Life and death in the embryos of seeds

Daphne J. Osborne and I. I. Boubriak

Oxford Research Unit, The Open University, Foxcombe Hall, Boars Hill, Oxford OX1 5HR, UK

Abstract

This article discusses the ageing of seeds and the embryos they contain, and seeks to understand why the life span of a viable seed is so dependent upon the conditions it experiences after harvest. It contains new information on the different nuclease activities that are brought into action at different levels of seed moisture content and explores the integrity and degradation of DNA and the appearance of nucleosomes as indicators of a programme to cell death and loss of seed quality. The results show the importance of hydration levels in the ageing processes of stored seeds and provide a caution for the priming procedures that are used in the seed industry.

Additional key words: *DNA integrity, moisture content, nucleases, nucleosomes*

Introduction

Different parts of plants have very different life spans: the reproductive parts are the most ephemeral - hours or days - whilst those of leaves and stems may survive for a year or more. By far the longest living cells are those of the embryos of seeds whose viable life-time can extend to decades or centuries. Whereas in the aerial parts, the processes of cell senescence and death resulting from ageing, temperature extremes, desiccation or pathogen attack take place quite rapidly once started and are usually complete within a matter of days, the degenerative changes that accompany the loss of viability in a stored seed are slow and continuous throughout the period the seed embryo survives in the dry, ungerminated state.

Life Spans

Perhaps the oldest authentic dry stored seeds known to germinate are those of the Canna lily which were inserted into growing walnuts so that when the nut was mature the seed freely rattled in the closed nut case. In 1972 Canna seed from such rattle necklaces found in the archaeological sites of pre-Inca tombs at Santa Rosa de Tastil in Argentina were coaxed to germinate and their rhizomatous offspring survive today at the University of La Plata. The walnut cases were carbon dated at 620 ± 60 years so the seed must have been at least that age (Lerman and Cigliano, 1971). Even older seed of the Sacred Lotus from the mud of the Palantien Lake in China that has been carbon dated at 1,288 ± 271 years has germinated but it is not clear whether this seed has had periods of imbibition when DNA repair and other metabolic processes could take place (Shen-Miller *et al.*, 1995). Wheat stored at Petra in silos of the 'rose red city half as old as time' was said to germinate after 30-40 years, whilst rye survives viable for up to 3-4 years.

For studies of the factors that control the ageing process in embryos, these life spans are too great to permit extensive or continued biochemical study on single seed lots so various attempts have been made to 'accelerate age' seed. Most methods depend upon holding the seed at an enhanced moisture content and an elevated temperature. Wheat for example held at 35 °C and 14.5 % moisture content (SMC) will survive for less than 28 days compared with the 30-40 years at Petra (Dell'Aquila, 1994).

Viability and Vigour

The modern seed industry demands much of the performance of any seed lot. It must show high percentage germination (viability) but that alone is not enough. It must also show synchronous and rapid germination and an ability to withstand considerable levels of stress with a minimum impairment of speed and uniformity of behaviour. Two lots of seeds with equal percentage germination in standard tests under optimal conditions can show quite different performance in the field when subjected to the vagaries of variable temperatures and water availability (Redfearn and Osborne, 1997). This inherent property of any seed embryo to perform under the conditions it experiences at germination reflects the vigour of that seed, and this deteriorates and becomes progressively diverse as the seeds age. For this reason, any improvement in our understanding of the ageing process and the means that

might be adopted to circumvent these ageing changes are central to the better future of the seed industry.

Many functions deteriorate in a seed embryo as it ages. Membranes lose integrity (hence high levels of solute leakage on imbibition), enzymes lose function (e.g., mitochondrial dehydrogenases, which are the basis of the tetrazolium test for viability), and ribosomal RNA is slowly cleaved by ribonuclease action, though the ribosomes themselves remain intact (Roberts *et al.*, 1973). However, perhaps the most important of all is the continued and accumulated fragmentation of DNA by the chromatin-associated endonucleases (Cheah and Osborne, 1978) which remain functional even at very low moisture contents (< 8-9 %). These are water contents of embryo cells of seeds held under the normal best conditions of seed storage, though frequently seeds are, by necessity, stored at much higher moisture contents than this (up to 16 %). Because the maintenance and fidelity of genetic information is crucial to successful germination and seedling establishment, many of our past investigations have centred upon a study of the integrity of DNA during ageing and the efficiency of the DNA repair system that operates when an embryo is first supplied with water (Elder *et al.*, 1987; Elder and Osborne, 1993; Osborne and Boubriak, 1997).

