IMPACT OF FOOD GRADE ANTIOXIDANTS ON PEANUT PODS AND SEEDS MYCOFLORA IN STORAGE SYSTEM FROM CÓRDOBA, ARGENTINA

M.A. PASSONE[1], R. BLUMA[1], A. NESCI[1], S. RESNIK[2] and M.G. ETCHEVERRY[1,3]

[1]*(CONICET) Departamento de Microbiología e Inmunología*
Facultad de Ciencias Exactas Físico Químicas y Naturales
Universidad Nacional de Río Cuarto
Córdoba, Argentina

[2]*(CIC) Departamento de Industrias*
Facultad de Ciencias Exactas y Naturales
Universidad Nacional de Buenos Aires
Buenos Aires, Argentina

Accepted for Publication November 7, 2007

ABSTRACT

A 5-month (2005) study was conducted in Argentina to determine the effect of food-grade antioxidants (butylated hydroxyanisole [BHA], butylated hydroxytoluene [BHT] and propyl paraben [PP]) alone and in mixtures on fungal spoilage from stored peanuts. Five experimental containers were filled with 200 kg of bulk peanuts. Four of them were treated with BHA (10 mM), BHA-PP mixture (10 + 10 mM) and BHA-PP-BHT mixtures (10 + 5 + 10 mM) and (10 + 10 + 10 mM). Fungal counts from peanuts treated with binary and ternary mixtures were often significantly reduced. A total of 10,997 fungal isolates were identified from the control pod and seed tissues in the six samplings done compared to 5,164, 1,469, 1,217 and 1,228 fungi isolated from silos 2, 3, 4 and 5, respectively. Ninety-eight percent of all fungi isolated were Deuteromycetes and Ascomycetes. Fungal isolation was greater from pod (91.9%) than from seed tissues. The most common fungi identified included Penicillium, Aspergillus, Monascus, Eurotium *and* Fusarium *spp. Isolation frequencies of* Penicillium *spp. from both peanut tissues treated with antioxidant mixtures were reduced about of 82.5%. Along the storage period, the reduction percentages of* Aspergillus *spp. counts by antioxidant mixtures were estimated in 74.5%. The fungal genus more sensitive to the antioxidants action was* Fusarium *spp.*

[3] Corresponding author. TEL: 54-0358-4676113; FAX: 54-0358-4676231; EMAIL: metcheverry@exa.unrc.edu.ar

Journal of Food Safety **28** (2008) 550–566.

PRACTICAL APPLICATIONS

These results indicate that the application of food-grade antioxidant mixtures, butylated hydroxyanisole (BHA)-propyl paraben (PP) and BHA-PP-butylated hydroxytoluene, on peanuts for human consumption at storage level could preserve peanut quality by significant fungal reductions at the allowed exporting level.

INTRODUCTION

Peanuts (*Arachis hypogaea* L.) are the most important oleaginous that are being cultivated in the world. In Argentina, the annual production is about 350,000 Mt and it found Third World peanut exporting. Most of the crop is exported to the European Union (EU) and the U.S.A. and the rest is devoted to the internal market (SAGPyA 2004). The activities of peanut sellers produce a very important influence on the socioeconomic and cultural aspects of the region, contributing to millionaire receipts on the order of $100 million (Soave *et al.* 2004). Nevertheless, the sown field was reduced; this fact is attributed to a complex crisis, indicating that the diseases caused by fungi are one of the reasons for the desertion of this crop (Busso *et al.* 2004). The close contact between the soil and the pods of growing peanuts is an optimal situation that allows fungal colonization. Such harvested peanut seeds contain fungal mycelia and spores that can result in a significant decrease in seed quality when they are stored. During storage, a microorganism's succession is development on the grains, which is determined by physical–chemical (moisture, temperature, pH, levels of O_2, chemical additives and storage time) and biological conditions (interactions with other microorganisms, presence of insects and rodents) that allow transitory or permanent changes in the fungal population (Lacey and Magan 1991). Studies conducted in the U.S.A. demonstrated that the genera more frequently associated with peanut pods are *Aspergillus*, *Alternaria*, *Curvularia*, *Fusarium*, *Lasiodiplodia*, *Mucor*, *Nigrospora*, *Phoma*, *Rhizoctonia* and *Trichoderma* spp. Species of *Fusarium*, particularly *F. solani* and *F. oxysporum*, were the most common with a frequency of isolation of 42% (Baird *et al.* 1993). Although the pod acts as a physical barrier and protects the seeds from the majority of fungal invasion, the even fungi can enter when it ruptures (Pitt and Hocking 1997b). Horn (2005) showed that the predominant fungal genera in peanut seeds are *Alternaria*, *Aspergillus*, *Cladosporium*, *Clonostachys*, *Emericella*, *Eupenicillium*, *Eurotium*, *Fusarium*, *Paecilomyces*, *Penicillium*, *Talaromyces*, *Cunninghamella*, *Rhizopus*, *Syncephalastrum* and *Thermomucor* spp. Fungal contamination causes a reduction in grain quality through the utilization of stock

carbohydrates and proteins, also producing oxidative mellowness of the seeds and contaminating the storage seeds with mycotoxins (Lacey and Magan 1991). In 2004, peanut losses in Argentina caused by biotic disease-causing agents at the post-harvest stage were estimated at 28,000 Mt of seeds, or 6 to 8% of total production; this represented $6.1 million lost income for producers (SAGPyA 2004). The rejection of the commodity with fungal contamination levels higher than 1,000 colony forming units (cfu)/g of peanuts was recently regulated by the EU (Atlanta Poland 2008). Recent studies conducted by Magnoli *et al.* (2006) showed a mycological survey of 47 peanut seed samples. Results indicated the presence of three genera of filamentous fungi (*Aspergillus*, *Penicillium* and *Eurotium* spp.) with counts higher than the allowed exporting level.

