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Conditions that regulate the growth of moulds inoculated into bottled mineral water

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Abstract

The influence of different storage conditions (temperature, illumination, brand of mineral water and storage time) on growth of mould spores was studied. *Alternaria alternata*, *Penicillium citrinum* and *Cladosporium cladosporioides* spores were inoculated in bottles of mineral and mineralised water, packaged in polyethylene terephtalate (PET). The bottles were incubated under different storage conditions. The strains had been isolated from bottled mineral water in a previous study. Storage time was the parameter that had the most important influence in mould growth. The spores grew into visible colonies after 5 month of incubation in bottles just filled, and in a month in bottles that had been stored for 5 month. This could be due to the migration of compounds from PET packaging material into mineral water. This compounds could be used as nutrients (organic matter) for mould growth. The plasticizer additive di-*n*-butyl phthalate (DBP) concentration in recently bottled mineral water and in 5-month stored bottles was measured. An increase of 20% of DBP concentration was observed. *A. alternata* and *P. citrinum* strains were toxicological characterised. Both strains produced mycotoxins in vitro, and *P. citrinum* produced citrinin in mineral water, posing a potential health risk for consumers.

Keywords: Fungi; Bottled mineral water; Mycotoxin

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1. Introduction

Several studies of microbiology of distribution systems and bottled water were carried out (Bischofberger et al., 1990; Morais and Da Costa, 1990; Mavridou, 1992; Hunter, 1993; Fewtrell et al., 1997; Tsai and Yu, 1997; Croci et al., 2001), some related to fungi growth in water distribution systems (Nagy and Olson, 1982; Hinzelin and Block, 1985; Frankova and Horecka, 1995; Zacheus and Martikainen, 1995), and a few of them regarding fungi spoilage in bottled mineral water (Fujikawa et al., 1997; Cabral and Fernández Pinto, 2002). However, during the last years, some problems related to the growth of mould in noncarbonated polyethylene terephtalate (PET) bottled mineral water have been detected. The moulds most frequently isolated from those products were *Penicillium* spp., *Cladosporium cladosporioides* and *Alternaria alternata* (Cabral and Fernández Pinto, 2002).

Notwithstanding that filamentous fungi in water are commonly thought to own no potential hazards to public health because most of these microorganisms are not human pathogen, there are species that cause illness in humans. One of the mechanisms of producing disease by the fungi is the production of mycotoxins. These metabolites are extremely toxic and can induce tumour in several animals. Some of fungi that were isolated from mineral water, e.g., *A. alternata* and *Penicillium citrinum*, do have some toxigenic potentiality (Cabral and Fernández Pinto, 2002). Fortunately, the presence of spores or even the growth of certain fungi is not always followed by toxin production. The conditions which allow toxin production are more restricted than those which give way to growth (Northolt et al., 1996). *A. alternata* produces several mycotoxins, tenuazonic acid (TA) being the most important. Other less toxic compounds are alternariol (AOH) and alternariol methyl ether (AME). *P. citrinum* just produces citrinin, a nephrotoxin of moderate activity (Comerio et al., 1998).

It is generally believed, however, that moulds do not grow into visible bodies in mineral water products containing very low amounts of nutrients (organic matter) for the organism (Fujikawa et al., 1999). According to bottle tags, mineral water contains only calcium, magnesium, sodium, potassium, chlorate, bicarbonate, sulphate and nitrates. A possible origin of organic matter for the growth of mould is the new beverage bottling material known as polyethylene terephtalate (PET). It is prepared from terephtalic acid or its esters, and ethylene glycol. Plasticizers are added to these polymers to give some properties to the final product. Di-*n*-butyl phthalate (DBP) is one of the most used plasticizer. During storage, PET releases organic matter (e.g., several plastic additives, residues from the polymerisation process, degradation compounds, etc.) that could provide additional substrate for the microbial growth (Evandri et al., 2000).

