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# Death of embryos from 2300-year-old quinoa seeds found in an archaeological site

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ABSTRACT

In the 1970s, during excavations at Los Morrillos, San Juan, Argentina, quinoa seeds were found within ancient pumpkin crocks protected from the light and high temperatures, and preserved in the very dry conditions of the region. The radiocarbon dates confirmed the age of these seeds at around 2300 years. Sectioning of some of these seeds showed reddish-brown embryos, different from the white embryos of recently harvested quinoa seeds. The ancient seeds did not germinate. The structure of the embryo cells was examined using light and transmission electron microscopy; proteins were analyzed by electrophoresis followed by Coomassie blue and periodic acid Schiff staining and fatty acids by gas chromatography. The state of nuclear DNA was investigated by TUNEL assay, DAPI staining, ladder agarose electrophoresis and flow cytometry. Results suggest that, although the embryo tissues contained very low water content, death occurred by a cell death program in which heterochromatin density was dramatically reduced, total DNA was degraded into small fragments of less than 500 bp, and some proteins were modified by non-enzymatic glycation, generating Maillard products. Polyunsaturated fatty acids decreased and became fragmented, which could be attributable to the extensive oxidation of the most sensitive species (linolenic and linoleic acids) and associated with a collapse of lipid bodies.


## 1. Introduction

Quinoa (*Chenopodium quinoa* Willd.) is a pseudo-cereal that has been grown in the Americas, especially along the Andes, for over 5000 years. The species has numerous genotypes, which are well adapted to extreme environmental conditions with regards to altitude, soil salinity, amount of annual precipitation and minimum temperatures [1,2]. The species is valuable because of the exceptional balance of amino acids in its proteins and nutritionally favorable lipids stored in the tissues of its embryos as well as due to the starch stored in its perisperm [3].

In this study, we analyzed archaeological seeds of quinoa found during an excavation at Los Morrillos, San Juan, Argentina, site identified as Gruta 1, BF, 40–50 [4–6]. In the cave, seeds were found stored within ancient pumpkin crocks protected from light, humidity and high temperature. In this region, the weather is mostly desert-like with little rainfall and marked temperature ranges, with remarkable differences in temperature between night and day as well as between summer and winter, with a significant solar radiation [4–6]. Here, we compared these ancient seeds with quinoa seeds of two currently cultivated genotypes: Utusaya and PRJ. These genotypes were selected as controls because they are usually grown in highly contrasting environments and thus were expected to have constitutive differences in the characteristics of their seeds (probably covering a wide range of variations). The genotype Utusaya is found in the Bolivian Altiplano, at 3600–4000 m altitude, and is adapted to the very arid conditions characteristic of the Altiplano, with less than 250 mm of annual rain and a minimum temperature of −1 °C; the genotype PRJ grows at sea level in the southern region of central Chile, and is adapted to more humid conditions (800 to 1500 mm of annual rain), and temperatures above 5 °C.

An orthodox seed usually exhibits its maximum germination potential soon after harvest, and as storage time increases, it loses

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vigor and finally dies. The rate of physiological ageing generally increases with increased moisture content and temperature. The ability of an orthodox seed [7] to survive at very low intracellular water content is the basis for its longevity, maintaining viable embryos over centuries [8] or even millennia [9–11]. However, there are no studies about the type of cell death in tissues of orthodox seeds preserved under gene banks conditions, whose embryos are constituted by storage tissue i.e. cells with de-differentiated organelles, absence of vacuoles, and consequently very low water content, with protein and lipid bodies occupying most of the cellular lumen. It is known that embryos of orthodox seeds always maintain a certain low level of metabolic activity, which varies depending on the longevity of the seed [12,13]. Low metabolism delays ageing but does not prevent seeds from eventually dying. According to Kranner et al. [14], the mechanisms underlying seed ageing and death are less understood than seed longevity as a function of water content and temperature.

The two modes of plant cell death, programmed cell death (PCD) and necrosis, differ fundamentally in their morphology, biochemistry and biological relevance [15]. Accurate identification of the mode of cell death occurring in a particular situation is a necessary prerequisite to understand the biological process taking place, specifically differences between PCD and necrosis during storage. PCD consists of an ordered sequence of cellular events, which includes the transcription of specific genomic sequences, synthesis of specific proteases, and activation of nucleases. Extensive literature exists regarding the associated molecular, biochemical and morphological changes involved [16–23].

