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Effect of processing for saponin removal on fungal contamination of quinoa seeds (*Chenopodium quinoa* Willd.)

Ursula Pappier, Virginia Fernández Pinto *, Gabriela Larumbe, Graciela Vaamonde

Laboratorio de Microbiología de Alimentos, Departamento de Química Orgánica, Area Química y Microbiología de Alimentos, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, (1428) Buenos Aires, Argentina

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ABSTRACT

Incidence of fungal contamination of quinoa seeds from three locations (Salar de Uyuni, Bolivia; Salta and Tucumán provinces, Argentina) was analyzed in samples with and without treatment to remove saponins (wet method). In processed samples, the percentage of infection was reduced. Distribution of the different fungal genera was not homogeneous in the three locations ($p<0.05$), although *Penicillium* and *Aspergillus* were the most prevalent contaminants, regardless the geographic origin of the samples. Other genera, such as *Eurotium*, *Fusarium*, *Phoma*, *Ulocladium*, *Mucor* and *Rhizopus* were less frequently isolated. *Absidia*, *Alternaria*, *Cladosporium*, *Dreschlera*, *Epicoccum* and *Monascus* were sporadically encountered. Significant differences ($p<0.05$) in the distribution of fungal genera in samples with and without saponins from each location were observed. In all cases, processing caused a decrease of *Aspergillus* incidence, while increased the proportion of *Penicillium*, *Eurotium*, *Mucor* and *Rhizopus* indicating that these genera were part of the internal mycota. *A. flavus* and *A. niger* were the dominating species of genus *Aspergillus*. A similar pattern of prevalent *Penicillium* species was observed in samples with and without saponins, since *P. aurantiogriseum, P. chrysogenum, P. citrinum* and *P. crustosum* were always present in high number, although their relative density was variable according to the geographic origin of samples. Mycotoxin-producing ability of most representative species was also determined. Toxigenic strains of *A. flavus* (aflatoxins and cyclopiazonic acid), *A. parasiticus* (aflatoxins), *P. citrinum* (citrinin) and *P. griseofulvum* (cyclopiazonic acid) were found. None of the *A. niger* isolates was ochratoxin A producer. The above mentioned mycotoxins were not detected in the samples analyzed.

1. Introduction

Quinoa (*Chenopodium quinoa* Willd.) is a pseudocereal currently recognized as a valuable food resource. It has been a major crop in the Andes Mountains since 3000 BC. There is a renewed interest in this plant, as well as in other ancient Americans crops like amaranth, because these small grains contain a protein of outstanding quality, better balanced in terms of aminoacids composition than that of most cereals (Tapia, 2000; Ruales and Nair, 1992; Galwey, 1995). The Food and Agricultural Organization (FAO) observes that quinoa is closer to the ideal protein balance than any other grain, being at least equal to milk in protein quality. Moreover, quinoa exhibits high level of resistance to several adverse factors like soil salinity, drought, frost, diseases and pests and adapts readily to ecologically extreme conditions whereas other grains have been not able to survive (Tapia, 2000).

The main producers and exporters of quinoa in the world are Bolivia and Perú, where the grain is cultivated between 3000 to 4200 masl. The production in Argentina is usually used for domestic consumption as seed and flour. Some European countries, such as Spain, are studying its adaptation to Mediterranean climates and others such as Denmark and Finland are interested in its cultivation (Vilche et al., 2003). Nutrition centers and health food stores are the main distribution places in the U.S., the European Union, Japan and other countries where it is appreciated as a natural and health food (Galwey, 1995).

The seeds of most varieties contain bitter tasting constituents, chiefly water-soluble saponins, located in the outer layers of the seed coat. Saponins are being studied for their insecticidal, antibiotic, fungicidal and pharmacological properties, but seem to be free from significant oral toxicity in humans (Dini et al., 2001). Anyhow, saponins are considered an antinutritional factor and have to be removed from the quinoa seeds by washing or dry polishing, in order to decrease possible biological negative effects and bitterness. Commercial methods for both processes have been developed (Ruales and Nair, 1992). The wet method for removing saponins keeps the nutritional characteristics of the quinoa grains, which could be affected by the other process based on abrasive dehulling of the seeds to remove the outer layers (Tapia, 2000).