The question is now addressed of whether the nuclear events associated with embryo death are similar in the dry state to those that take place at higher moisture contents and whether unusual changes occur in the DNA of seed embryos that are already dead when they too are exposed to elevated moisture levels.

The experiments described here show that the loss of integrity of DNA as a seed ages is dependant upon the moisture content at which the seed is stored and that accelerated ageing procedures do not produce the same DNA fragmentation patterns as those arising under the conditions of normal dry storage. As a result, these experiments provide an insight into the potential hazards that can arise when priming procedures are used to improve seed quality.

Materials and Methods

Seed source

Two seed lots of rye (*Secale cereale*, Var. Rheidol) were used. The first had grains of 96 % viability and was a gift from Dr P I Payne of Plant Breeding International, Cambridge, UK. The second had been dead for seven years and gave 0 % germination in 1991 after a 4-year period of dry storage at ambient temperature. Embryos were hand-dissected from the dry grain and used at once for each experiment.

Accelerated ageing of seeds

Accelerated ageing was achieved by suspending the seeds in muslin bags over a saturated NaCl solution (giving 75 % relative humidity) in closed Kilner jars. The jars were held in an incubator at 40 °C. Isolated embryos were weighed before and at different times after hydration for calculation of water uptake at each collection time.

Irradiation

Isolated embryos from dry seed and nucleosome lysis extracts were gamma-irradiated with 500 and 750 Gy respectively (dose rate 0, 14 Gy/sec) from a ^{137}Cs-source of a Gravitron RX 30/55 Irradiator (Gravatom UK).

Extraction and fractionation of DNA

For DNA isolation a commercial Genomic DNA kit (InViSorb™, Bioline, UK) was used. The DNA content of each isolated sample was quantified at 260/280 nm using a CE-440 spectrophotometer (Cecil Instruments, UK) or at 550 nm in a Luminescence Spectrometer L550B (Perkin Elmer, UK) against standards of the DNA specific dye PicoGreen™ (Molecular Probes Europe, The Netherlands). Equal DNA samples (5-7 µg) were fractionated on 0.8 % agarose gels by electrophoresis at constant current (45 mA) for 2.0 h, then visualised by the addition of 0. 5 µg/mL ethidium bromide.

Nucleosome quantification

Nucleosome contents were measured using a Nucleosome ELISA kit (Oncogene Research Products, Calbiochem, USA). For this, 55 mg of embryos were ground in dry ice and lysed in 750 µL of kit buffer. Samples were diluted 1:8 and 1:16 for immunological analysis against anti-H_3 histone (rabbit) as primary antibody and peroxidase-linked anti-rabbit as secondary antibody. Data are presented as nucleosome units per 1 mL of original lysis solution calculated from kit standards. One nucleosome unit per mL is defined as the amount of nucleosomes from 444 UV-treated Daudi cells/ml and the limit of detection of the assay is equivalent to 67 irradiated Daudi cells. DNA contents per mL between samples did not differ by more than 2 %.

Nuclear DNA values

Embryos were fixed overnight in ethanol:glacial acetic acid (3:1 v/v), then hydrolysed in 5 M HCl for 30 min at room temperature, rinsed in water, stained in Feulgen reagent (BDH, UK) for 60 min at 26 °C, rinsed (x 3) with 0.5 % potassium metabisulphite in 0.05 M HCl, then with water, followed by 10 min in 45 % acetic

acid. After rinsing in water, embryos were squashed on slides, air-dried and mounted in Canada Balsam (Sigma, UK). The density and area of at least 200 nuclei for 5 embryos was measured on a Vickers M85 Scanning Microdensitometer at 550 nm, mask size 4, against adjacent non-nuclear cytoplasmic areas. Values are expressed in relative DNA units.