The application of traditional fungicides at storage level is rejected because of their toxic effects on human health (Klaassen 1996). Thus, the storage sector company needs to implement the use of new inhibitors without risks for human health. Studies *in vitro* carried out in our laboratory demonstrated that the application of mixtures of food-grade antioxidants inhibited total fungal growth in peanut seeds for 35 days (Passone *et al.* 2005).

Therefore, the objectives of this research were to identify the fungal genera from pods and seeds of peanuts stored for 5 months and to determine if the applications of food-grade antioxidants added with good storage practices are able to reduce fungal colonization in peanut seeds to the levels allowed for exportation.

MATERIALS AND METHODS

Antioxidants

All the antioxidant chemicals were obtained from the Eastman Chemical Company (Kingsport, TN). These were benzoic acid, 2(3)-*tert*-butyl-4 hydroxyanisole (BHA); n-propyl *p*-hydroxybenzoate (PP) and 2,6-di (*tert*-butyl)-p-cresol (BHT). Stock solutions of BHA, PP and BHT (5 and 10 mmol/L) were prepared in ethyl alcohol-distilled water (95:5 v/v) and the appropriate concentration was sprayed to in-pod peanuts.

Fitting-Out and Treatment of the Peanut

Trials were conducted in 2004–2005 in the Storage Company in the south of Córdoba, Argentina. One ton of bulk peanut conventional cultivar (Runner variety) harvested in 2003–2004 for human consumption was distributed in five containers (big bag). At the same time, the silos were filled, in-pod peanuts were treated with different antioxidant formulations using a dosage measure

system. A control container without antioxidants was made, namely silo 1, whereas silo 2 was treated with BHA (10 mM/1,802 g/kg), a binary mixture of BHA-PP (10 + 10 mM/1802 + 1802 g/kg) M1 was applied on silo 3 and treatments with ternary mixtures of BHA-PP-BHT (10 + 5 + 10 mM/ 1,802 + 901 + 2,204 g/kg) M2 and (10 + 10 + 10 mM/1,802 + 1,802 + 2,204 g/kg) M3 were applied on silos 4 and 5, respectively. The five experimental units were put in the stockpiled cell to maintain the treated and control peanuts in the same environmental conditions than that of the peanut storage system.

Sampling Procedures

Ten 500-g samples from each silo were randomly selected of each experimental unit (200 kg) using a device, which enabled samples to be taken from different depths. The first sampling was made at the start of the assay and then every 30 days during a 5-month period. The samples were collected in polyethylene bags, brought to the laboratory and analyzed immediately for mold contamination. Peanut seeds and pods were separated and were analyzed separately.

Environmental Dates

Temperatures were measured with distance-reading thermometers; temperature changes were registered every week. Water contents of peanut grains from each sampling were determined by measurement of water activity (a_W) with a Thermoconstanter Novasina TH 200 (Novasina, Zurich, Switzerland). Previous to storage, peanut humidity was reduced up to 9% by using a continuous dryer that insufflated air at 45C. Positive aeration was sporadically applied on stored peanuts to prevent temperature increase and a_W in the grains. pH values were determined by macerating 5 g of milled peanut seeds with 20 mL of distilled water to 25C using a pH electrode (Hanna Instruments pH 211 Microprocessor pH Meter, Sarmeola di Rubano, Italy).

Laboratory Procedure

The colonization of the peanuts was assessed as cfu/g of peanut pods and seeds. Subsamples (50 g) from each treatment were finely ground in a Buehler laboratory mill and analyzed by shaking for 30 min with 450 mL of peptone-distilled water (0.1% w/v) plus 0.06 g/L of Tween 80. Serial decimal dilutions until 10^{-4} for control samples and until 10^{-3} for treated samples were done. A 0.1 mL aliquot of the three last serial decimal dilution of each treatment was spread on the surface of two solid media, dichloran/rose bengal/ chloramphenicol (DRBC) and dichloran/glycerol 18% (DG18) by duplicate

(Pitt and Hocking 1997a). Plates were incubated in darkness at 28C for 5–7 days. At the end of the incubation period, the average number of colonies (duplicate) was done in that plates that ranged between 10 and 100 cfu. The identification of fungal genera was done according to Samson *et al.* (2002). *Aspergillus* and *Penicillium* sections were identified following the identification keys of Pitt (2000) and Klich (2002).

Statistical Analyses

Statistical analyses were made using SigmaStat program Version 3.10. Copyright© 2004 Systat Software, Inc. Means data on fungal populations grew on DRBC and DG18 media were determined by analyses of variance ($P < 0.001$). To evaluate the significant differences between the control and the treatments Tukey test ($P < 0.05$) was used.

RESULTS

Fungal Populations

Fungal counts were similar in all silos in the first sampling, but in the subsequent days, the fungitoxic effect of antioxidant mixtures became statistically significant ($P < 0.05$) (Table 1). Fifty-two percent of the total fungal isolates were from peanuts without antioxidants compared to 31.4, 9.8, 3.8 and 4.0% from peanuts treated with BHA (10 mM), binary mixture M1 (10 + 10 mM) and ternary mixtures M2 (10 + 5 + 10 mM) and M3 (10 + 10 + 10 mM), respectively, regardless of peanut tissue analyzed.

Isolation from pod tissue yielded more fungi than from seeds, regardless of the treatment applied. In the control samples, 85% of the fungi isolated were from pod tissue, whereas the remaining fungi (15%) were from seeds. In the same way, a mycological survey from peanut treated with BHA (10 mM), M1, M2 and M3 revealed that 94, 92, 95 and 90% were isolated from pod tissue.