Studies on microbiological and toxicological aspects related with quinoa are very scarce. Like cereals and oilseed, quinoa seeds are

* Corresponding author.
E-mail address: virginia@qo.fcen.uba.ar (V. Fernández Pinto).

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susceptible to fungal growth and mycotoxin contamination, but little information is available on the contaminating fungi. Aims of the present work were to identify the mycota of quinoa seeds from three different regions with special reference to toxigenic fungi and to evaluate the effects of a process for saponin removal on fungal contamination.

2. Materials and methods

2.1. Samples

Thirty representative samples of 500 g of quinoa grains (*C. quinoa* Willd.) cultivated in different geographic places were analyzed. Ten of them were collected from the Salar de Uyuni (Bolivia), 10 from Tucumán province (Argentina) and 10 from Salta province (Argentina). Bolivian seeds in this research corresponded to Real variety of quinoa and Argentinean seeds belonged to Sajama variety. They were stored frozen (−18 °C) up to the moment of analysis.

Five samples from each locality were seeds with saponins and in the other five samples saponins were removed. In Bolivian samples saponins were taken off by the industrial wet method, in which the quinoa grains were placed into a water vapor jacketed tank, provided with agitation, washed using abundant water in a relation of 4 to 5 parts of water to 1 part of grains and then dried for 4–5 h to a moisture content less than 10% (wet basis). In the Argentinean samples the saponins were removed at laboratory by agitation during 10 min with sterile distilled water at 50 °C. The grains were dried aseptically in a laminar flow cabinet for 4–5 h to moisture content less than 10% (wet basis). The saponin content of Sajama variety was about 0.8% and in the Real Variety was 2.6%. The saponin content decrease to less than 0,06% in seeds treated by industrial process. In those seeds washed only with water at 50 °C the saponins were about 20% of the original content (Bertoni y Cattáneo, 1990; Bacigalupo y Tapia, 2000; Nieto y Valdivia, 2001).

2.2. Isolation and identification of fungi

The direct plating method of Pitt and Hocking (1997) was used both for counting and isolation of fungi.

Two hundred seeds (approx 1 g) of each sample were placed in 10 Petri dishes (20 seeds per plate) on Dichloran–Chloramphenicol–Peptone Agar (DCPA) and in 10 Petri dishes with Dichloran 18% Glycerol Agar (DG18) and incubated in the dark at 25 °C for 4–7 days. The resulting colonies were sub-culturated on Czapek Yeast Agar (CYA) and stored at 5 °C.

Isolates were identified at genus level according to Pitt and Hocking (1997) and Ellis (1971) for the dematiaceous fungi. *Penicillium* species were identified according to Pitt (2000), *Aspergillus* species according to Klich and Pitt (1998) and *Eurotium* species according to Pitt and Hocking (1997).

The percentage of infection (*I*), the isolation frequency (Fq) and the relative fungal density (RD) of genera and species were calculated as follows:

$$I\% = \text{Number of seeds with occurrence of fungi/Total number of seeds} \times 100 \quad (1)$$

$$Fq\% = \text{Number of isolates of a genus/Total number of fungi isolated} \times 100 \quad (2)$$

$$RD\% = \text{Number of isolates of a species/Total no of isolates of the genus} \times 100. \quad (3)$$

2.3. Mycotoxin production by fungal isolates

The agar plug methods for extracellular (Filtenborg and Frisvad, 1980) and intracellular mycotoxins (Filtenborg et al., 1982) were used for toxin extraction of colonies grown on CYA.