Results and Discussion

The moisture contents of the embryos held dry at the start of the experiments were below 9 % for both the viable and the 7-year dead material. On transferring seed from the dry to accelerated ageing conditions (75 % relative humidity at 40 °C) for 15 to 19 days, the moisture contents increased to 13-14 % for both rye embryo samples (Table 1). After 13 days in this temperature and humidity, the embryos of originally viable seed ceased to germinate and were therefore considered dead. No detectable changes were found in the total nuclear DNA contents of the live or dead seeds after accelerated ageing; no DNA increase occurred in the live embryos before they died and no losses took place from the 7-year dead material (Table 2). Irrespective of the state of integrity of the DNA therefore, the total DNA contained within the nuclear membrane remains unaltered as judged by Fuelgen staining.

There is already ample evidence from neutral and alkaline gel electrophoresis that the DNA of embryos of

Table 1. The percentage moisture contents of viable and 7-year dead embryos of rye grains held dry or under conditions of accelerated ageing.

	Viable	7-year dead
Air dry	8.6 ± 0.1	5.9 ± 0.2
Accelerated aged	13.5 ± 0.5	12.7 ± 0.9

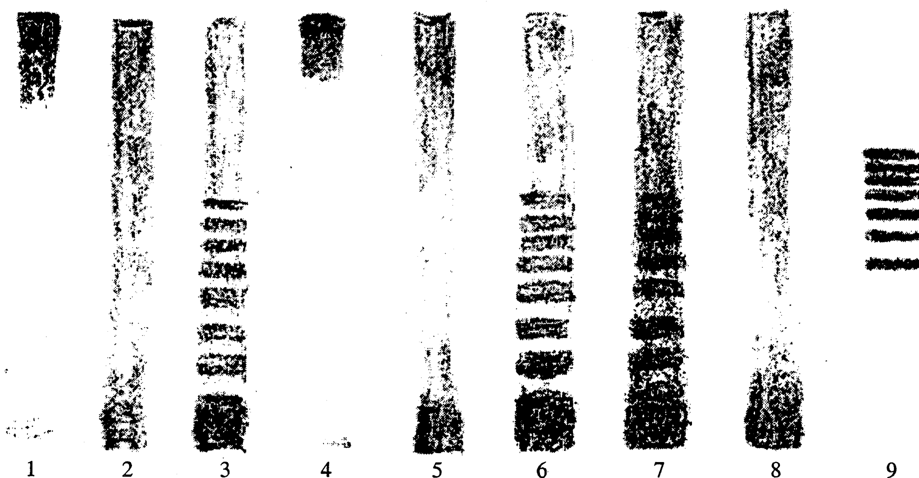
Table 2. DNA content of 2C nuclei in embryos of viable and 7-year dead seed held in dry, accelearated aged and fully imbibed conditions.

	Viable	7-year dead
Air dry	22.5 ± 5.0	27.1 ± 4.4
19 d - Accelerated aged[1]	22.5 ± 6.7	-
19 d - Accelerated aged[1] and Imbibed for 20h	25.8 ± 6.4	23.3 ± 5.6

[1]After 13d in accelerated aged conditions, embryos are dead.

low viability or non-viable, dead seed is highly fragmented (Cheah and Osborne, 1978; Elder *et al.*, 1987; Vázquez-Ramos *et al.*, 1988) and that on imbibition of water by dead embryos, further fragmentation proceeds apace so that by 3 h little high molecular weight DNA remains (Cheah and Osborne, 1978). What is of special interest, is the fragmentation pattern of DNA that results when seeds are held under a more restricted level of hydration that precludes germination (13-14 % SMC). In addition to the random sized fragments that progressively accumulate with time at 8-9 % moisture content (Fig. 1, lane 2), another fragmentation pattern with multiple repeats is initiated (Fig. 1, lane 3). As long as embryos remain fully viable, there is little DNA fragmentation in the dry state (primarily single-strand breaks with some double-strain breaks; Fig. 1, lane 1) and after any period of imbibition (in water) of 30 min or more, these low molecular weight fragments are religated *in situ* through the efficient operation of a nuclear DNA repair system (Dandoy *et al.*, 1987; Elder *et al.*, 1987; Vázquez *et al.*, 1991; Elder and Osborne, 1993). Fragmentation patterns in fully viable dry embryos or in those that are germinating are always seen as random, even in those of low viability (Elder and Osborne, 1993) or in those of low vigour (Boubriak, unpublished). The efficiency of the repair of single-strand breaks is highest in the highest viability and vigour seeds as is shown by the rapidity of these embryos to religate breaks in the DNA introduced by γ-irradiation of the dry embryos (Elder *et al.*, 1993). Within 30 min of imbibition, the majority of DNA in rye embryos is already restored to high molecular weight and by 1.5 h is fully restored (Elder and Osborne, 1993). A fast and efficient restoration of genomic integrity is an essential requirement for rapid and successful germination. In low viability seeds, the DNA repair system is already impaired, so that religation of accumulated DNA breaks is then slow and germination and emergence of the radicle retarded (Elder *et al.*, 1987; Elder and Osborne, 1993).