Most of the fungi were isolated in the last sampling period from control samples. The total counts in the first sampling were 4.7×10^4 and 3.4×10^3 cfu/g, while after a 5-month storage period, the count increased to 7.2×10^4 and 1.7×10^4 cfu/g in pods and seeds, respectively. While an increase of temperature from 7.6 to 18.7C was observed after 7 weeks of storage, a_W levels tended to be low (0.724 to 0.673 a_W) at the end of the storage period and pH values were maintained relatively stable (mean: 6.7) (Fig. 1a–c). On the contrary, a reduction of fungal colonization of about 80, 52, 89 and 85% was observed at the end of storage period in peanut samples of silos treated with food-grade antioxidants.

TABLE 1.
EFFECT OF ANTIOXIDANTS ON TOTAL MYCOFLORA (UFC/G^{-}) FROM PEANUT PODS AND SEEDS STORED IN EXPERIMENTAL UNITS DURING FIVE MONTHS

Time (months)	cfu/g ± S.D.				
	Silo 1	Silo 2	Silo 3	Silo 4	Silo 5
Pods					
July	$3.1 \times 10^3 \pm 2.5 \times 10^3$ a	$3.6 \times 10^4 \pm 3.5 \times 10^4$ a	<b	<b	<b
August	$1.2 \times 10^5 \pm 1.1 \times 10^4$ a	$5.7 \times 10^4 \pm 2.4 \times 10^4$ a	$2.7 \times 10^4 \pm 2.9 \times 10^4$ b	$2.4 \times 10^4 \pm 2.9 \times 10^4$ b	$1.5 \times 10^4 \pm 1.2 \times 10^4$ b
September	$8.5 \times 10^4 \pm 1.8 \times 10^5$ a	$1.3 \times 10^4 \pm 1.1 \times 10^4$ b	$1.1 \times 10^4 \pm 7.3 \times 10^3$ b	$3.0 \times 10^3 \pm 3.0 \times 10^3$ c	$3.8 \times 10^3 \pm 3.8 \times 10^3$ bc
October	$2.4 \times 10^4 \pm 1.2 \times 10^4$ a	$1.4 \times 10^4 \pm 8.8 \times 10^3$ a	$8.2 \times 10^3 \pm 1.3 \times 10^4$ b	$1.5 \times 10^3 \pm 8.5 \times 10^2$ b	$2.7 \times 10^3 \pm 1.5 \times 10^3$ b
November	$2.6 \times 10^4 \pm 2.4 \times 10^4$ a	$3.8 \times 10^4 \pm 3.2 \times 10^4$ a	$1.0 \times 10^4 \pm 1.1 \times 10^4$ a	$2.3 \times 10^4 \pm 3.6 \times 10^4$ a	$3.1 \times 10^3 \pm 4.5 \times 10^3$ b
December	$6.1 \times 10^4 \pm 3.5 \times 10^4$ a	$6.3 \times 10^3 \pm 4.2 \times 10^3$ b	$3.1 \times 10^3 \pm 2.5 \times 10^3$ bc	$3.1 \times 10^3 \pm 4.0 \times 10^3$ bc	$2.0 \times 10^3 \pm 2.8 \times 10^3$ c
Seeds					
July	$3.4 \times 10^3 \pm 2.6 \times 10^3$ a	$1.7 \times 10^3 \pm 2.5 \times 10^3$ a	<b	<b	<b
August	$6.6 \times 10^3 \pm 7.2 \times 10^3$ a	$2.3 \times 10^3 \pm 2.6 \times 10^3$ ab	$4.8 \times 10^3 \pm 7.4 \times 10^3$ab	$1.3 \times 10^3 \pm 2.0 \times 10^3$ bc	<c
September	$2.7 \times 10^3 \pm 3.7 \times 10^3$ a	$3.3 \times 10^3 \pm 3.9 \times 10^3$ a	<b	<b	<b
October	$1.6 \times 10^4 \pm 2.7 \times 10^4$ a	$2.0 \times 10^3 \pm 2.8 \times 10^3$ ab	<bc	<c	<bc
November	$9.4 \times 10^3 \pm 2.0 \times 10^4$ a	$2.1 \times 10^3 \pm 3.9 \times 10^3$ b	<b	<b	<b
December	$1.7 \times 10^4 \pm 2.3 \times 10^4$ a	<b	<b	$1.0 \times 10^3 \pm 2.0 \times 10^3$ b	<b

Data not sharing a common letter in the same group are significantly different according to Tukey Test ($P < 0.05$).
Silo 1 (Control); Silo 2 (BHA 10 mmol/g); Silo 3 (BHA-PP 10:10 mmol/g); Silo 4 (BHA-PP-BHT 10:5:10 mmol/g); Silo 5 (BHA-PP-BHT 10:10:10 mmol/g).
<; minor to 10^3 cfu/g.

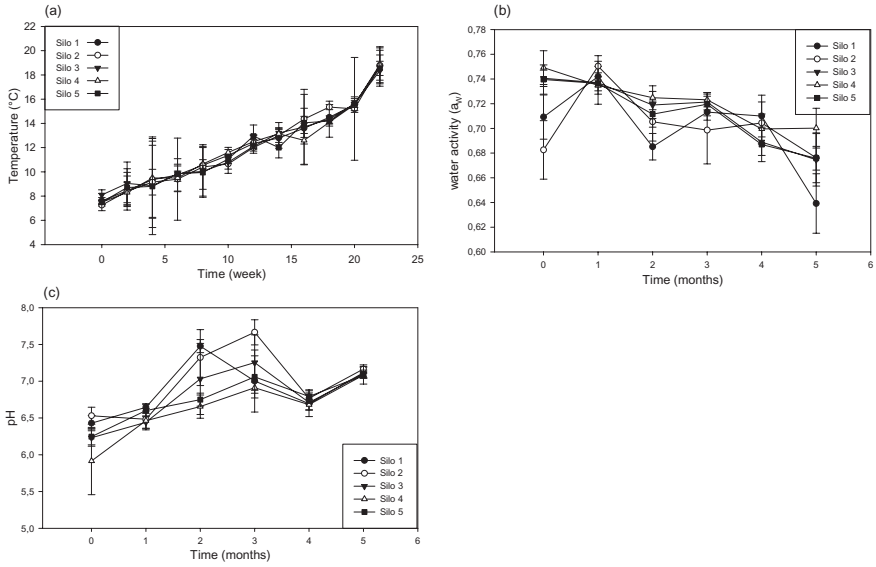

FIG. 1. ENVIRONMENTAL CHANGES – (A) TEMPERATURE; (B) A_W; AND (C) PH – REGISTERED FROM PEANUT STORED IN SILO 1 (CONTROL), SILO 2 (BHA 10 MM), SILO 3 (BHA-PP 10 + 10 MM), SILO 4 (BHA-PP-BHT 10 + 5 + 10 MM) AND SILO 5 (BHA-PP-BHT 10 + 10 + 10 MM) DURING 5 MONTHS