Mycotoxin detection was performed using thin-layer chromatography on silica gel G 60 plates (20×20 cm, 0.25 thick, Merck 5721) at room temperature in a non saturated chamber. For cyclopiazonic acid (CPA) and citrinin the plates were previously dipped in a 2% solution of oxalic acid in methanol followed by air drying to avoid tailing.

Standards of aflatoxins (B_1: 0.5 μg/ml; B_2: 0.1 μg/ml; G_1: 0.5 μg/ml; G_2 :0.1 μg/ml), citrinin (2,5 μg/ml), cyclopiazonic acid (25 μg/ml) and ochratoxin A (OTA: 1 μg/ml) from Sigma Chemical Co., St. Louis, M.O. (USA), all in chloroform as solvent, were used.

Chloroform/acetone (90:10) was used as developing solvent for aflatoxins and ethyl acetate/2-propanol/ammonium hydroxide (40:30:20) for CPA. Citrinin and OTA were developed using toluene/ethyl acetate/formic acid 90% (50:40:10).

Aflatoxins were visualized under longwave UV light (366 nm) and confirmed by spraying with 25% (wt/vol) H_2SO_4 solution. CPA was visualized in daylight after treatment of the plates with Ehrlich's Reagent (1 g of 4-dimethylaminobenzaldehyde in 75 ml ethanol and 25 ml concentrated HCl), with subsequent development of blue spots. Citrinin was identified under longwave UV light (366 nm) as a yellow fluorescent spot and OTA was visualized as a bluish-green fluorescent spot under UV light (366 nm). Confirmation assays were carried out by treating the spots with ammonia (OTA bluish-green fluorescence spots change into deep blue and citrinin yellow fluorescence spots disappeared).

2.4. Analysis of samples to detect natural occurrence of mycotoxins

For aflatoxin detection the samples were mixed with methanol–water (55:45), hexane and ClNa, blended at high speed for 1 min and extracted with chloroform in a separatory funnel according to the AOAC method BF 970.45 (AOAC, 1995). CPA analysis was performed by the method of Fernández Pinto et al. (2001) by mixing the sample with methanol: 2% sodium hydrogen carbonate (7:3), blended at high speed for 3 min, centrifugated at 1500 rpm and filtered. The filtrate was defatted with hexane, then 10% KCl solution was added and the mixture was acidified to pH 2 and extracted with chloroform. Citrinin analysis was performed by the method described by Comerio et al. (1998). Samples were shaken with a mixture of acetonitrile: 4% solution KCl: H_2SO_4 (180:20:2), filtered and extracted with water–chloroform (25:50). All these methods were TLC based. Plates, developing solvents and confirmation were the same as for mycotoxin production for fungal isolates. The detection limits were: 1 μg/kg for aflatoxins, 50 μg/kg for CPA and 2 μg/kg for citrinin.

2.5. Statistical analysis

All statistical analyses were performed using Infostat software (Grupo Infostat, Universidad Nacional de Córdoba, Argentina). A Chi square test was applied to the distribution of the different genera in the three locations and for the effect of the treatment to remove saponins. A value of $p<0.05$ was considered statistically significant.

3. Results

3.1. Effect of saponin removal on fungal contamination

All samples with saponins showed 100% of seeds with fungal contamination (Percentage of infection=100%). Samples from the three different places showed a greater reduction of % infection due to saponin removal when plated on two culture media, DCPA and DG18 (Fig. 1). Samples from Tucumán province held relatively high percentages of infection (ranging between 36,5 and 88,5%) while samples from Salta province and from Uyuni showed a bigger reduction of percentages of infection (ranging between 4–21% and 4–36% respectively).

