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Chromosome stability in cryopreserved germplasm of *Cyrtopodium hatschbachii* (Orchidaceae)

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Cyrtopodium hatschbachii is a recently-described orchid species distributed in northeastern Argentina. Natural populations of this species require conservation because they have a very restricted size and distribution and are in danger of destruction. Cryopreservation in liquid nitrogen is the most commonly-applied technique for *ex situ* conservation of plant genetic resources, but genetic stability after cryopreservation is an important consideration. Chromosome number and morphology are primary cytogenetic parameters that must remain stable after cold storage. We analyzed plants of *C. hatschbachii* cytogenetically in order to determinate their stability level. The plants from cryopreserved encapsulated seeds were stable at chromosome and phenotypic level, but showed more limited condensation of the chromatin during the first stages of their development. The chromosome number and karyotype analysis of *C. hatschbachii* is presented for the first time.

Key words: cryopreservation, *Cyrtopodium*, encapsulation-dehydration, karyotype, orchid germplasm

Introduction

The destruction and alteration of natural environments and over-collection of plants are the main pressures that threaten the conservation of orchid diversity. The world orchid diversity status is still poorly recognized and the Red List does not register extinct orchids, but does indicate 150 threatened species (IUCN Red List 2004). Johnson (2001) indicates 21 extinct species and 1799 others with different degrees of threat. In Argentina, the province of Misiones has a great diversity of orchids with 129 species, whereas Corrientes has 76 species (Zuloaga *et al*. 1999). There is no registration of extinct or endangered species for Argentina, even though many species of its flora have ornamental value, through showy flowers or attractive vegetative characteristics. They are constantly under threat of over-collection by local settlers and must therefore be considered for conservation (Johnson 2001).

Cyrtopodium hatschbachii is a recently-described orchid species from northeast Argentina (Insaurralde *et al*. 1997), where it occurs on low-lying moist soils, a type of habitat still not protected in Argentina. Due to the fragility of these ecosystems and the meagre knowledge of natural populations of *C. hatschbachii*,

their conservation *in situ* and *ex situ* is of great importance. As a method of *ex situ* conservation, cryopreservation has been considered to be important for the long-term storage of plant germplasm (Engelmann 2000). Previous studies have established the value of cryogenic techniques to preserve orchid seeds (Popova *et al*. 2003, Popov *et al*. 2004, Hirano *et al*. 2005). Our experience with *C. hatschbachii* indicates that immature seeds germinate more readily *in vitro* than mature seeds. Thus establishment of a method for preservation of immature seeds is interesting for conservation of germplasm and for the propagation of various orchid species.

In the cryopreservation process (culture–cryoprotection–regeneration) some genome alteration may be induced, so it is necessary to assess the genetic integrity of the plants regenerated after cryogenic treatment (Harding 2004). Many cryopreservation protocols have included analysis of genetic stability at different levels (Harding 2004), but the number and morphology of the chromosome complement are among the most important cytogenetic characters that must remain stable after recovery from liquid nitrogen (LN) storage.

Previous cytogenetic studies have indicated that the chromosome numbers in subtribe Cyrtopodiinae are 2n = 44, 2n = 46 and 2n = 92, with 2n = 46 the most frequent number within *Cyrtopodium* (Aoyama 1989, Felix & Guerra 2000). However, there are still no cytogenetical data available for *C. hatschbachii* and previous studies have not considered the chromosome stability of orchid plants obtained from seeds after warming from LN storage. The aim of the present study was to analyze plants of *C. hatschbachii* produced *in vitro* to determine the number and morphology of the chromosome complement and its degree of stability after the cryopreservation process.

Material and methods

Plant material

Adult plants of *C. hatschbachii* were collected from the Campus Universitario area of the Universidad Nacional de Misiones, Argentina (UNaM). They were cultivated at the Laboratorio de Fisiología Vegetal of the Facultad de Ciencias Agrarias (UNNE). The plants were self-pollinated and immature pods were collected four months after pollination. Each pod was surface-sterilized with a sodium hypoclorite solution (1% available chlorine) containing a few drops of Tween-20 for 10 min and washed twice in sterile water. Immature seeds were removed from the pods and used for the study.