Over 20,000 fungal isolates were obtained from peanut pod and seed tissues corresponding to control and four treated silos during a 5-month storage period. The most frequently occurring fungi are presented in Tables 2 and 3. Ninety-eight percent of the fungal isolates were Deuteromycetes and Ascomycetes, and the remaining were Zygomycetes.

Penicillium, *Aspergillus* and *Fusarium* spp. were the genera most commonly isolated from the two peanut tissue types and five silos. The fungal genera that showed a relatively low frequency of isolation and that were not important mycotoxin producers were all included in the filamentous fungi group. Sources of variation for predominant peanut fungal genera have been combined and are presented as pod and seed tissues (Tiss), sampling periods (Sam) and antioxidant treatments (Treat) in Table 4. There was statistical significance in isolation from tissue type and antioxidant treatments over storage months for *Penicillium*, *Aspergillus*, *Fusarium* spp. and the filamentous fungi group.

Effects of Antioxidants on *Penicillium* Species

Penicillium spp. had the greatest mean frequency levels in control pod tissue during the research period with a count of around 1.8×10^4 cfu/g

TABLE 2.
FUNGAL GENERA IN POD TISSUE FROM CONTROL AND TREATED SILOS

Fungal genera†	cfu/g ± SD*				
	Silo 1	Silo 2	Silo 3	Silo 4	Silo 5
Sterile mycelium	58,167 ± 113,499.63	14,167 ± 21,581.63	2,333 ± 5,715.48	233 ± 408.25	167 ± 314.11
Zygomycetes					
Absidia	3,500 ± 6,442.05	10,000 ± 13,160.55	2,450 ± 4,251.47	167 ± 265.83	917 ± 1,273.45
Mucor	12,000 ± 16,994.12	25,667 ± 25,256.02	6,500 ± 7,611.31	2,167 ± 1,894.91	2,317 ± 1,295.25
Rhizopus	167 ± 408.25	6,833 ± 9,537.64	700 ± 809.94	167 ± 408.25	483 ± 523.13
Ascomycetes					
Eurotium	833 ± 2,041.24	1,333 ± 16,293.15	233 ± 393.28	583 ± 1,144.41	117 ± 240.14
Monascus	49,333 ± 39,154.40	14,083 ± 28,904.62	10,700 ± 21,450.03	3,533 ± 4,722.57	3,250 ± 5,382.84
Deuteromycetes					
Alternaria	7,333 ± 17,962.92	4,667 ± 7,474.40	1,433 ± 3,510.94	0 ± 0.00	17 ± 40.82
Aspergillus					
Section *Candidi*	0 ± 0.00	17 ± 40.82	5 ± 8.37	2 ± 4.08	2 ± 4.08
Flavi	3,650 ± 1.57	2,572 ± 1,905.79	1,642 ± 2,275.56	553 ± 503.81	452 ± 330.48
Flavipedes	0 ± 0.00	0 ± 0.00	17 ± 40.82	2 ± 4.08	3 ± 8.16
Fumigati	0 ± 0.00	333 ± 508.70	25 ± 43.24	32 ± 29.94	10 ± 15.49
Nigri	3,452 ± 29.55	1,950 ± 1,158.66	1,473 ± 1,815.64	1,490 ± 2,716.25	752 ± 727.75
Ornati	0 ± 0.00	0 ± 0.00	2 ± 4.08	7 ± 12.11	2 ± 4.08
Terrei	17 ± 40.82	283 ± 694.02	0 ± 0.00	2 ± 4.08	2 ± 4.08
Fusarium	2,125 ± 5,173.56	2,123 ± 4,764.32	279 ± 1,087.94	66 ± 229.91	335 ± 1,323.64
Penicillium					
Section *Divaricatum*	144 ± 388.39	232 ± 750.49	182 ± 309.30	788 ± 2,780.47	626 ± 1,285.86
Furcatum	2,317 ± 4,163.39	831 ± 2,577.42	1,442 ± 2,987.61	304 ± 831.65	486 ± 1,888.45
Simplicia	15,730 ± 19,999.39	9,733 ± 11,734.76	1,952 ± 4,630.11	1,385 ± 3,915.84	1,790 ± 5,710.70
Trichoderma	167 ± 408.25	2,333 ± 5,240.87	100 ± 200	283 ± 449.07	233 ± 338.62
Total filamentous fungi ($\times 10^6$)	4.90 ± 0.026	3.53 ± 0.017	1.08 ± 0.006	0.71 ± 0.007	0.45 ± 0.003

* Mean + SD based on 120 independent pod samples.
† Deposition of genera and section into group according to Pitt (2000), Klich (2002), Samson *et al.* (2002).