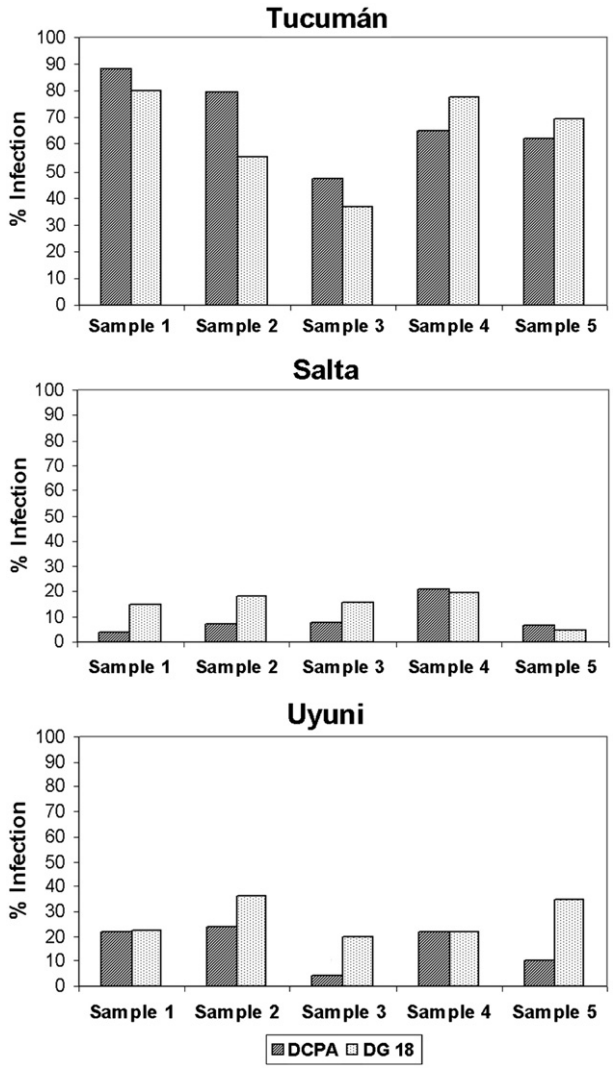

Fig. 1. Percentage of infection of quinoa seeds after removal of saponins.

3.2. Effect of saponin removal on the incidence of fungal genera and species

Table 1 shows the incidence of mold genera in samples from the three locations, with and without saponins. *Penicillium* and *Aspergillus* were the most prevalent contaminants; they were present in all analyzed samples, regardless of their geographic origin. Results of the Chi Square Test ($p<0.05$) indicated that the distribution of the different genera is not homogeneous in the three locations. In samples from Tucumán province incidence of *Penicillium* was significantly higher than that of *Aspergillus*, in samples with saponins (61.5% *vs.* 26.4%) as well as in those without saponins (66.8% *vs.* 18.8%). Conversely, samples from Salta province showed a strong predominance of *Aspergillus*, 90.6% and 66.1%, in comparison with the low incidence of *Penicillium*, 8.0% and 11.0%, in samples with and without saponins respectively. In samples from Uyuni *Penicillium* is predominant, showing 55.2% and 62.2% of incidence in seeds with and without saponins, but *Aspergillus* incidence is also high (40.0% and 27.5% respectively). Other genera, such as *Eurotium*, *Fusarium*, *Phoma*, *Ulocladium, Mucor* and *Rhizopus* were less frequently isolated. *Absidia*, *Alternaria*, *Cladosporium*, *Dreschlera*, *Epicoccum* and *Monascus* were sporadically encountered.

Regarding to the effect of the treatment to remove saponins, statistical analysis of data indicated significant differences ($p<0.05$) in the distribution of fungal genera in samples with and without saponins from each location. In all cases, processing caused a decrease of *Aspergillus* incidence, while increased the proportion of *Penicillium*, *Eurotium, Mucor* and *Rhizopus*.