The aim of the present study was to investigate under what environmental conditions can contaminating fungi grow and become visible in bottled mineral water. We also attempt to establish whether toxic metabolites could be released into the water under storage conditions. Finally, we also studied if DBP could migrate from PET and could be a carbon source available for mould growth.

2. Materials and methods

2.1. Mould spore preparation

Four mould isolates, *A. alternata* BAFC cult. 17, *A. alternata* BAFC cult. 805, *P. citrinum* BAFC cult. 1078, *C. cladosporioides* BAFC cult. 806, which were isolated from bottled mineral water, were used in this study (Cabral and Fernández Pinto, 2002). The strains were incubated on potato dextrose agar (PDA) plates (Merck, Art. 64271, Darmstadt, Germany) at 25 °C for a period of 15 days. In order to harvest spores, cultures plates were flooded with sterile water with Lutensol AP 10 as surfactant. The spore suspensions were collected in sterile Erlenmeyer flasks. *A. alternata* spores of strains 17 and 805 were mixed. By using a haemocytometer, the spore concentration was then adjusted to 1×10^5 spores ml^{-1}.

BAFC denotes the culture collection of Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina.

2.2. Mineral water samples

A total of 96 samples of uncarbonated bottled mineral water and 96 samples of bottled uncarbo-

nated mineralised water were purchased from local retail stores in 0.5 l PET bottles. At this moment, all the bottles had 2 days of bottling. To Argentine legislation (CAA) and the European Community (EC, 1980), natural mineral water is the potable water that originates from an underground table or deposit, emerging from a spring tapped at one or more natural or bore hole exits. All natural mineral water must be recognised by the local authority, and hydrological, physical, chemical and physio-chemical surveys must be undertaken. Mineralised water is potable water with added salts and must meet the same microbiological criteria as natural water (CAA, 1998). In contrast, the US legislation does not distinguish between bottled water and bottled mineral water, classing them both as bottled water (EPA, 1980). The mineral content in the mineral water was calcium 19 mg/l, magnesium 14 mg/l, sodium 164 mg/l, potassium 10 mg/l, fluoride 0.7 mg/l and bicarbonate 450 mg/l. While the mineral content in the mineralised water was calcium 51.5 mg/l, magnesium 5.2 mg/l, sodium 79.6 mg/l, fluoride 0.5 mg/ml and bicarbonate 225.3 mg/l.

2.3. Sample inoculation

A 1-ml aliquot of spore suspensions from each mould species was inoculated into 12 bottles of mineral and 12 of mineralised water (assay A), obtaining 36 inoculated bottles.

The bottles inoculated with the same species were divided into two groups: six bottles were stored at 25 °C and the other six at 30 °C, in temperature-controlled rooms. Each group was also divided into two further groups: three bottles were stored in constant darkness and the other three in 12 h of photoperiod.

For each species and each condition, one bottle without inoculate was incubated as control.

The experiment was repeated 5 months later, inoculating bottles belonging to the same lot that those bottles used in the previous experiment, it means have 5 months since they were full (assay B). The bottles had been stored in the dark.

In both experiments, fungal foreign bodies in the bottles were examined by the naked eye every 6 days during 28 weeks.

2.4. Mycotoxin analysis in vitro

2.4.1. Penicillium citrinum

Citrinin production was studied using the method of Filtenborg and Frisvad (1980). *P. citrinum* was incubated on potato dextrose agar (PDA) at 25 °C for 7 days. Using a sterile tube, small plugs were transferred to the TLC plate (Silica gel 60 G, Merck Art.5721). The plugs were removed from the TLC plate when a liquid front appeared. The spots were allowed to dry, and the TLC plate was developed in toluene-ethyl acetate-formic acid (TEF; 5:4:1). The mycotoxin was detected directly by its yellow fluorescence under long-wave UV light (366 nm). The yellow fluorescence disappeared when the spots were submitted to ammoniac vapour. Citrinin was identified by comparing it with a standard solution (Sigma, St. Louis, MO, USA; 2.5 μg/ml in chloroform).