TABLE 3.
FUNGAL GENERA IN SEED TISSUE FROM CONTROL AND TREATED SILOS

Fungal genera†	cfu/g ± SD*				
	Silo 1	Silo 2	Silo 3	Silo 4	Silo 5
Sterile mycelium	8,100 ± 12,208.85	933 ± 1,953.12	50 ± 83.67	0 ± 0.00	50 ± 83.67
Zygomycetes					
Absidia	183 ± 299.44	17 ± 40.82	33 ± 81.65	0 ± 0.00	67 ± 163.30
Mucor	200 ± 209.76	450 ± 714.84	117 ± 160.21	117 ± 147.20	50 ± 83.67
Rhizopus	0 ± 0.00	83 ± 116.91	67 ± 121.11	0 ± 0.00	17 ± 40.82
Ascomycetes					
Eurotium	0 ± 0.00	167 ± 408.25	0 ± 0.00	0 ± 0.00	17 ± 40.82
Monascus	1,217 ± 1,766.82	50 ± 83.67	100 ± 154.92	50 ± 122.47	167 ± 287.52
Deuteromycetes					
Alternaria	167 ± 136.63	17 ± 40.82	33 ± 51.64	0 ± 0.00	33 ± 51.64
Aspergillus					
Section *Candidi*	0 ± 0.00	2 ± 4.08	0 ± 0.00	0 ± 0.00	0 ± 0.00
Flavi	195 ± 155.27	355 ± 238.64	113 ± 99.13	17 ± 26.58	39 ± 48
Flavipedes	0 ± 0.00	0 ± 0.00	0 ± 0.00	2 ± 4.08	0 ± 0.00
Fumigati	0 ± 0.00	33 ± 81.65	33 ± 81.65	5 ± 12.25	0 ± 0.00
Nigri	208 ± 351.31	78 ± 82.80	33 ± 58.54	23 ± 29.44	13 ± 16.33
Ornati	0 ± 0.00	12 ± 28.58	0 ± 0.00	2 ± 4.08	0 ± 0.00
Fusarium	201 ± 636.89	33 ± 145.62	9 ± 57.97	34 ± 172.72	8 ± 37.98
Paecilomyces	0 ± 0.00	0 ± 0.00	33 ± 81.65	0 ± 0.00	0 ± 0.00
Penicillium					
Section *Divaricatum*	37 ± 60.78	79 ± 535.25	8 ± 14.22	53 ± 151.97	371 ± 988.47
Furcatum	104 ± 145.99	67 ± 201.88	60 ± 173.44	78 ± 195.13	355 ± 854.05
Simplicia	1,407 ± 989.65	525 ± 1,370.16	136 ± 169.25	168 ± 306.85	154 ± 447.31
Trichoderma	17 ± 40.82	0 ± 0.00	17 ± 40.82	17 ± 40.82	0 ± 0.00
Total filamentous fungi ($\times 10^4$)	60.97 ± 0.507	16.34 ± 0.109	3.89 ± 0.028	5.07 ± 0.053	7.78 ± 0.078

* Mean + SD based on 120 independent seed samples.
† Deposition of genera and section into group according to Pitt (2000), Klich (2002), Samson *et al.* (2002).

TABLE 4.
SIGNIFICANCE OF POD AND SEED TISSUES (TISS) STORAGE PERIOD (SAM), ANTIOXIDANT TREATMENTS (TREAT) AND THEIR INTERACTIONS ON THE NUMBER OF FUNGAL ISOLATES

Source of variation	DF†	*Aspergillus spp.*		*Penicillium spp.*		*Fusarium spp.*		Filamentous fungi	
		MS‡	*F* value	MS	*F* value	MS	*F* value	MS	*F* value
Tiss	1	732.08	669.45**	532.95	337.49**	86.60	95.19**	716.41	568.89**
Sam	5	47.24	43.20**	96.94	61.39**	11.77	12.94**	44.01	34.94**
Tiss × tam	5	25.04	22.90**	24.87	15.75**	1.45	1.60	26.79	21.28**
Treat	4	36.21	33.11**	107.36	67.99**	15.28	16.79**	23.40	18.58**
Tiss × treat	4	3.60	3.29*	7.33	4.64**	6.90	7.59**	11.71	9.30**
Sam × treat	20	6.29	5.75**	17.97	11.38**	10.85	11.93**	7.07	5.61**
Tiss × sam × treat	20	2.85	2.60**	5.43	3.44**	5.26	5.78**	4.75	3.77**
Error	1,200	1.09		1.58		0.91		1.26	

* Significant at $P < 0.05$.
** Significant at $P < 0.001$.
† DF = degrees of freedom.
‡ MS = mean squares.

(Table 2). In pod samples from silos 3, 4 and 5, a decreased *Penicillium* spp. count in 1 log unit was observed after antioxidant application, and counts were 3.6×10^3, 2.5×10^3 and 2.9×10^3 cfu/g, respectively, during the storage period. In the same way, a high frequency of *Penicillium* spp. isolation (1.5×10^3 cfu/g) from peanut seeds was observed in the control samples (Table 3). Antioxidant mixtures showed an inhibitory, effect on *Penicillium* spp. during the entire storage period; the reduction percentages ranged from 8 to 28%. *Penicillium* species sorted in three sections – *Divaricatum*, *Furcatum* and *Simplicia* – were isolated from both tissue types and five silos during the storage period. The highest frequency of isolation corresponded to the *Simplicia* section (79.4%), followed by the *Furcatum* (14.5%) and *Divaricatum* sections. However, a predominance of the *Divaricatum* over the *Furcatum* section was observed in the samples from silos 4 and 5.

Effects of Antioxidants on *Aspergillus* Species

Aspergillus spp., a common peanut contaminant, was isolated from both pod and seed tissues in control and treated samples at the six sampling periods (Tables 2 and 3). The cfu counts were 7.1×10^3 and 4.0×10^2 cfu/g in pod and seed control samples, respectively. A decrease of *Aspergillus* spp. counts between 9 and 20% was observed in peanut pods treated with the preservative mixtures. In the same way, ternary antioxidant mixtures, M2 and M3, reduced *Aspergillus* spp. counts from seeds by one log unit. Six sections of *Aspergillus* genus were identified from pod and seed tissues, while the *Aspergillus* section

Terrei were not found in seeds. The *Aspergillus* section *Flavi* had the greatest mean frequency in the five experimental units (48.2%), followed by the section *Nigri* (47.6%). The *Aspergillus* section *Fumigati* (2.4%) was isolated from both tissue types but only in the silos treated. The *Aspergillus* sections *Candidi*, *Flavipedes* and *Ornati* showed a low frequency of isolation of about 0.1% and were mainly present in peanuts treated with the antioxidant mixtures.