Isolates from genera *Penicillium, Aspergillus* and *Eurotium* were identified at species level and the relative density of each species in samples with and without saponins was calculated (Tables 2 and 3). In samples with saponins, *Aspergillus* is almost exclusively represented by *A. flavus* (potential producer of aflatoxins and cyclopiazonic acid) and *A. niger* (potential producer of ochratoxin A). The latter is predominant in samples from Tucumán province, while in samples from Salta province and Uyuni there is a high incidence of *A. flavus.* Treatment to remove saponins does not modify the relative density of both species in Tucumán samples. In samples from Salta province, internal mycota shows a high incidence of aflatoxigenic species (*A. flavus* and *A. parasiticus*). In samples from Uyuni, removal of saponins cause a decrease of *A. flavus* incidence while increases *A. niger. P. aurantiogriseum, P.chrysogenum, P. citrinum* and *P. crustosum* were always present in high numbers in samples with and without saponins, although their relative density is variable according to the geographic origin of samples. *P. griseofulvum* was also present in high number in samples from Tucumán province.

3.3. Effect of saponin removal on incidence of toxigenic species

Several known toxigenic species of *Aspergillus* and *Penicillium* were detected in samples from the different places. To evaluate the mycotoxin-producing potential of most representative species, *A. flavus* strains were screened for aflatoxins and cyclopiazonic acid (CPA) production, *A. parasiticus* for aflatoxins, *A. niger* for ochratoxin A (OTA), *P. citrinum* for citrinin and *P. griseofulvum* for CPA.

Aspergillus flavus was frequently isolated from samples of the three geographical regions. Table 4 shows the ability to produce CPA and aflatoxins of *A. flavus* isolates from samples with and without saponins. None of the isolates produced only aflatoxins, while a high proportion produced only CPA. In samples from Salta province without saponins the co-production of CPA, aflatoxin B_1 and aflatoxin B_2 was observed in 22/154 (14.3%) of the isolates. All aflatoxigenic *A. flavus* strains produced simultaneously CPA. Rate of aflatoxin-producing *A. flavus* was variable in the three locations, being higher in Tucumán province (44%) and lower in samples from Uyuni (11.7%). *A. parasiticus* was less frequently isolated; only 9 isolates were detected in samples without saponins from Uyuni and Salta, but all of them produced type B and G aflatoxins. *A. niger* was detected in high number in samples from the three regions with and without saponins (101 isolates from Salta, 390 isolates from Tucumán and 69 isolates from Uyuni) but none of the strains was OTA producer. This species was recognized as ochratoxigenic since 1994 (Abarca et al., 1994), but the percentage of toxigenic isolates is generally low (Abarca et al., 2001; Romero et al., 2005). Other species from *Aspergillus* section *Nigri*, such as *A. carbonarius,* are stronger OTA producers, but they were not found in quinoa seeds. Among toxigenic species of *Penicillium*, *P. griseofulvum* and *P.citrinum* were the most relevant and they were present in samples with and without saponins. All strains of *P. griseofulvum* ($n=83$) produced CPA and all *P. citrinum* isolates ($n=63$) produced citrinin.

3.4. Analysis of samples to detect natural occurrence of mycotoxins

When the samples were analyzed, no mycotoxins were found below their respective limits of detection.

4. Discussion

Our results are quite different from those reported previously in the literature. Boerema et al. (1977) referred the occurrence of *Ascochyta*

Table 1
Incidence of fungal genera in samples with and without saponins in three locations