2.4.2. Alternaria alternata

Erlenmeyer flasks (250 ml) containing 25 g of raw rice adjusted to 45% moisture content were sterilised by autoclaving for 15 min at 120 °C. Each flask was inoculated with a 6-mm disk from an actively growing colony margin of *A. alternata* strains 17 and 805 and incubated at 25 °C for 21 days.

The production of AOH, AME and TA were determined following the method described by Visconti et al. (1986) with some modifications. The rice cultures (25 g) were extracted in a blender with 37.5 ml methanol, and later filtered. Forty milliliter of 20% aqueous ammonium sulphate was added to 20 ml of the filtrated extract, and again filtered. Forty-five milliliter of filtrate was extracted twice with 2.5 ml chloroform, and the combined extracts were evaporated to dryness by means of a rotatory evaporator. The residue was redissolved in methanol and analysed for AME, AOH by TLC. The aqueous solution remaining after chloroform extraction was acidified to pH 2 with concentrated hydrochloric acid and extracted twice with 25 ml chloroform. TA was extracted from the combined organic phases with 15 ml of 2.5% aqueous sodium bicarbonate, then reconverted to its acidic form by adjusting to pH 2 with 1 N hydrochloric acid and extracted twice with 15 ml chloroform. The chloroform extracts were combined, washed with 12.5 ml of water and evaporated to dryness. The residue was redissolved

in methanol, and the toxin was analysed by TLC. Silica gel G-60 plates (0.25 mm) were used for the analysis (Merck Art.5721). TEF (5:4:1) was used as development solvent. AOH, AME and TA were confirmed by visual comparison with standard solutions (Sigma). AOH and AME were visualised by spraying the plate with 2% ferric chloride in methanol and TA with UV light as blue fluorescence spots.

2.5. Mycotoxin analysis in situ

Citrinin production was determined in *P. citrinum*-inoculated bottled mineral water with visible fungal foreign bodies, with the method of Prasongsidh et al. (1998) with slight modifications. The mycelia were filtered trough filter paper (Whatman N°1). Twenty-five milliliter methanol–2% sodium hydrogen carbonate mixture (7:3) was added to 25 ml of filtrate. The water–methanol mixture was acidified to pH 3 with 6 N HCl. The mycotoxin was extracted twice with 100 ml chloroform. Organic phase was evaporated to dryness and redissolved in 5 ml chloroform. Citrinin was analysed by TLC using Silicagel G 60 plates previously immersed in 10% oxalic acid in methanol, dried and activated at 110 °C for 10 min. TEF (5:4:1) was used as developing solvent. The spots were observed under UV light (366 nm) as a yellow fluorescent spot that disappeared with ammoniac vapour.

2.6. Analysis of phthalates in mineral water bottled

In order to establish the origins of organic matter for the growth of mould in bottled mineral water, we measured the concentration of DBP in samples of 0.5 l PET bottled mineral water with and without 5 months of storage.

Five hundred milliliter of water of each bottle was saturated with 20 g of sodium chloride and extracted twice with 30 ml hexane. Hexane extracts were combined, washed twice with 10 ml of water, dried with anhydrous sodium sulphate and evaporated to dryness. The aqueous phase remaining after hexane extraction was extracted with 2×20 ml dichloromethane. The organic phases were combined, washed with 2×10 ml of water, dried with anhydrous sodium sulphate and evaporated to dryness. Then, the aqueous phase was extracted with 2×30 ml ethyl acetate. Ethyl acetate extracts were combined, washed twice with 10 ml of water, dried with anhydrous sodium sulphate and evaporated to dryness. The three extracts were analysed by gas–liquid chromatography (GLC) with a FID detector, using a Hewlett-Packard-P5890 gas chromatograph with an HP-5 capillary column (50 m×0.32 mm) and HP-17 (10 m×0.53 mm). The column temperature was programmed from 150 to 250 °C at 5 °C/min. Injector and detector temperatures were 250 and 300 °C, respectively. DBP were confirmed by comparison with standard solutions (Sigma).

