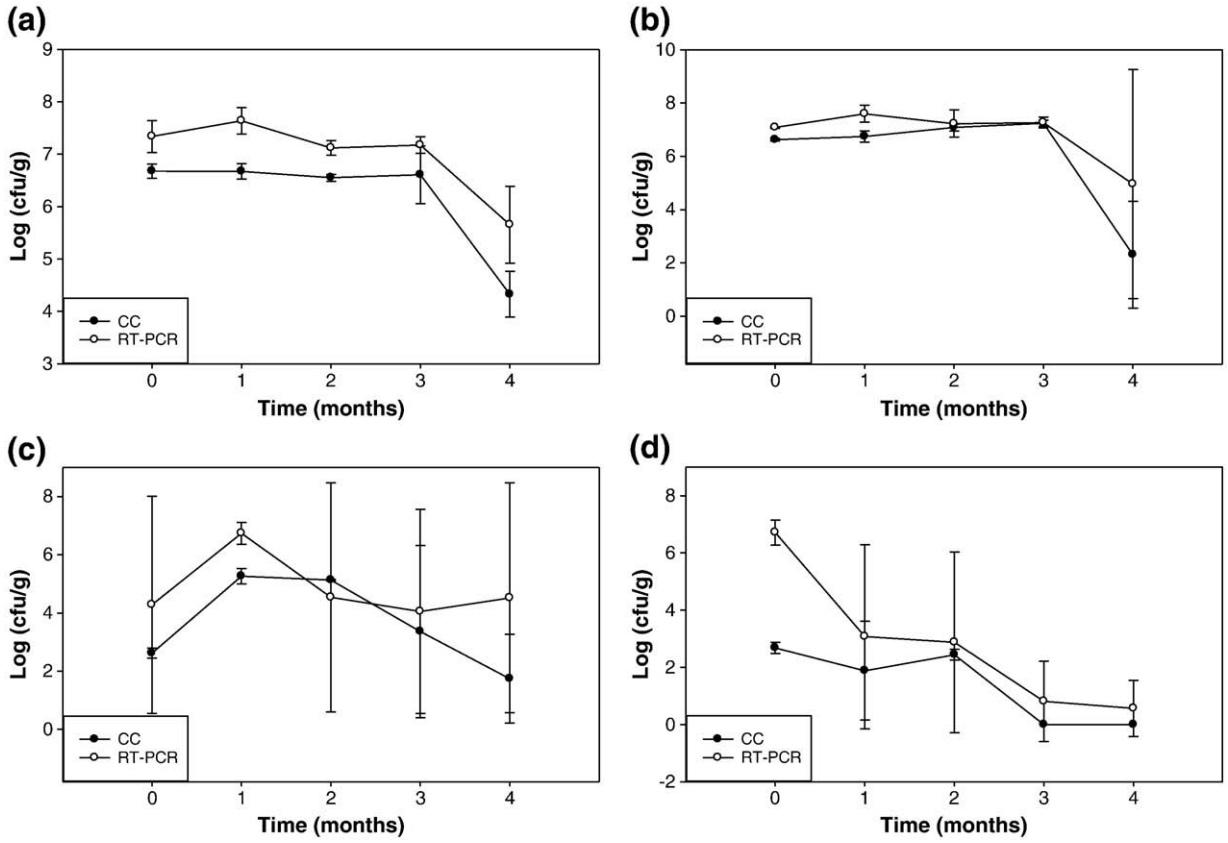

Fig. 2. Comparison of the colony forming units per gram of peanut (log cfu/g ± DS) of *Aspergillus* section *Flavi* spp. present in peanut samples from big bags 1 (a), 2 (b), 3 (c) and 4 (d) during a 4-month storage period measured by conventional count (CC) and molecular (RT-PCR) methods.

1.0×10^7 cfu/g (CC) and 2.6×10^7 cfu/g (RT-PCR), for big bags 1 and 2 respectively. A reduction of *Aspergillus* section *Flavi* spp. counts (2.3–4.6 log units (CC) and 1.7–2 (RT-PCR)) was observed at the end of the storage period. In peanut samples proceeding from big bag 3, *Aspergillus* section *Flavi* spp. counts were relatively constant (7.9×10^5–7.1×10^6 cfu/g) during the whole storage period. Similar to the results observed in big bags 1 and 2, *Aspergillus* section *Flavi* spp. counts were reduced at about 51% by the end of the storage period. In samples taken at the third and fourth months of storage from big bag 4, RT-PCR was able to detect *nor*-1 copies estimated at 92 and 17 cfu/g, respectively, while the conventional method gave <100 cfu/g.

3.4. Natural occurrence of aflatoxins from peanut kernels

Aflatoxin B_1, B_2, G_1 and G_2 concentrations of the one-hundred peanut samples collected are shown in Table 1. Aflatoxin levels in samples from big bags 1 and 2 increased with the time of storage, although with different time rates. When samplings of the first and fourth months were compared, toxin increases were 57 and 97% for big bags 1 and 2, respectively. The mean value of total aflatoxins obtained in samples from big bag 3 at the third sampling was significantly higher (89%) than from those obtained in the first and second samplings ($p<0.05$). Aflatoxins were not detected at any analyzed peanut sample from big bag 4. Statistical analysis showed a very poor negative correlation between cfu and aflatoxins accumulation ($r=-0.514$; $p=0.05$) in peanut samples.