When fully viable rye seeds held dry (8-9 % SMC) are exposed to accelerated ageing conditions, such that the once fully viable embryos are dead by 13 days, the DNA extracted from them (after 15 days) shows a multiple repeat fragment pattern on electrophoresis in neutral agarose gels (Fig. 1, lane 6). These fragmentation repeats are similar to those of the 7-year dead material when put into water. On return of these once viable seeds to their previous dry storage conditions (8-9 % SMC) for 70 days, the multiple repeats in DNA remain stable and are retained, whilst further endonuclease cleavage to random length fragments



Legend

Lane	
1	dry, viable
2 & 5	dry, 7-year dead
3	7-year dead, imbibed 3 h
4	viable, imbibed 1.5 h
6	viable, accelerated aged 15 d
7	viable, accelerated aged 15 d then stored dry, 70 d
8	7-year dead, accelerated aged 15 d
9	molecular markers, 2000-800 bp (Hybaid Bioladder^{TM} 200)

Figure 1. Distribution of DNA from viable and 7-year dead embryos of rye in neutral agarose gels following accelerated ageing of seed and imbibition of the embryos.

9 % SMC) for 70 days, the multiple repeats in DNA remain stable and are retained, whilst further endonuclease cleavage to random length fragments proceeds as seen from the increased density of the overall random fragmentation (Fig. 1, compare lane 7 with lane 6). No fragment banding occurs when the 7-year dead material is accelerated aged (Fig. 1, lane 8).

When viable seed material is first put under accelerated ageing conditions, the embryo cells are potentially fully capable of all the metabolic events that lead to germination provided sufficient water is available. Even at 40 °C nucleic acid and protein synthesis is possible if the cells have access to unlimited water. At this restricted level of water availability under accelerated ageing (13-14 % SMC) protein synthesis is not possible (a minimum of 16-18 % SMC is required for polysome formation in wheat, Marcus *et al.*, 1966) and sufficient ATP generation is doubtful (Hourmant and Pradet, 1981). In the 7-year dead material, no metabolic activity occurs at any time after the original loss of viability. The activation of hydrolytic enzymes present in the embryo in an inactive state from the time of harvest, can however occur by non-metabolic means. Present examples are the operation of an endo-nuclease that will cleave the nuclear DNA of dead embryos when these are exposed to full hydration in water, and an endonuclease in viable embryos when these are held at moisture contents (13-14 %) that preclude protein synthesis.

From the banding patterns seen on the gels (Fig. 1) these accumulated repeat DNA fragments coincide with 200 base pair multimers as judged from the molecular markers (Fig. 1, lane 9) run with them.

That the DNA of dry rye embryos is composed of multimeric 200 base pair units but of different fragment

shown that the 140 base pair nucleosome core repeat and the 60 base pair nucleosome linker region were identical to that in DNA of rat liver. Those findings indicated a high degree of stability of the overall DNA-histone complex which is still retained in dry long-dead seeds despite the continuous random length fragmentation that proceeds in the dry state.

Direct analysis for the presence and accumulation of nucleosomes has substantiated these early findings and has revealed that accelerated ageing conditions will activate a nucleosome-generating nuclease activity in viable embryo cells when the seeds are brought to 13-14 % SMC. This does not occur with the 7-year dead material. No nucleosomes are generated in the dead embryo cells when these seeds are held at 13-14 % SMC although the nucleosome-generating nucleases must already be present in an inactive form (they cannot be newly synthesised) and are activated readily at full hydration when embryos are placed in water (Table 3).

It is very clear that with respect to the integrity of DNA, the conditions of accelerated ageing described here for viable rye do not reflect those taking place in the dry state and that accelerated ageing procedures cannot be used as a quick method to study changes linked to the slow cell death of seeds that are held dry.