Effects of Antioxidants on *Fusarium* Species

Isolation frequency of *Fusarium* spp. was sporadic throughout the study. The mean counts for this genus were 2.1×10^3 and 2.0×10^2 cfu/g in the control pod and seed tissues, respectively (Tables 2 and 3). The application of the three antioxidant mixtures M1, M2 and M3 to peanuts inhibited the growth of *Fusarium* spp. from pods; mean reduction percentages were 27, 45 and 24%, respectively. In the same way, a marked impact of namely mixtures on this fungal genus from peanut seeds was observed; count decreases ranged from 34 to 61%.

Effects of Antioxidants on Filamentous Fungi Group

Results for isolation of *Monascus* spp. from pod and seed tissues and from treated and untreated silos in 5 months are shown in Tables 2 and 3. *Monascus* spp. was consistently isolated from pods and seeds of the five silos during the last 3 months of the assay. It is notable that *Monascus* spp. was isolated at numerically greater levels from control peanut pods (97.6%) than seeds (2.4%) during the study. The antioxidant mixtures markedly affected *Monascus* spp. counts from pod tissue; reduction percentages ranged from 14 to 25%. Similarly, *Monascus* spp. growth in seeds was reduced by around 36% by binary and ternary antioxidant mixtures; this effect was markedly extended along the storage time.

Sterile mycelium, *Absidia*, *Mucor*, *Rhizopus*, *Alternaria*, *Eurotium*, *Peacilomyces* and *Trichoderma* spp. were all isolated in low frequency during the 5-month storage (Tables 2 and 3). In peanuts treated with BHA (10 mM), counts of filamentous fungi group from pods and seeds (mean: 3.3×10^4 cfu/g) were similar to the control samples (4.5×10^4 cfu/g) in the entire storage period. However, the antifungal effects of binary and ternary mixtures M1, M2 and M3 reduced counts of the filamentous fungi group from pods and seeds in the order of 16 to 35%, 28 to 46% and 26 to 40%, respectively. The Zygomycetes such as *Absidia*, *Mucor* and *Rhizopus* spp. were consistently isolated from pod and seed tissues from the five silos at the first two sampling periods, whereas the incidence of *Monascus* spp. and *Eurotium* spp. increased at the end of the storage time. *Alternaria* and *Trichoderma* spp. were isolated with low levels during the assay. Counts of

Alternaria spp. were considerably affected by antioxidant mixtures: M2 inhibited totally their growth and M3 reduced it about 50%. However, counts of *Trichoderma* spp. were higher in treated than untreated peanuts.

DISCUSSION

In the present work, mycelial growth of fungal populations was found to be significantly influenced by peanut tissues, sampling periods, antioxidant treatments and their interactions. Fungal counts in five silos were higher from pods (95%) than from seeds. The low incidence of fungal colonization in seeds supports earlier research showing the importance of injury for invasion by microorganisms and the role of the seed coat as a barrier for invasion (Carter *et al.* 1973). Tannins, waxes, amino compounds and structural features in the peanut seed coat have been implicated in resistance to invasion by *A. flavus* and *A. parasiticus* (LaPrade *et al.* 1973; Zambettakis and Bockelee-Morvan 1976; Amaya-F *et al.* 1977; Sanders and Mixon 1978). As well as the in-pod stored peanuts offer additional safety, we consider it necessary to evaluate the impact of antioxidant mixtures on fungal contamination in in-pod peanuts, owing to the fact that earlier studies have demonstrated that even fungi enter when it ruptures (Jackson 1964; Pitt and Hocking 1997b).

The mycological survey from two peanut tissues and five silos analyzed showed a prevalence of *Penicillium*, *Aspergillus*, *Monascus*, *Eurotium* and *Fusarium* spp., whereas sterile mycelium, *Absidia*, *Mucor*, *Rhizopus*, *Alternaria*, *Paecilomyces* and *Trichoderma* spp. were isolated in low percentages during the storage period. The prevalent genera (*Penicillium*, *Aspergillus*, *Fusarium* and *Eurotium* spp.) have been reported previously from freshly harvested peanut seeds as dominant fungi (Pettit and Taber 1968; Joffe 1969; Hanlin 1973; Adebesin *et al.* 2001; Magnoli *et al.* 2006). For the first time, reduction of fungal counts from peanut pods and seeds by antioxidant mixtures were estimated in 18 and 27%, respectively. Similarly, Marín *et al.* (2000) reported that total fungal populations from maize grains were equally inhibited by 0.5 and 1 g/kg propionate. Recent studies conducted by Nesci *et al.* (2008) demonstrated that BHA-PP mixture (20 + 20 mM) applied on natural maize grains exhibited an inhibitory effect on total mycoflora during a 6-month storage period. On the other hand, the selective effect of antioxidants on peanut mycoflora has been demonstrated. For example, populations of *Penicillium*, *Aspergillus* and *Fusarium* spp. decreased by around 18, 45 and 68%, respectively, when preservative mixtures were applied, showing that *Fusarium* was the genus more sensitive to the antioxidant application. Furthermore, the tolerance of some fungal species to certain doses of antioxidants has been shown. For example, populations of *Eurotium* and *Rhizopus* species increased

