Storage of date palm pollen during ten years

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The date palm (Phoenix dactylifera) is a heterozygot and dioecious species. It is frequent that the periods of maturity of male and female flowers are not synchronized, due to the necessity to conduct the conservation end the storage of the pollen.

Twelve pollen samples coming from the experimental station of Biskra (Algeria) have been stored in the pollen bank in the laboratory of Palynology (Paris).

A particular Freeze drying method (60 °C and 6.10-³atm) has been used and three experimental temperatures (+4°C, -20°C and -80°C) have been applied.

The results indicate that after ten years of storage several samples shoed a viability higher than 80 % under the temperature of -20°C.

Key words: Pollen, Date palm, Storage, Freeze drying, Pollen bank, Viability, in vitro germination, Algeria.

Spontaneous diploidization occurs after nuclear fusion during *in vitro* maize induced microspore embryogenesis

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Pollen embryogenesis leads to the formation of multicellular structures which differentiate apical meristems and are able under suitable growth conditions to generate homozygous lines useful for plant breeding programmes. During this type of embryogenic development chromosome doubling may occur spontaneously, specially in cereals, or may be induced with the help of drugs such as antimitotic agents or herbicides. It has been suggested that the spontaneous chromosome doubling occurs during the early stages of microspore development, but little is known about the mechanism and the precise stage at which this occurs. The knowledge of the processes which govern natural chromosome doubling is of interest for fundamental research and for establishing effective methods to produce true homozygotes for breeding purposes.

In maize, the spontaneous chromosome doubling rate during induced microspore embryogenesis is more efficient (>40% of the regenerated plants) than in other species, therefore, microspore embryogenesis has been induced in isolated microspores of maize to determine when and how chromosome doubling occurs. *In situ* characterisation by ultrastructural, cytochemical and immunocytochemical methods at specific stages has been performed. At 5-7 days of culture multinuclear-enlarged microspores display two domains which have been called " embryo-like domain " and " endosperm-like domain " due to specific structural and molecular features. After 12-14 days, the multicellular structures are released into the medium and develop into " embryos" which are able to generate plantlets

In this work, a pluridisciplinary study has been made in order to analyse the spontaneous chromosome doubling process during the early stages of *in vitro* maize microspore embryogenesis. At different developmental stages, chromosome number was determined from squashed cells, and DNA content was measured by cytometry. In parallel, an ultrastructural analysis of the microspore derivatives demonstrated the occurrence of a nuclear fusion process. It seems likely that nuclear fusion ensures chromosome doubling at early stages of induced microspore embryogenesis, the process occurring at the 5/7 day stage in the embryo domain. These results will

shed light to the correct knowledge of this process ,which will be of great importance in the future to increase the percentage of homozygous plants for crop improvement.

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Poster session a3

POLLEN FOR IN-VITRO PRODUCTION OF HAPLOIDS

Influence of medium Fe concentration upon embryogenic microspores induced by mannitol in barley (*Hordeum vulgare* L.)

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Barley androgenesis is one of the most efficient methods of producing double haploids, which constitutes a very valuable system for breeding programs. Great efforts have been made to improve the culture conditions for inducing androgenesis using either anther or isolated microspore cultures (KASHA *et al.* 1990). The culture conditions for microspore embryogenesis are generally derived from those developed for anther culture. In either method it is essential to supplement the medium with Fe not only to obtain embryogenic structures but also to reduce the production of albino plants (KASHA et al. 1990). To date no studies have been made to compare the cytological changes which occur during the different phases of the androgenic process induced by anther and microspore culture in barley. We report here the results of a comparative cellular and ultrastructural analysis applied to the study of early androgenesis in isolated microspore and anther cultures.

We applied mannitol pre-treatments to induce androgenesis in both anther (CASTILLO *et al.* 2000) and isolated microspore cultures (OLSEN 1991) using the highly responsive barley cultivar Igri. Anthers and isolated microspores at different stages of androgenesis were treated for light and electron microscopy. Samples were fixed in 0.25% glutaraldehyde and 4% paraformaldehyde in 0.5M cacodylate buffer, then dehydrated in ethanol series and embedded in Unicryl. Very similar cell behaviour was found in both culture systems: non-synchronous or homogeneous response, symmetrical divisions together with some asymmetrical divisions, formation of embryos inside the pollen wall and enlargement of the intine wall. The main difference to be seen was the formation of electron-dense inclusions in the wall of the embryogenic microspores obtained from isolated microspore culture, which were not visible after anther culture.

To discover the nature of these inclusions different treatments, including proteinase K and EDTA, were applied to the ultrathin sections. The inclusions were not digested after the enzyme treatment, which lead us to

believe that they were not proteins. They did disappear however with the EDTA treatment, indicating that they might be cations, which were chelated by EDTA. The electron-dense inclusions were also analysed by X-Ray microanalysis at TEM level and found to be made up mainly of iron. To find out more about the influence of the concentration of Fe in the microspore culture medium we made isolated microspore cultures with no Fe, 0.015x, 0.5x, 0.75x, 1x and 2.5xFe (1xFe being the initial concentration of $FeNa_2$ EDTA 37.3 mg/l used in the culture medium of both anthers and isolated microspores). The presence of electron-dense inclusions on the cell wall and the number of embryo-like structures (ELS) and green and total plants obtained with these different concentrations were evaluated. Inclusions were not found in the cell wall when 0.75x or lower Fe concentration were used. When higher concentrations of Fe were used in the culture medium, however, inclusions were visible in the walls. The Fe deposits accumulated in the intine demonstrated that we were using an excessive concentration of Fe in the isolated microspore culture medium, which may well be toxic to the embryogenic development of the microspores. The evaluation of the number of ELS, green and albino plants regenerated from microspore cultures using different concentrations of Fe suggest that the optimum concentration is between 0.75x and 1x.