When the seeds of rye (and presumably other cereal crops too) mature on the parent plant, both ribo- and deoxyribo-nucleases are conserved as potentially active enzyme molecules within the dehydrating cells of the embryo. Nucleases are perhaps some of the most stable proteins; active nucleases have been demonstrated in the extracts of the 7-year dead embryos (Cheah and Osborne, 1978) and reportedly active also in the leaves of long dead herbarium material.

Table 3. Nucleosome contents of embryos of viable and 7-year dead seed held in dry, accelerated aged and fully imbibed conditions.

	Viable	7-year dead
Dry	nd[1]	nd
Imbibed 20 h	nd	13.3 ± 0.9
Accelerated aged[2] (15 d)	5.9 ± 1.0	-
Accelerated aged[2] (19 d)	8.7 ± 2.0	nd
Accelerated aged[2] (19 d) Stored dry 70 d	10.3 ± 1.0	-
Accelerated aged[2] (19 d) Imbibed 20 h	21.1 ± 3.4	15.6 ± 3.0

[1] nd = not detectable
[2] After 13 d in accelerated aged conditions, embryos are dead.

What is apparent from the present study is the evident multiplicity of nuclease activities and the conditions under which these enzymes can function in the endo-cleavage of DNA. At water contents of less than 9 %, the DNA is reduced to random length oligo- and poly-nucleotide fragments such that a smear of these different fragment lengths is seen in gels following electrophoresis. The absence of nucleosomes under these conditions is confirmed by direct assay for these in both the viable and the 7-year dead embryo material (Table 3, and Fig. 1, lanes 1, 2 and 5). If the storage conditions become wholly unfavourable (accelerated aged, 40 °C and 75 % relative humidity, when the moisture content is raised to 13-14 %), nucleosomes are generated in the once viable embryos before they reach the stage of becoming non-viable. The 7-year dead material cannot generate nucleosomes at this moisture content as seen by the absence of any nucleosome multimers when the embryo DNA is fractionated in agarose gels (Fig. 1, lane 8) or when the DNA is assayed directly for the presence of nucleosomes (19 d accelerated ageing of the 7-year dead rye embryos, Table 3). In viable material, therefore, a nuclease activity can occur at levels of hydration (13-14 % SMC) which are too low for new protein synthesis but are sufficient to elicit either a change in the conformational state of the enzyme, or a change in the conformational state of the nuclear chromatin such that the DNA becomes accessible to the endonucleolytic cleavage in the histone H_1 linker region of the molecule. Since these events occur only in viable embryos at 13-14 % SMC and not in the 7-year dead embryos at 13-14 % SMC it can be assumed that it is unlikely that the conversion of chromatin at $<$ 8-9 % SMC to another more accessible state is the reason for the lack of nucleosome formation at 13-14 % SMC. In the viable embryo cells, another factor, not simply a higher state of water activity, must operate to activate the nuclease that cleaves at the site of the histone H_1 link. One possibility is that the immobile cytoplasmic water present in the glassy state of dry embryo cells (Seewaldt *et al.*, 1981; Bruni and Leopold, 1991) can be converted to one in which weakly bound (or free) water exists only in live cells held at 14 % SMC. In dead material, the cytoplasmic organisation and ultrastructure is already so changed (Hallam *et al.*, 1973) that even higher levels of water availability must be provided (by imbibition directly in water) to achieve to the same result. Only then will the nucleosome-forming DNA degradation proceed in the once dry, dead cells of the 7-year dead rye (compare Fig. 1, lanes 3 and 8; Table 3, 20 h imbibed with 19 d accelerated aged).

Conclusions

What do these results mean for the seed industry?

Firstly, they mean that seeds (specifically cereal seeds, but it may apply to other seed types also) that show a DNA pattern that contains nucleosome multimers have, in all probability, been stored under unfavourable conditions for some period of time after harvest and are hence likely to be poor performers in the field. In other words their vigour has been impaired for at present there is no evidence that nucleosome breaks, linked to apoptotic cell death, are repaired.

This also raises the question of whether there is a commonality between priming and accelerated ageing? We suggest that once the water activity in the cell reaches that permissive for DNA repair of random breaks for however short a period (30 min is sufficient) an embryo will start to restore its genomic integrity. But if this restoration is then overtaken by an enhanced nucleosome DNAase activity which is not repairable, a decline in viability will then follow and the cells will become progressively apoptotically aged.