with the application of BHA (10 mM), while *Aspergillus* and *Mucor* species were unaffected by this treatment. It has been reported that subinhibitory doses, together with inadequate distribution of chemicals, could favor fungal growth on the treated material with an initial low level of contamination (Smith and Moss 1985; Lacey 1989). Marín *et al.* (2000) demonstrated that *Penicillium* populations from maize grain were reduced about 0.5 to 1 log unit by the application of different doses of propionate, whereas *Aspergillus* spp. counts were stimulated by these treatments. With in-shell peanuts, 0.3 and 0.5% ammonium propionate was demonstrated to exert a selective effect on the natural mycoflora, with *Eurotium* spp. representing more than 50% of the total fungal counts. This effect was evident with 0.3% propionic acid and controlled potentially aflatoxigenic fungi up to day 14 of incubation (Calori-Dominguez *et al.* 1996). Similarly, previous studies by Farnochi *et al.* (2005) showed that PP concentrations resulted in a stimulation of *Aspergillus* populations, whereas this preservative often inhibited *Fusarium* spp.

Efficacy of preservatives such as propionic acid is pH dependent, the best antimicrobial activity occurs with 50% dissociation. The efficacy of these acids, therefore, depends on the dissociation constant, pKa that is 4.87 (De Boer and Nielsen 1995). The pH of peanuts in this study ranged from 5.92 to 7.67; consequently, in most of the cases, the percentage of dissociation was between 93 and 99%. On the one hand, this pH variation probably did not exert a great influence on preservative efficacy; on the other hand, pH levels were optimum for the fungal growth (Magan and Lacey 1984; Wheeler *et al.* 1991). The most important environmental determinants on fungal growth are a_W and temperature (Pitt 1975; Troller and Christian 1978). In this study, temperature values were not a limiting factor for the fungal development since from the seventh week of storage, the level was the minimum enough (15C) to allow the growth of mesophilic fungi. However, a_W values were low, with a mean level of about 0.724 at the beginning of the assay, tending to reduce (0.673) at the end of the storage. This fact, limited fungal development such as *Fusarium* spp. and filamentous fungi group (Beuchat 1983), allowed growth of only the xerophilic fungi that dominated in this study: *Penicillium*, *Aspergillus* and *Monascus* spp.

CONCLUSIONS

This is the first study that evaluated the effect of food grade antioxidants on peanut mycoflora at storage scale-up level. Although good storage conditions were registered along the assay, these were not enough to maintain the total fungal counts in untreated peanut seeds with a mean level below 1,000 cfu/g (Atlanta Poland 2008). This result was achieved by the application

of antioxidant mixtures M1, M2 and M3, added with the application of good storage practices. This study showed that BHA-PP and BHA-PP-BHT mixtures at concentrations of 5 to 10 mM have been showed to be suitable to apply in a traditional storage system of peanuts in Argentina for a period of up to 5 months, because it effectively inhibited the growth of potentially toxigenic fungi.

ACKNOWLEDGMENTS

This research received financial support from Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto (Res. N° 077/03, granted during 2003–2004), FONCYT PICT (N° 08–14551) and FONCYT PID (N° 440).

REFERENCES

ADEBESIN, A.A., SAROMI, O.T., AMUSA, N.A. and FAGADE, S.O. 2001. Microbiological quality of some groundnut products hawked in Bauchi, a Nigerian City. J Food Technol. Afr. *6*, 53–55.

AMAYA-F, J., YOUNG, C.T., MIXON, A.C. and NORDEN, A.J. 1977. Soluble amino and carbohydrate compounds in the testa of six experimental peanut lines with various degrees of *Aspergillus flavus* resistance. J. Agric. Food Chem. *25*, 661–663.

ATLANTA POLAND. 2008. Specyfikacja Surowca (Raw Material Specification), Orzechy Arachidowe (Peanuts). Available at http://www.atlantapoland.com.pl (accessed September 8, 2008).

BAIRD, R.E., BRENNEMAN, T.B., BELL, D.K., CULBREATH, A.K. and MULLINIX, B.G. 1993. The peanut shell mycobiota of detached vs. mechanically harvested pods either treated or not treated with flutolanil. Plant Dis. *77*, 405–408.

BEUCHAT, L.R. 1983. Influence of water activity on growth, metabolic activities, and survival of yeasts and molds. J. Food Prot. *46*, 135–141.

BUSSO, G., CIVITARESI, M., GEYMONAT, A. and ROIG, R. 2004. Situación socioeconomica de la produccion de mani y derivados en la region centro-sur de Córdoba. Universidad Nacional of Río Cuarto Press, Córdoba, Argentina.

CALORI-DOMINGUEZ, M.A., FONSECA, H. and DE CAMARGO, M.R.T. (eds.) 1996. Effect of propionic acid on fungal growth and aflatoxin production in moist inshell groundnuts. Rev. Microbiol. *27*, 151–161.

CARTER, J.B.H. 1973. The influence of the testa, damge and seed dressing on the emergence of groundnut (*Arachis hypogaea*). Annu. Appl. Biol. *74*, 315–323.

DE BOER, E. and NIELSEN, P.V. 1995. Food preservatives. In *Introduction to Food-borne Fungi* (R.A. Samson, E.S. Hoekstra, J.C. Frisvad and O. Filtenborg, eds.) pp. 289–294, Cetraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands.

FARNOCHI, M.C., TORRES, A.M., MAGAN, N. and CHULZE, S.N. 2005. Effect of antioxidants and competing mycoflora on *Fusarium verticillioides* and *F. proliferatum* populations and fumonisin production on maize grain. J. Stored Prod. Res. *41*, 211–219.

HANLIN, R.T. 1973. The distribution of peanut fungi in the southeastern United States. Mycopathol. Mycol. Appl. *49*, 227–241.

HORN, B.W. 2005. Colonization of wounded peanut seeds by soil fungi: Selectivity for species from *Aspergillus* section. Flavins Mycol. *97*, 202–217.