Cryopreservation and culture conditions

Immature seeds were cryopreserved using the encapsulation-dehydration technique suggested by Sakai (1995). The seeds were suspended in a sterile 3% (w/v) sodium alginate solution and dropped into 0.1 M $CaCl_2$ solution, after 1 min in the $CaCl_2$ solution each bead (4–5 mm in diameter) containing a mean of 50 seeds was recovered. The encapsulated seeds were subsequently pretreated in sucrose solutions as follows: 0.08 M (24 h), 0.15 M (24 h), 0.25 M (24 h), 0.5 M (24 h) and 0.75 M (24 h). Then, they were dehydrated for 5 h by exposure to sterile dry silica gel. Finally, the beads were placed into cryotubes and immediately plunged into LN. Samples were stored in LN for at least 12 h, after which the cryotubes were thawed in a water bath at 30 °C for 2 min and the beads were subjected to the same sucrose series as above, but in reverse. The encapsulated seeds were then sown on 0.6% agar-solidified Murashige and Skoog (1962) medium (MS) with 3% sucrose and cultured at 27 °C under 116 μmol m^{-2} s^{-1} of light intensity and a photoperiod of 14 h.

Cytogenetic analysis

The determination of the number and morphology of the chromosomes was made on 20 *in vitro* plants 195 days after encapsulated seeds were cultured (control) and on 20 plants *in vitro* obtained from encapsulated and cryopreserved seeds. These plants were selected randomly from 100 of each treatment. Root-tips were treated with 0.002% 8-hydroxyquinoline solution for 5 h at 25 °C, fixed in 5:1 mixture of lactic acid:

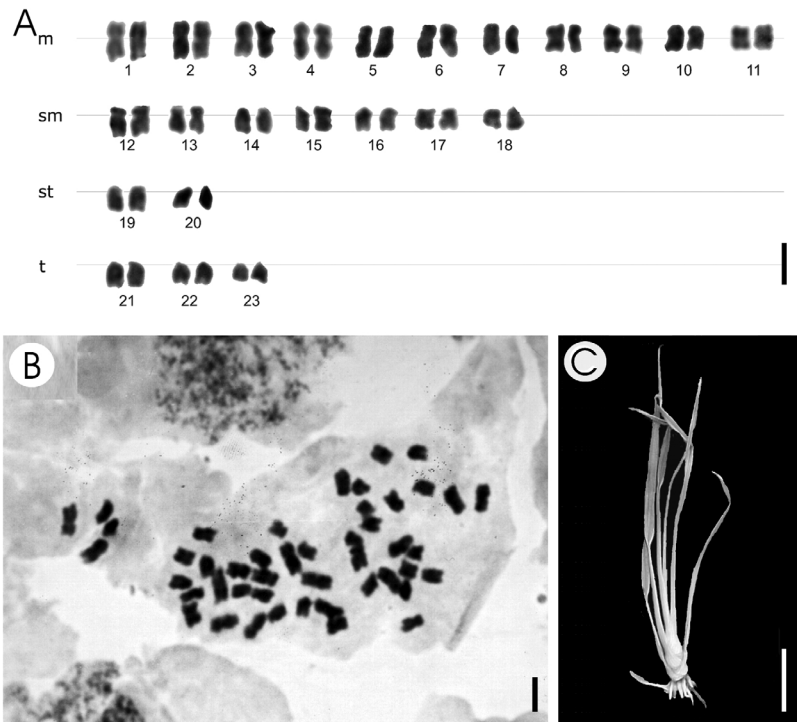

Fig. 1. Chromosomes of plants obtained from non-frozen seeds of *Cyrtopodium hatschbachii* (control). — **A**: Karyotype, 2n = 46, 22m + 14sm + 4st + 6t. — **B**: Mitotic metaphase plate, 2n = 46. — **C**: *In vitro* plant at 195 days from which the karyotype was made. Scale bars: 3 µm for **A** and **B**, 3 cm for **C**.

ethyl ethanol for 12 h at 4 °C and kept in 70% ethanol at 4 °C. For chromosome staining, the root-tips were hydrolyzed in 1N HCl at 60 °C for 8 min, stained with Schiff's solution (Feulgen reaction) and then squashed in acetic orcein. Microscope slides were examined at 1000× magnification and drawings of chromosomes were made on an optical microscope with a camera lucida (3400×). The chromosomes were classified according to Levan *et al*. (1964). Karyotypes and idiograms were composed with regard to the centromeric index (CI) (short arm/total length × 100). According to this parameter, the chromosomes were classified as metacentrics (CI = 50–37.5), submetacentrics (CI = 37.5–25), subtelocentrics (CI = 25–12.5) and telocentrics (CI = 12.5–0).