Genera	Tucumán				Salta				Uyuni			
	WS[a]		WOS[b]		WS		WOS		WS		WOS	
	IN[c]	Fq%[d]	IN	Fq%	IN	Fq%	IN	Fq%	IN	Fq%	IN	Fq%
Absidia	–	–	1	0.1	–	–	–	–	–	–	–	–
Alternaria	–	–	–	–	–	–	–	–	–	–	1	0.5
Aspergillus	269	26.4	205	18.8	271	90.6	162	66.1	150	40.0	61	27.5
Cladosporium	–	–	2	0.2	–	–	–	–	–	–	–	–
Dreschlera	–	–	–	–	–	–	–	–	–	–	1	0.5
Epicoccum	1	0.1	–	–	–	–	–	–	–	–	–	–
Eurotium	11	1.1	15	1.4	–	–	37	15.1	2	0.5	12	5.4
Fusarium	6	0.6	1	0.1	1	0.3	–	–	3	0.8	–	–
Monascus	–	–	1	0.1	–	–	–	–	–	–	–	–
Mucor	36	3.6	77	7.1	–	–	12	4.9	–	–	–	–
Penicillium	626	61.5	726	66.8	24	8.0	27	11.0	207	55.2	138	62.2
Phoma	9	0.9	1	0.1	–	–	–	–	5	1.3	3	1.4
Rhizopus	48	4.7	52	4.8	–	–	3	1.2	–	–	–	–
Ulocladium	11	1.1	6	0.6	3	1.0	4	1.6	8	2.1	6	2.7
Total	1017		1087		299		245		375		222	

WS[a]: with saponins.
WOS[b]:without saponins.
IN[c]: isolates number.
Fq%[d]: frequency %.

hyalospora in seeds from *C. quinoa* from Bolivia. This fungus caused leaf spots and stem necroses in different *Chenopodium* species. Spehar et al. (1997) carried out the mycological analysis of different genotypes of *C. quinoa* cultivated in Brazil. According to these authors, *Alternaria, Phoma, Fusarium , Bipolaris, Cladosporium* and *Pyronochaeta* were the major fungal genera associated with this pseudocereal. The mycoflora of quinoa seeds from genotypes of Peruvian and Czech origin was determined by Drímalková (2003). *Fusarium, Ascochyta* and *Alternaria* were isolated from seeds cultivated in the Czech Republic, while *Ascochyta* and *Alternaria* were isolated from seeds of Peruvian origin. *Penicillium* and *Aspergillus* were isolated only in minor proportion. We have found a wider diversity of seedborne fungi in *C. quinoa* from two South-American countries (Argentina and Bolivia). Most of the fungi isolated from quinoa seeds in the present study belong to xerophilic species, capable of growth at low water activity levels, which could cause spoilage of improperly stored grains. Field fungi (e.g. *Alternaria, Cladosporium and Fusarium*) were almost absent. *Mucor* and *Rhizopus* were significant only in samples from one of the locations (Tucumán province, Argentina) probably due to more humid climatic conditions.

Toxigenic fungi were isolated from both type of samples, including those treated to remove saponins, probably meaning that they are part of the internal mycota and would be capable of producing mycotoxins during all steps of quinoa seeds postharvest process such as cleaning, classification, debittering, drying, transport and storage. When saponins are removed by the wet method, drying of grains to moisture content less than 10% (wet basis) is a critical control point to prevent

Table 2
Relative density of prevalent identified fungi on quinoa seeds with saponins

Species	Tucumán		Salta		Uyuni	
	IN[a]	RD %[b]	IN	RD %	IN	RD %
Aspergillus						
A. flavus	49	18.2	170	62.7	114	76.0
A. fumigatus	–	–	–	–	1	0.7
A. niger	220	81.8	101	37.3	34	22.7
A. orizae	–	–	–	–	1	0.7
Total	269		271		150	
Eurotium						
E. amstelodami	1	9.1	–	–	1	50.0
E. rubrum	10	90.9	–	–	1	50.0
Total	11		0		2	
Penicillium						
P. aurantiogriseum	132	21.1	2	8.3	12	5.8
P. brevicompactum	6	1.0	–	–	–	–
P. chrysogenum	376	60.1	12	50	30	14.5
P. citrinum	7	1.1	10	41.7	14	6.7
P. commune	6	1.0	–	–	–	–
P. crustosum	33	5.3	–	–	144	69.6
P. griseofulvum	29	4.6	–	–	–	–
P. hirsutum	–	–	–	–	1	0.5
P. polonicum	15	2.4	–	–	3	1.5
P. solitum	15	2.4	–	–	3	1.5
P. viridicatum	7	1.1	–	–	–	–
Total	626		24		207	

IN[a]: isolates number.
RD %[b]: relative density %.