JACKSON, C.R. 1964. Location of fungal contamination or infection in peanut kernels from intact pods. Plant Dis. Rep. *48*, 980–933.

JOFFE, A.Z. 1969. The mycoflora of fresh and stored groundnut kernels in Israel. Mycopathol. Mycol. Appl. *39*, 255–264.

KLAASSEN, C. (ed.) 1996. *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 5th Ed., MacMillan Publishing Company, New York.

KLICH, M.A. (ed.) 2002. *Identification of Common Aspergillus Species*. Centraalbureau Voor Schimmelcultures, Utrecht, The Netherlands.

LACEY, J. 1989. Prevention of mould growth and mycotoxin production through control of environmental factors. In *Mycotoxins and Phycotoxins, Bioactive Molecules 10* (S. Natori, K. Hashimoto and Y. Ueno, eds.) pp. 161–169, Elsevier Science, Amsterdam, The Netherlands.

LACEY, J. and MAGAN, N. 1991. Fungi in cereal grain: Their occurrence and water and temperature relationship. In *Cereal Grains Mycotoxins, Fungi and Quality in Drying and Storage* (J. Chelkowski, ed.) pp. 77–118, Elsevier Science Publisher B.V, Amsterdam, The Netherlands.

LAPRADE, J.C., BARTZ, J.A., NORDEN, A.J. and DEMUYNK, T.J. 1973. Correlation of peanut seed-coat surface wax accumulations with tolerance to colonization by *Aspergillus flavus*. J. Amer. Peanut Res. Educ. Soc. *5*, 89–94.

MAGAN, N. and LACEY, J. 1984. Water relations of some *Fusarium* species from infected wheat ears and grain. Trans. Brit. Mycol. Soc. *83*, 281–285.

MAGNOLI, C., ASTORECA, A., PONSONE, L., FERNÁNDEZ-JURI, M.G., CHIACCHIERA, S. and DALCERO, A. 2006. Ochratoxin A and the occurrence of ochratoxin A-producing black aspergilli in stored peanut seed from Córdoba. Argent. J. Sci. Food Agric. *86*, 2369–2373.

MARÍN, S., MAGAN, N., ABELLANA, M., CANELA, R., RAMOS, A.J. and SANCHIS, V. 2000. Selective effect of propionates and water activity on maize mycoflora and impact on fumonisin B_1 accumulation. J. Stored Prod. Res. *36*, 203–214.

NESCI, A., FERRARI, L. and ETCHEVERRY, M. 2008. Effect of synthetic antioxidants on stored maize grain mycoflora in situ. J. Sci. Food Agric. *88*, 797–804.

PASSONE, M.A., RESNIK, S.L. and ETCHEVERRY, M.G. 2005. Chemical control of *Aspergillus* section *Flavi* in peanut grains using food grade antioxidants. Biocell *29*, 232.

PETTIT, R.E. and TABER, R.A. 1968. Factors influencing aflatoxin accumulation in peanut kernels and the associated mycoflora. Appl Microbiol *16*, 1230–1234.

PITT, J.I. 1975. Xerophilic fungi and the spoilage of foods of plant origin. In *Water Relation of Food* (R.B. Duckworth, ed.) pp. 273–307, Academic Press, London.

PITT, J.I. 2000. *A Laboratory Guide to Common Penicillium Species* (3rd E). CSIRO, Division of Food Research, Academic Press, Sydney, Australia.

PITT, J.I. and HOCKING, A.D. 1997a. Methods for isolation, environmental and identification. In *Fungi and Food Spoilage* (J.I. Pitt and A.D. Hocking, eds.) pp. 469–488, CSIRO Division of Food Science and Technology, Academic Press, Sydney, Australia.

PITT, J.I. and HOCKING, A.D. 1997b. Spoilage of fresh and perishable foods. In *Fungi and Food Spoilage* (J.I. Pitt and A.D. Hocking, eds.) pp. 469–488, CSIRO Division of Food Science and Technology, Academic Press, Sydney, Australia.

SAGPyA 2004. *Secretaría de Agricultura, Ganadería, Pesca y Alimentación. Estimaciones Agrícolas Cereales*, Dirección de Coordinación de Delegaciones, Buenos Aires, Argentina.

SAMSON, R.A., HOEKSTRA, E.S., FRISVAD, J.C. and FITENBORG, O. 2002. *Introduction to Food-and Airborne Fungi* (R.A. Samson and E.S. Hoekstra, eds.) Posen & Looyen, Wageningen, The Netherlands.

SANDERS, T.H. and MIXON, A.C. 1978. Effect of peanut tannins on percent seed colonization and *in vitro* growth by *Aspergillus parasiticus*. Mycopathologia *66*, 169–173.

SMITH, J.E. and MOSS, M.O. 1985. Control of mycotoxins. In *Mycotoxin: Formation, Analysis and Significance* (J.E. Smith and M.O. Moss, eds.) pp. 127–142, John Wiley & Sons, Chichester, U.K.

SOAVE, J.H., BIANCO, C.A. and KRAUS, T.A. 2004. Descripción de dos nuevos cultivares de maní (*Arachis hypogaea* L. subsp hypogaea var hypogaea). Agrisciencia *2*, 85–88.

TROLLER, J.A. and CHRISTIAN, J.H.B. 1978. Water activity and food. In *Microbial Growth* (J.A. Troller and J.H.B. Christian, eds.) pp. 87–105, Academic Press, London.

WHEELER, K.A., HURDMAN, B.F. and PITTS, J.I. 1991. Influence of pH on the growth of some toxigenic species *Aspergillus, Penicillium* and *Fusarium*. Int. J. Food Prot. *54*, 375–377.

ZAMBETTAKIS, C. and BOCKELEE-MORVAN, A. 1976. Recherches sur la structure du – Tegument seminal de la graine d'arachide et son influence sur la penetration de l'*Aspergillus flavus*. Oléagineux *31*, 219–228.