Table 1
Aflatoxins production by species of *Aspergillus* section *Flavi* isolated from stored peanut kernels.

Sampling	Aflatoxins concentration ng/g (mean ± SE)[a]		
	Big bag 1[b]	Big bag 2	Big bag 3
July	86.8 ± 27.8 a	3.9 ± 0.2 c	1.1 + 1.1 b
August	158.2 ± 38.1 a	50.7 ± 3.8 bc	1.2 + 0.1 b
September	139.6 ± 28.7 a	82.6 ± 21.8 ab	10.6 + 0.3 a
October	153.4 ± 47.5 a	83.6 ± 41.2 ab	13.8 + 3.5 a
November	200.4 ± 69.1 a	140.9 ± 14.9 a	12.5 + 3.0 a

[a] Mean levels of total aflatoxins ($AFB_1+AFB_2+AFG_1+AFG_2$) from peanut kernel samples. SE: standard error, $n=5$. Detection limit: 1 ng/g.
[b] Big bag 1 0.94 ± 0.01 a_W; big bag 2 0.88 ± 0.01 a_W; big bag 3 0.84 ± 0.01 a_W. Values in columns with no letters in common are significantly different ($p<0.05$) according to Fisher LSD test.

3.5. Variations in temperature and water activity of peanut samples

The determination of physical properties of the samples revealed marked differences in water activity (a_W) and temperature among individual samples in the four big bags throughout the storage period (Fig. 3a–b). Water activity levels in peanut samples from big bags 1 and 2 decreased relatively faster during the first three months of storage, from 0.92 ± 0.01 a_W to 0.76 ± 0.00 a_W and from 0.88 ± 0.01 a_W to 0.67 ± 0.03 a_W, respectively and thereafter at a slower rate until the end of storage period (mean = 0.65 ± 0.01 a_W). However, a_W levels in peanuts from big bag 3 were lower during the four months of storage (from 0.84 ± 0.01 a_W to 0.60 ± 0.04 a_W). Peanut samples a_W from big bag 4 significantly decreased ($p<0.05$) during the second month of storage and thereafter remained relatively constant until the end of storage period, reaching similar values (0.66 ± 0.01 a_W) to those registered in big bags with the highest initial a_W. Due to the gradual increase of ambient temperature at last spring warming under the Argentinean conditions, temperatures registered in peanuts from the four big bags showed a constant increase during the four months of storage, from 12.7 ± 0.45 °C to 29.2 ± 0.6 °C. These environmental variations significantly affected fungal density ($F=584.413$; $p<0.001$) and total

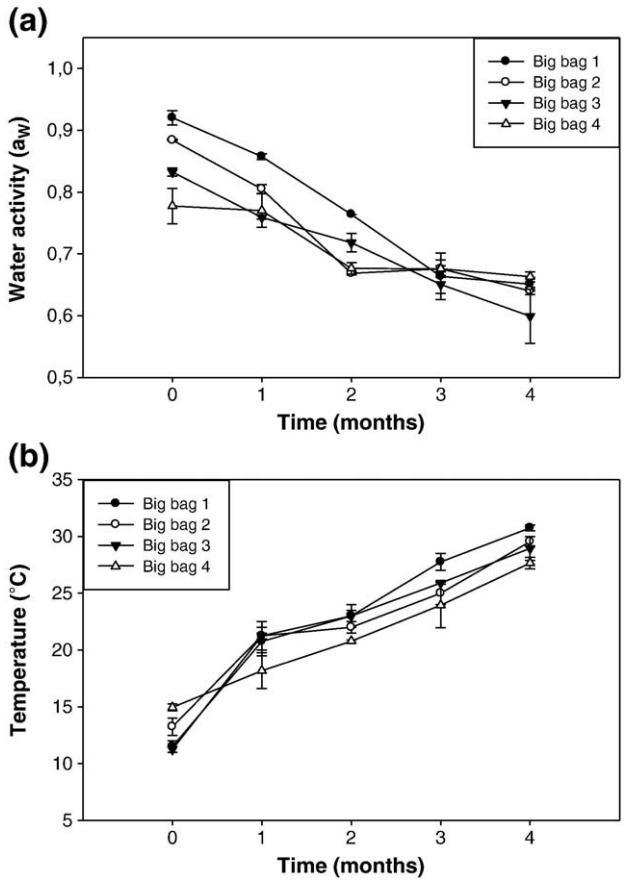

Fig. 3. Environmental changes a_W (a) and temperature (b) registered from peanuts stored during 4 months and conditioned at different initial a_W: big bag 1 at 0.94 ± 0.01 a_W; big bag 2 at 0.88 ± 0.01 a_W; big bag 3 0.84 ± 0.01 a_W; big bag 4 at 0.76 ± 0.02 a_W.

aflatoxin accumulation ($F = 32.475$; $p < 0.001$). Statistical analysis carried out with data registered from big bags 1, 2 and 3 showed a negative correlation between a_W and toxin accumulation ($r = -0.643$; $p = 0.031$) and a positive correlation between temperature and total aflatoxins ($r = 0.658$; $p = 0.0225$).