3. Results

3.1. Growth of mould spores during storage

P. citrinum and *A. alternata* growth was observed after 5 months from the inoculation in the assay A and after a month in assay B (Figs. 1 and 2). *P. citrinum* grew only in mineral water, while *A. alternata* grew in natural mineral and mineralised water. *C. cladosporioides* grew at 23 weeks in assay A and at 6 weeks in B (Fig. 3). This strain grew less than the others ones.

No difference was observed in the growth of fungi mycelia between the samples incubated at 25 and 30 °C, neither between those incubated in light nor dark.

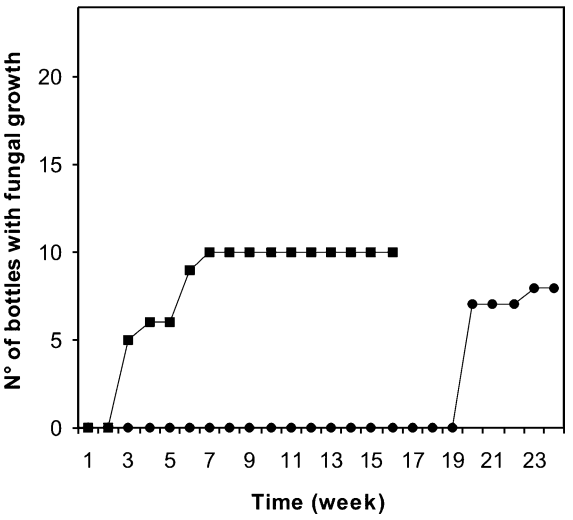

Fig. 1. Growth of *P. citrinum* in bottled mineral water. (●) Assay A: 2 days after bottling inoculated bottles; (■) Assay B: 5 months after bottling inoculated bottles.

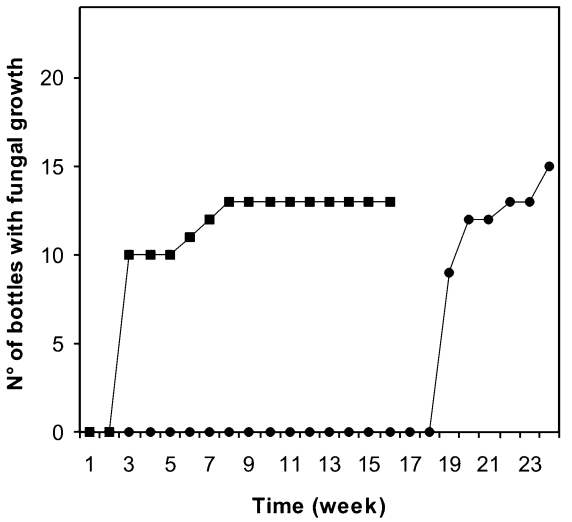

Fig. 2. Growth of *A. alternata* in bottled water. (●) Assay A: 2 days after bottling inoculated bottles; (■) Assay B: 5 months after bottling inoculated bottles.

3.2. Mycotoxin analysis

Citrinin was produced by *P. citrinum* when it was incubated on PDA for 7 days.

AOH and AME were produced by *A. alternata* strains 17 and 805 when they were incubated on rice for 21 days. TA was only produced by *A. alternata* strain 805. No TA production by *A. alternata* strain 17 was detected during the incubation period.