Table 3
Relative density of prevalent identified fungi on quinoa seeds without saponins

Species	Tucumán		Salta		Uyuni	
	IN[a]	RD %[b]	IN	RD %	IN	RD %
Aspergillus						
A. flavus	35	17.1	154	95.1	22	36.1
A. niger	170	82.9	–	–	35	57.4
A. parasiticus	–	–	8	4.9	1	1.6
A. sidowii	–	–	–	–	3	4.9
Total	205		162		61	
Eurotium						
E. amstelodami	2	13.3	–	–	–	–
E. chevalieri	8	53.3	16	43.3	3	25
E. repens	4	26.7	3	8.1	7	58.3
E. rubrum	1	6.7	18	48.6	2	16.7
Total	15		37		12	
Penicillium						
P. aurantiogriseum	54	7.4	3	11.1	12	8.7
P. brevicompactum	24	3.3	–	–	–	–
P. canescens	–	–	–	–	1	0.7
P. chrysogenum	312	43.0	5	18.5	25	18.1
P. citrinum	6	0.8	16	59.3	10	7.3
P. corylophilum	5	0.7	1	3.7	–	–
P. crustosum	237	32.6	–	–	81	58.7
P. griseofulvum	52	7.2	–	–	2	1.5
P. janczewskii	2	0.3	–	–	1	0.7
P. polonicum	–	–	–	–	3	2.2
P. solitum	25	3.4	2	7.4	3	2.2
P. viridicatum	9	1.2	–	–	–	–
Total	726		27		138	

IN[a]: isolates number.
RD %[b]: relative density %.

Table 4
Aflatoxins and CPA production by *A. flavus* isolates from quinoa seeds with and without saponins

A. flavus producers of	Tucumán				Salta				Uyuni			
	WS[a]		WOS[b]		WS		WOS		WS		WOS	
	TI[c]	%	%		%		%		%		%	
CPA*	35/49	71.4	28/35	80	102/170	60	126/154	81.8	64/114	56.1	17/22	77.3
CPA + AFB_1	19/49	38.8	18/35	51.4	17/170	10	63/154	41	6/114	5.3	10/22	45.5
CPA + + AFB_1 + AFB_2	0/49	0	0/35	0	0/170	0	22/154	14.3	0/114	0	0/22	0

WS[a]: with saponins.
WOS[b]: without saponins.
TI[c]: toxigenic isolates: number of toxin producers/number of isolates examined.

mycotoxin contamination. This moisture content would be suitable for safe storage according to data from quinoa sorption isotherms (Tolaba et al., 2004).

It was observed that after the treatment to remove saponins, the proportion of toxigenic isolates increased, mainly of aflatoxin producers. It could be hypothesized that saponins may have an inhibitory effect on the toxigenic ability of the isolates.

The high proportion of *A. flavus* strains capable of producing CPA (almost 70% of the isolates) was in concordance with results from previous surveys carried out on other crops from our country, such as peanuts, wheat, soybeans (Vaamonde et al., 2003; Pildain et al., 2005) and corn (Resnik et al., 1996).

According to the composition of the quinoa seeds mycota, aflatoxins, CPA and citrinin were the mycotoxins of higher risk of contamination in this substrate. However, when the samples were analyzed, no mycotoxins were found as natural contaminants. It could be due to the fact that harvest, drying and storage of seeds were performed in good conditions. Perhaps, moisture content and temperature were not favorable for mycotoxins biosynthesis. It seems that quinoa behaves like other small grains (e.g. amaranth) which have been reported as less susceptible to mycotoxins contamination than bigger size grains, like corn or peanuts (Smith and Moss, 1985; Bresler et al., 1998). Other factors such as chemical composition and the presence of saponins on the surface of the seeds could also be involved and deserve further investigations.

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