Statistical analysis

The data were subjected to analysis of variance (ANOVA) and the means were compared using the Tukey test.

Results

Cryopreservation of seeds and *in vitro* plants

Encapsulated seeds treated cryogenically developed into normal seedlings after 195 days culture on MS medium. The plants obtained from cryopreserved encapsulated seeds and those obtained from material without cold treatment were not different at the phenotypic level (Figs. 1C and 2C).

Cytogenetic analysis

The somatic chromosome numbers of non-cryopreserved plants (control) and those of the cryopreserved ones were all 2n = 46, which indicates that the ploidy level remained stable during the culture cryoprotection-regeneration process. Karyotypes of control and cryopreserved-seeds plants are presented in Figs. 1 and 2.

The karyotypic formula, 22 metacentrics +

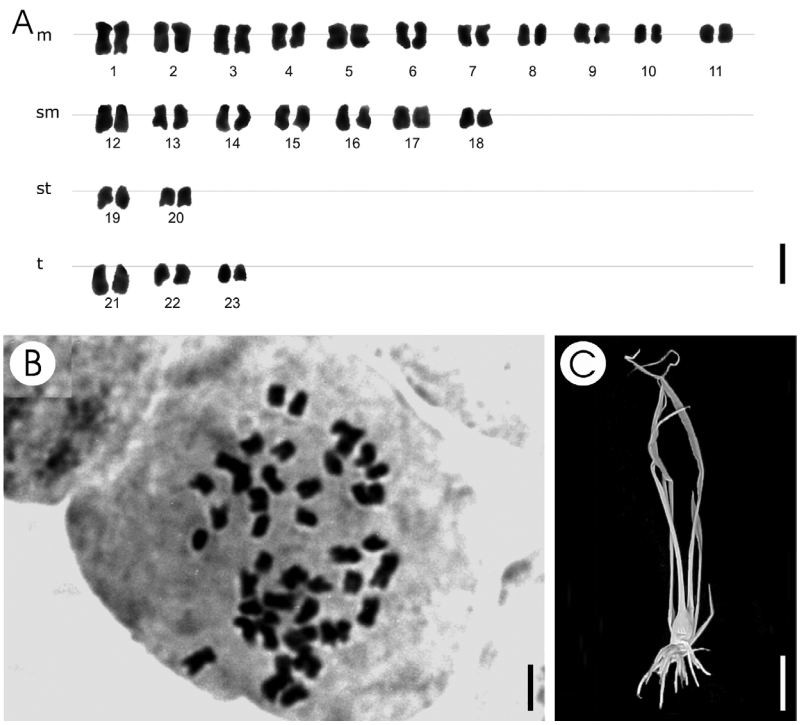

Fig. 2. Chromosomes of plants obtained from cryopreserved seeds of *Cyrtopodium hatschbachii*. — **A**: Karyotype, 2n = 46, 22m + 14sm + 4st + 6t. — **B**: Mitotic metaphase plate, 2n = 46. — **C**: *In vitro* plant at 195 days from which the karyotype was made. Scale bar: 3 µm for **A** and **B**, 3 cm for **C**.

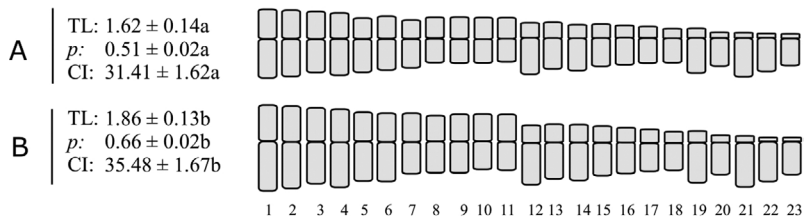

Fig. 3. Idiograms of *Cyrtopodium hatschbachii* (n = 23). Chromosome parameters comparison between control plants obtained from (**A**) non-cryopreserved seeds and (**B**) plants obtained from cryopreserved seeds. TL = average total length (µm), *p* = average shorter arm length (µm), CI = centromeric index. Data represent the mean of twenty mitotic metaphases. Values followed by the same letter are not significantly different at 0.05 level.