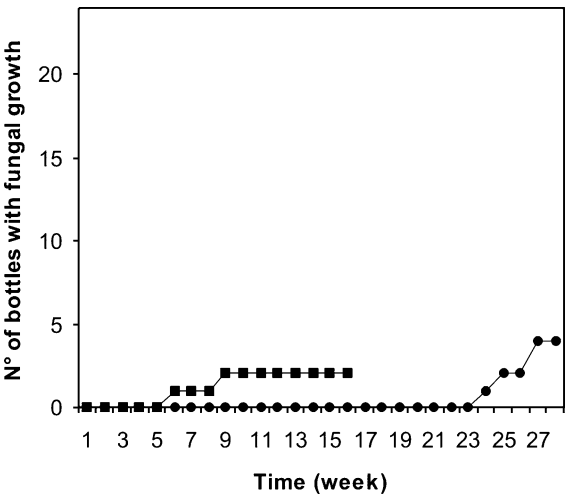

Fig. 3. Growth of *C. cladosporioides* in bottled water. (●) Assay A: 2 days after bottling inoculated bottles; (■) Assay B: 5 months after bottling inoculated bottles.

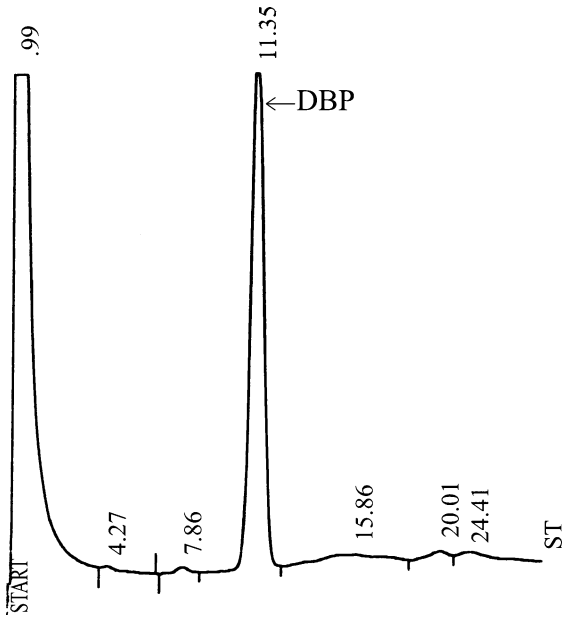

Fig. 4. Chromatograms of DBP standard.

On the other hand, *P. citrinum* produced citrinin levels of 0.517±0.202 μg/ml (N=3) in inoculated bottled mineral water with visible fungal colonies after 5 month of storage.

3.3. Phthalate in bottled mineral water

DBP was detected by GLC analysis in bottles with and without storage in the ethyl acetate extract (Fig. 4).

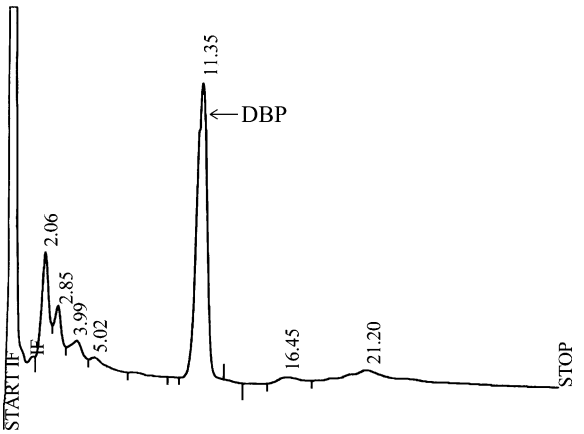

Fig. 5. Chromatograms of ethyl acetate extract of mineral water without storage.