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Influence of aluminum treatments on the androgenic development of wheat microspores

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The *in vitro* microspore selection is a widely used method to improve stress tolerance of plants. Investigations were also performed from wheat microspores to develop aluminium-tolerant dihaploid plants (Karsai et al, 1994; Barnabas et al. 2000). In spite of this, till now, no exact information is available about the effects of Al during microspore development. Here, data are presented on the cell division pattern, the viability of the wheat microspores, the embryoid formation and their green plant regeneration under Al stress condition.

For this, wheat anthers containing mainly LU microspores were cultured in liquid MMS3 at pH 5.8 and at pH 4.0 without Al (for controls) and with 300 or 800 µM $Al_2(SO_4)_3$. The cultures were incubated at 26 °C under 16h dim light (50 µmol m^{-2} s^{-1}). FDA and DAPI staining were applied for determining the viability and the developmental stages of the microspore derived structures. The cytological structure of the microspores was observed by TEM. Since Al toxicity strongly depends on pH of the culture medium due to changing its solubility, it was necessary to determine the pH and the free Al(III) concentration – by using Morin-spectrofluorometry – of the culture media.

No significant difference was found in the microspore development during the cultivation period, when Al was not present in the cultures either at pH 5.8 or at pH 4.0.

At the 6th day after initiation of anther culture, 20-30% of the microspores were viable showing typical bicellular structure with star-like cytoplasm. No significant difference was found in the viability between the Al-treated and control cultures, probably because the Al could not penetrate through the intact anther walls. By the 13th day, when most cells of the anther walls were perished, the ratio of viable microspore derived structures were 20% less in the cultures containing Al than in the controls. After this time, however, due to the intensive cell metabolism and proliferation, the pH of the culture medium was increased and by the end of 4th week approximately pH 5.0 was reached even in the culture media containing Al. This mechanism can contribute to eliminate the toxic effects of Al. Since at pH above than 4.5, the precipitation of Al is predictable resulting in a decrease of Al toxicity, the amount of free (toxic) Al(III) form was checked in the culture medium. Respectively, 37 and 295 µM free Al(III) could be measured in the MMS3 culture media containing 300 and 800 µM $Al_2(SO_4)_3$ at the beginning of the experiments (which are still higher than the toxic intracellular concentration). During the culture period, this amount of free Al(III) decreased to zero due to the precipitation of Al into non-toxic Al forms (mainly $AlPO_4$, $Al(OH)_3$).

In conclusion, it seems that the effect of Al on the microspore development in anther culture, could be effective during the short disruption period of the anther walls, since before, the anther wall can protect the microspores, and later the microspore development resulted in an increase of pH eliminating the toxic effects of Al. This is also confirmed by the fact that, when the half part or the total amount of the culture media were replaced weekly to fresh solution containing 37 µM free Al(III), the microspore development reduced dramatically by the 13th day: the viability was 40-50% lower in the treated cultures than in the controls and the microspores developed into only not more than 4 celled structures with thick walls and large central vacuole.

Further studies are needed to discover the entire defensive strategy of cultured microspores against Al-toxicity.

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In vitro selection of maize microspores to improve oxidative stress tolerance in plants

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Nowadays, one of the most important aims in agriculture is to improve abiotic and biotic stress tolerance of crops. It is possible by increasing the antioxidant capacity of plants because most abiotic and biotic stresses induce oxidative damage. Since the genes of most antioxidant enzymes are also expressed during androgenesis, the uninucleate microspores are suitable for selecting oxidative stress resistant lines. In the Agricultural Research Institute of the Hungarian Academy of Sciences, efficient, reproducible anther culture methodologies for maize and wheat have been elaborated (Barnabás 2003). In maize this method was used to carry out *in vitro* microspore selection in the presence of various oxidative stress stimulators (paraquat, t-buthylhydroperoxide (BHP), menadione, riboflavin+ methionine).

The selective compounds (in various concentrations) were applied both in the induction and plant regeneration phases of anther culture. In each treatment 2000 anther were used.

Superoxide radicals derived from paraquat (Pq, used at 0.5, 1 and 5 µM concentration) significantly reduced both the haploid induction and the regeneration capacity. In the developing microspores less starch accumulation was observed. In the case of paraquat 5 µM concentration was lethal for the microspores. Using 0.5 or 1 µM Pq 15 fertile, dihaploid plants could be regenerated. The superoxide radicals produced by menadion at 50, 100µM and 1mM concentrations, affected especially the degeneration of the nuclei of microspores, which caused low anther induction and regeneration. Riboflavin was also applied at 1,10, and 100 µM concentration along with methionine in light condition. No effect was observed at 1 and 10 µM, but considerable reduction was detected at 100 µM. But in these plants due to an assynchronous flowering abnormal plant regeneration was observed. At 10 µM riboflavin, however, 10 fertile dihaploid plants could be produced. The t-BHP, a lipid peroxide, had to be applied at 0.1, 1 and 10 mM concentration to reach an efficient selection, resulting in 10 fertile dihaploid plants.

Further investigations, especially ultrastructural analysis, are carried out to get new information about the effects of different selecting compounds, on microspore development.

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