4. Discussion

For many years there has been much interest focused on detection and quantification of *Aspergillus* species from section *Flavi* which are responsible for the contamination of peanuts with aflatoxins (Bhattacharya and Raha, 2002; Gonçalez et al., 2008; Nakai et al., 2008). In the present study, a rapid, specific and sensitive RT-PCR assay for the detection and quantification of *Aspergillus* section *Flavi* on stored peanuts was developed. To achieve this, a simplified and economic procedure for DNA extraction directly from milled peanuts was used. The RT-PCR data, although correlated with cfu values obtained with the conventional count methodology ($r = 0.613$; $p < 0.0001$) were generally higher than CC data, showing a higher sensitivity of the molecular technique. The major difference (4 log units) was observed in big bag 4 at the start of the storage period. This result could be due to the long-time drying process needed to reduce water availability of in-pod peanuts at 0.76 a_W. This process probably killed a large portion of fungal population preventing thus aflatoxin accumulation in this big bag and resulting in a reduction of cfu values obtained with CC methodology. On the other way, Mayer et al. (2003) suggested that the difference observed between RT-PCR and CC data might be due to several reasons, since cfu determination mainly reflects the number of spores or propagules present in the sample. Mycelial fragments will give rise to only one colony, even if they consist of many cells. In addition, the spores of Aspergilli are usually uninucleate, whereas mycelial cells are multinucleate (Jennings and Lysek, 1996; Kaminskyj and Hamer, 1998). Each nucleus of the target organism carries at least one *nor*-1 gene, all of them contributing to the number of *nor*-1 copies. For this reason mycelial fragments, if present, increase the *nor*-1 copy number at a much higher rate than the cfu number obtained with CC. In addition, dead cells can contribute to the number of *nor*-1 copies. Moreover, sensitivity testing demonstrated that DNA representing one conidium of *A. parasiticus* RCP08300 can be detected, confirming that RT-PCR approach is 100 times more sensitive that CC method (Goebes et al., 2007). A similar relationship was found in a RT-PCR assay applied to quantitatively measure the infection of maize, pepper and paprika with *A. flavus* (Mayer et al., 2003). These authors also found differences between CC and RT-PCR data of *A. flavus*, the latter resulting between 0.5 and 3.5 log units higher.

Stored peanuts represent a complex ecosystem in which seed spoilage by fungi is determined by a range of factors which can be classified into four groups; intrinsic nutritional factors, and extrinsic, processing and implicit factors (Magan et al., 2004). Alone or in combination among them, these factors affect the composition of the fungal population, inducing changes throughout the storage period. In our study, reductions of peanut a_W and increases of temperatures were registered in all big bags throughout the storage period and these physical changes were reflected by a reduction of fungal growth, independently of the quantification methodology applied. When the CC method was used, *Aspergillus* section *Flavi* count reductions were estimated in 35.2, 65.1, 33.6 and 100% for big bags 1, 2, 3, and 4, respectively. It is important to emphasize that similar results were obtained with RT-PCR assays, whose cfu reductions were around 36.0%.

The aflatoxin concentrations in peanuts from big bags 1, 2 and 3 increased toward the end of storage period, the increases in the second bimonthly ranging between 15.8 and 55.1%, which is about 2 times lower than those registered in the first two months. These results appear related to the reduction of the *Aspergillus* section *Flavi* population throughout the storage period. It is possible that a balance occurs in the closed silos environment, between the cumulative increase in aflatoxins concentration and the lower marginal efficiency in their production and/or release, due to the fungus population decrease. Despite the fungal population reduction observed at the end of the storage period, we suggested that ambient stress could produce an increase in aflatoxin levels. Gunterus et al. (2007) reported that no correlation was observed between inoculum level and aflatoxin accumulation. Aflatoxin concentration increased with decreasing inoculum size. The lowest inoculum (10^2 conidiospores/g peanuts) generated highest aflatoxin levels (1.000 μg/g peanuts).

Several *in vitro* studies to quantify mycotoxigenic fungi that were artificially inoculated in natural matrixes such as cereals, grapes, coffee and spices by applying molecular methods have been reported (Atoui et al., 2007; Gil-Serna et al., 2009; Mayer et al., 2003; Nicolaisen et al., 2009; Selma et al., 2008). This is the first *in situ* study that evaluates the applicability of economic, rapid and sensitive RT-PCR method for monitoring *Aspergillus* section *Flavi* in peanuts stored in plant conditions during a 4-month period. The application of this RT-PCR assay would contribute to improve food safety as well as to allow the prediction of the main source of aflatoxin contamination in peanuts. Therefore, the information derived from these tests would allow informed decisions about storage life of peanuts and the prevention strategies eventually needed.

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