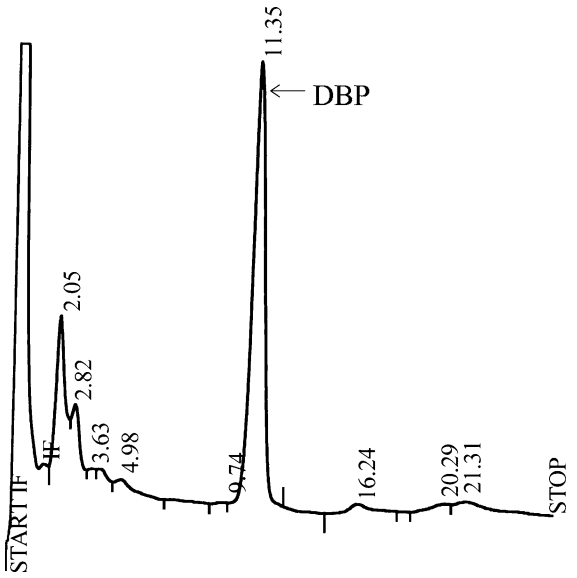

Fig. 6. Chromatograms of ethyl acetate extract of mineral water with 5-month storage.

Calibration curves in the range of 0.01–2.5 mg/l in ethyl acetate were constructed, obtaining a correlation coefficient of 0.9993.

The DBP concentration present in bottles without storage was 2.0±0.12 ppb (N=5, RSD=1.3; Fig. 5), and with 5-month storage, it was 2.4±0.22 ppb (N=5, RSD=0.83; Fig. 6).

No DBP concentration was detected neither in hexane extract nor in dichloromethane extract.

4. Discussion

This is the first report in Argentina and one of the few in the world that clearly demonstrates that fungi spores can grow into visible mycelia in mineral and mineralised water which contained a very low organic matter concentration.

When recently, bottling water was inoculated with spores' suspensions, the fungi growth was observed only after several months of incubation. On the other hand, when the water was inoculated after 5 month of storage, the detection of the growth was faster. These results support the hypothesis that, during the storage, the PET releases organic matter into the water, providing additional substrate for microbial growth. In order to prove this hypothesis, we measured DBP in bottles recently filled and with a 5-month storage packing observing a 20% DBP increase. However, when flasks with sterile water with different concentration of DBP (including those founded in the CGL study) were inoculated with *P. citrinum* spore suspensions, no fungi growth was observed. It is likely that DBP was not the only requirement for growth. Besides carbon source, fungi also need nitrogen, phosphorus, magnesium and potassium in order to grow. These elements could be supplied by the salts that mineral water contains. Tsai and Yu (1997) speculate that the metabolites or decomposition products of contaminating bacteria provide nutrients for the support of other organisms. However, Fujikawa et al. (1999) clearly demonstrated that water-contaminating bacteria suppress the growth of the spore inoculated. They showed that when mould spores contaminate mineral water products already colonised with bacteria, the spores can survive but cannot grow to visible foreign bodies due to the competition among microorganisms. Furthermore, we examined the microbiological quality of bottled water with and without storage, and we found less than 10 colony forming units ml^{-1} in both. So, it is difficult to think that the autochthonous bacteria were supplying nutrients that permitted the mould growth.

Differences in growth of mould at different temperature and illumination conditions of incubation were not observed. Light and temperature parameters seem to not be an influence in mould's growth. Generally, fungi are able to grow in wide ranges of temperature, and the light, being important for their morphogenesis and germination, is not a somatic requirement for them. These findings are different to those of bacteria, where a difference in growth according to the incubation temperature was verified (Croci et al., 2001). However, differences in growth according to the type of water exist. *P. citrinum* only grew in mineral water and not in mineralised water. Probably, the mineral and mineralised water nutrients are different, and the last ones are not appropriate for the *P. citrinum* growth.

The microbiological quality of mineral water should meet the local water standards, which may differ in each country. None of them take fungi into account because they are thought to lead to no public health problems. However, we have seen that *A. alternata* and *P. citrinum* strains isolated in mineral water by Cabral and Fernández Pinto (2002) were

producers of mycotoxins in culture, and that *P. citrinum* can produce citrinin in bottled mineral water. Paterson et al. (1997) have detected aflatoxins and identified *Aspergillus flavus* on water from a cold water storage tank.

It would be interesting then to count fungal propagules in routine microbiological studies of bottled mineral water and establish baselines to make these products safe and acceptable for consumers.

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