14 submetacentrics + 4 subtelocentrics + 6 telocentrics, was the same in control plants and in those from cryopreserved seeds. The respective idiograms of the two types of plants are presented in Fig. 3. In the control plants, the average chromosome size was 1.62 μm, ranging between 1.06 μm and 2.66 μm, and the mean centromeric index was 31.41. In plants from cryopreserved seeds the average chromosome length was 1.86 μm, varying between 1.15 μm and 2.81 μm, which is significantly greater than the control ($P \leq 0.05$). As a result, the average centromeric index was 35.48 (Fig. 3).

Discussion

The effects of cryo-injury upon the genome of an organism are often unknown. Because of the cryopreservation–tissue-culture–regeneration process, surviving plants may be subject to the effects of somaclonal variation thereby producing several differences in their genotype and phenotype profiles (Harding 2004). If cryopreservation is used to preserve relevant plant germplasm, the determination of genetic stability is fundamental after storage in LN.

Alteration of ploidy level is one of the most

frequent genetic variations in *in vitro* systems (Larkin & Scowcroft 1981). Chromosomal instability is also influenced by the genotype and tissue culture conditions. In this work we studied ploidy levels as well as chromosome size and morphology of *in vitro* plants after 195 days that were derived from cryopreserved seeds and non-frozen seeds respectively. The cells analyzed in the control plants as well as those obtained from cryopreserved seeds showed a stable somatic chromosome number, 2n = 46. The *ex vitro* donor plants showed the same diploid number, which indicates that the ploidy constitution remained stable during the culture–cryoprotection–regeneration process. This is the first report on genetic stability at chromosome level in cryopreserved orchid germplasm.

The cytological information available for *Cyrtopodium* is scarce and in most cases is limited to chromosome counts. The members of the genus analyzed to date are: *C. andersonii* (2n = 46), *C. punctatum* (2n = 46) (Aoyama 1989), *C. eugenii* (2n = 44), *C. gigas* (2n = 46), *C. paranaense* (2n = 46), *C. inaldianum* (2n = 46), *C. intermedium* (2n = 46) and *C. blanchetii* (2n = 92) (Felix & Guerra 2000). Our first chromosome study on *C. hatschbachii* (2n = 46) is in agreement with the available information for *Cyrtopodium*. These records suggest that *Cyrtopodium* has two basic chromosome numbers, x = 22 and x = 23, with a majority of taxa having diploid level. The results obtained here confirm the base number x = 23 for *Cyrtopodium*.

The mean chromosome total length of the cryopreserved-seed plants was significantly greater than those of the control plants ($P \leq 0.05$), which suggest a smaller degree of chromosome condensation in mitotic metaphases of plants from cryopreserved seeds at 195 days (Figs. 2 and 3). There are few reports on chromosome structure of cryopreserved plant germplasm. In *Swietenia macrophylla*, changes in chromatin conformation after recovery from LN were observed (Harding & Millam 2000), possibly due to changes in methylation status of the DNA. In apples, the methylation status of DNA was altered after storage in LN (Hao *et al*. 2001) and the changes were accompanied by an increase in the capacity of cryopreserved shoot tips for rooting. It is probable that a de-methylation of chromatin happens when *C. hatschbachii* seeds are recovered from LN. Evidence suggests that DNA methylation patterns are stable and inherited, resulting in the phenomenon of DNA imprinting (Shemer *et al*. 1996).

Although the DNA methylation status was not examined in this work, it is important to consider it in future investigations.

This work represents the first cytogenetic study in cryopreserved orchids and the first karyotype report for *C. hatschbachii*. The plants obtained from cryopreserved encapsulated-seeds are stable at chromosomal and phenotypic level, making this technique an efficient tool for the *ex situ* conservation of this and related species.

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