Kranner et al. (2011) reported internucleosomal DNA fragmentation during pea seed ageing and suggested that artificial ageing induces a complex process of interlinked programmed and non-programmed events, which lead to cell death. Anyway, the DNA laddering found in aged and dead pea seeds suggests that degradative processes during seed ageing at 12% water content involve enzymatic activity, including that of caspase-like proteins involved in cell death during dry ageing [14]. Likewise, it is well established that enzymatic and nonenzymatic products of polyunsaturated fatty acid oxidation are implicated in essential aspects of cellular aspects of cellular signaling, including the induction of PCD [24–26]. Glycation also has a fundamental role in many processes leading to cell death; glycans, either alone or complexed with glycan-binding proteins, can deliver intracellular signals or control extracellular processes, which induce the initiation and execution of cell death programs [25]. On the other hand, necrosis is typically an acute cell death response that develops rapidly, but is no longer considered an unprogrammed process [15]. At this time, necrosis remains poorly characterized at the biochemical and genetic levels, so there are as yet no molecular markers of necrosis [15,26,27].

Archaeological seeds degrade into small fragments over thousands of years, and the PCR product size decreases to less than 300 bp [28]. Different authors [29,30] have reported that the DNA from charred seeds is often of higher weight than that from specimens that are morphologically well-preserved, i.e. seeds die within a slow process of PCD. Then, DNA yield and molecular weight are dependent on the initial condition of the sample and storage conditions that prevent nuclease activity.

We have previously hypothesized that quinoa seeds, an orthodox seed [31–34] under the conditions of preservation of the cave where they were found, could have maintained their viability for a long time. If they finally died, then, it might be interesting to find out what kind of death occurred in the cells of embryo tissues of an orthodox seed, which lack vacuoles [3].

In the present study, we examined the embryo tissues of the ancient quinoa seeds found at Los Morrillos and embryos of two existing quinoa genotypes (PRJ and Utusaya), using a range of well-established markers of PCD such as: (i) morphological changes in nuclei, mitochondria, plastids, and storage reserves (lipid and protein bodies), (ii) DNA degradation, (iii) lipid oxidation, and (iv) protein degradation. The structure of the embryo cells was studied using light and transmission electron microscopy (TEM) and proteins were analyzed by electrophoresis followed by Coomassie blue and periodic acid Schiff (PAS) staining; fatty acid oxidation was studied by gas chromatography: the state of nuclear DNA was investigated by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL), DNA ladder and flow cytometry. Then, we discussed about the type of death that occurred in the embryos of these ancient seeds and finally concluded that it is a non-vacuolar type of PCD, so far not described in the literature.

## 2. Material and methods

### 2.1. Material

Archaeological quinoa seeds were gathered at a burial site from the Ansilta civilization located in an Andean cave in Los Morrillos, San Juan, Argentina from Los Morrillos, Gruta 1, BF, 40–50 [4–6]; (Fig. 1). The seeds (approximately 2.5 kg) were found within ancient pumpkin crocks protected from the light and high temperatures, and preserved in the very dry conditions of the region (annual rainfall ranging from 20 to120 mm; rains occur more often during the summer). Average temperatures range from 10 °C to 35 °C. The site is at 31°42′, 46.58′í south and 69°44′13.28′í west, at an altitude of 2935 m above sea level [4,5].

Likewise, seeds of two existing quinoa genotypes, Utusaya and PRJ, were analyzed as controls (Fig. 1A). The seeds of genotype PRJ were provided by the Bank of Germplasm INIA-Vicuña, Chile, whereas the seeds from genotype Utusaya were provided by the Bank of Germplasm, Universidad Mayor de San Andrés, La Paz, Bolivia.

### 2.2. Calculation of radiocarbon dates

The radiocarbon dates were measured by beta counting and Accelerator Mass Spectrometry (AMS). Beta counters measure the radioactivity of the sample, whereas AMS determines the ratio of the three different carbon isotopes in the sample.

### 2.3. Viability as determined by the 2,3,5-Triphenyl-2H-Tetrazolium chloride (TTC) test and confirmed by fluorescein diacetate staining

Seeds were soaked in water overnight, and then placed in a 0.1% tetrazolium chloride solution (2,3,5 triphyenyl tetrazolium chloride in water) for 3 h at 30 °C, according to the recommendation of The International Seed Testing Association (Supplements 2011 to ISTA Working Sheets on Tetrazolium Testing, Volume I and II).

Viability was also determined by Fluorescein Diacetate (FDA: Sigma-Aldrich Co. LLC, C-7521). The FDA stock solution was prepared by dissolving 5 mg of FDA in 1 ml acetone and solution was stored at −20 °C. Fresh sections were obtained with a razor blade and mounted on slides. Following addition of staining solution (50 μmol), sections were incubated at room temperature for 5 min in the dark. After that, the staining solution was removed with PBS and then analyzed with fluorescent microscopy. A FDA filter set (excitation 488 nm, emission 530 nm) was used to examine the samples.

### 2.4. Germination and humidification treatment (priming)

Seeds of the three genotypes were tested for germination on top of three pieces of filter paper moistened with 3.5 $cm^3$ distilled-