The borderline between these two activities will depend upon the water relations and temperatures that each embryo cell experiences. The continued maintenance of conditions that favour the activation of nucleosome cleavage processes will, we suggest, lead to overpriming and finally to apoptotic-like embryo cell death. Then both viability and vigour are lost.

Acknowledgments

This work was carried out during the tenure of an INTAS-UA 95-92 grant. D.J. Osborne acknowledges the award of a fellowship from the Leverhulme Trust, United Kingdom.

References

Bruni, F. and Leopold, A.C. 1991. Cytoplasmic glass formation in maize embryos. *Seed Science Research* ***2***, 251-253.

Cheah, K.S.E. and Osborne, D.J. 1977. Analysis of nucleosomal deoxyribonucleic acid in a higher plant. *Biochemical Journal* ***163***, 141-144.

Cheah, K.S.E. and Osborne, D.J. 1978. DNA lesions occur with loss of viability in embryos of ageing rye seed. *Nature* ***272***, 593-599.

Dandoy, E., Schyns, R., Deltour, R. and Verly, W.G. 1987. Appearance and repair of apurinic/apyrimidinic sites in DNA during early germination of *Zea mays*. *Mutation Research* ***181***, 57-60.

Dell'Aquila, A. 1994. Wheat seed ageing and embryo protein degradation. *Seed Science Research* ***4***, 293-298.

Elder, R.H. and Osborne, D.J. 1993. Function of DNA synthesis and DNA repair in the survival of embryos during early germination and imbibed dormancy. *Seed Science Research* ***3***, 53-63.

Elder, R.H., Dell'Aquila, A., Mezzina, M., Sarasin, A. and Osborne, D.J. 1987. DNA ligase in repair and replication in the embryos of rye, *Secale cereale*. *Mutation Research* ***181***, 61-71.

Hallam, N.D., Roberts, B.E. and Osborne, D.J. 1973. Embryogenesis and germination in rye (*Secale cereale L.*) III Fine structure and biochemistry of the non-viable embryo. *Planta* ***110***, 279-290.

Hourmant, A. and Pradet, A. 1981. Oxidative phosphorylation in germinating lettuce seeds (*Lactuca sativa*) during the first hours of imbibition. *Plant Physiology* ***68***, 631-635.

Lerman, J.C. and Cigliano, E.M. 1971. New carbon-14 evidence for six hundred years old *Canna compacta* seed. *Nature* ***252***, 568-570.

Marcus, A., Feeley, J. and Volcani, T. 1966. Protein synthesis in imbibed seeds. III Kinetics of amino acid incorporation, ribosome activation and polysome formation. *Plant Physiology* ***41***, 1167-1172.

Osborne, D.J. and Boubriak, I.I. 1997. DNA status, replication and repair in desiccation tolerance and germination. *In* Basic and applied Aspects of Seed Biology (eds. R.H. Ellis, M. Black, A.J. Murdoch and T.D. Hong), pp 23-32, Kluwer Academic Publisher, Dordrecht/Boston/London.

Redfearn, M. and Osborne, D.J. 1997. Effects of advancement on nucleic acids in sugar beet (*Beta vulgaris*) seeds. *Seed Science Research* ***7***, 261-267.

Roberts, B.E., Payne, P.I. and Osborne, D.J. 1973. Protein synthesis and the viability of rye grains. Loss of activity of protein-synthesizing systems *in vitro* associated with a loss of viability. *Biochemical Journal* ***131***, 275-286.

Seewaldt, V., Priestley, D.A., Leopold, A.C. and Feigenson, G.W. 1981. Membrane organization in soybean seeds during hydration. *Planta* ***152***, 19-23.

Shen-Miller, J., Mudgett, B., Schopf, J.W., Clarke, S. and Berger, R. 1995. Exceptional seed longevity and robust growth: ancient sacred lotus from China. *American Journal of Botany* ***82***, 1367-1380.

Vázquez, E., Montiel, F. and Vázquez-Ramos, J.M. 1991. DNA ligase activity in deteriorated maize-embryo axes during germination: a model relating defects in DNA metabolism in seeds to loss of germinability. *Seed Science Research* ***1***, 269-273.

Vázquez-Ramos, J.M., López, S., Vázquez, E. and Murillo, E. 1988. DNA integrity and DNA polymerase activity in deteriorated maize embryo axes. *Journal of Plant Physiology* ***133***, 600-604.