

## Karyotypic Stability in Asparagus (*Asparagus officinalis* L.) Cultivars Revealed by rDNA *in situ* Hybridization

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**Summary** Four asparagus cultivars were cytologically studied by conventional staining and *in situ* hybridization, in order to evaluate both the variability among cultivars and the distribution pattern of 18S–25S rRNA genes. The mitotic and meiotic analysis with conventional chromosome staining did not show heteromorphisms or differences between cultivars. *In situ* hybridization with 18S and 25S rDNA probes showed 6 chromosomes with 18S–25S rDNA sites in the 4 studied genotypes. The New Jersey, Waltam Washington and G4×14 cultivars presented 4 chromosomes with larger subterminal sites and 2 with interstitial sites of smaller size. In the Deco cultivar, heteromorphism was verified in the size of the rDNA sites, which were larger and subterminal in 3 chromosomes, and small and interstitial in 3 other chromosomes. The results suggest that asparagus cultivars may present only small differences in the number and distribution of rDNA loci.

Asparagus breeding programs have been stepped up in the last 30 years, bringing the development of a great number of new hybrids and cultivars (Roose and Stone 1999). Cultivar identification has been based mainly on agronomical and morphological characteristics (Geoffriau *et al.* 1992), although this may be hindered by environmental variations. Meanwhile, asparagus cultivars have also been characterized using biochemical or molecular techniques, such as isoenzyme markers (González-Castañón 1999), PCR-based DNA markers (Khandka *et al.* 1996) and restriction patterns of rDNA (Kanno and Kameya 1999).

Germplasm characterization has also been carried out by karyotype analysis based on chromosome size and morphology, number and location of secondary constrictions, heterochromatic bands and specific chromosome sites located by *in situ* hybridization (Forni-Martins and Guerra 1999, Moscone *et al.* 1999). Although such methods have been little explored, karyotype variation has been reported between cultivars of maize (Aguiar-Perecin and Vosa 1985), common beans (Moscone *et al.* 1999) and some others.

The mitotic chromosomes of asparagus ( $2n=20$ ) vary in length from 2.2 to 5.1  $\mu\text{m}$ , displaying metacentric to submetacentric morphology, with 1 to 4 pairs of satellited chromosomes (Löptien 1979). In another species of *Asparagus*, however, a variation in the number of satellited chromosomes between 2 and 6 pairs has been reported (Kar and Sen 1985). Recently, Reamon-Büttner *et al.* (1999) observed six sites of 45S rDNA and 2 sites of 5S rDNA in asparagus by *in situ* hybridization.

In the present work, 4 cultivars of asparagus were cytologically studied by conventional staining and *in situ* hybridization, in order to evaluate both the variability among cultivars and the distribution pattern of 18S–25S rRNA genes.

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## Material and methods

Rhizomes and seeds of 4 asparagus cultivars (Deco, New Jersey, Waltam Washington, G4×14) were obtained from the germplasm bank of Embrapa Semi-Árido, Petrolina, Brazil. The plants were maintained in the Experimental Garden at the Botany Department of the Federal University of Pernambuco, for cytological analysis.

Young root tips were pre-treated with 0.002 M 8-hydroxyquinoline at 4°C for 24 h, fixed in ethanol–acetic acid (3:1) overnight at room temperature and stored at –20°C. Floral buds were fixed directly in ethanol–acetic acid (3:1) for meiotic analysis. For conventional chromosome analysis, Giemsa staining was used (Guerra 1983). The chromosome sizes of 3 to 5 cells of each cultivar were estimated from amplified negative images using a micrometric scale of the same enlargement or by image processing using the Leica QWin program.

For *in situ* hybridization, the probes SK18S and SK25S, containing 18S and 25S rDNA of *Arabidopsis thaliana* were used (Unfried *et al.* 1989, Unfried and Gruendler 1990). They were kindly supplied by Prof. D. Schweizer of the University of Vienna. The *in situ* hybridization procedure was followed as described by Moscone *et al.* (1996). The probes were directly labelled with biotin-11-dUTP by nick translation (Life Technologies), detected using 2 groups of antibodies: anti-biotin produced in mouse (Dakopatts M0743, DAKO) and anti-mouse antibodies (Dakopatts R0270, DAKO) produced in rabbit conjugated with TRITC (tetramethyl-rhodamine isothiocyanate). All preparations were counterstained with 2 µg/ml DAPI (4',6-diamidino-2-phenylindole) and mounted in Vectashield H-1000 (Vector).

The chromosomes were photographed with a DMLB Leica epifluorescence microscope, using T-Max Kodak film ASA 400 for *in situ* hybridized chromosomes and Imagelink Kodak film ASA 25 for conventionally stained chromosomes.

## Results

The material analysed presented  $2n=20$  relatively small chromosomes, varying in size from 1.29 to 2.68 µm. According to the nomenclature of Löptien (1979), they can be classified into 3 groups based on their average size: L chromosomes, 4 longer pairs with  $\approx 2.4$  µm; M chromosomes, 2 medium pairs with  $\approx 1.9$  µm; and S chromosomes, 4 smaller pairs with  $\approx 1.5$  µm. Fig. 1 presents an idiogram with the chromosome size, arm ratio and chromosome classification of the Deco cultivar. The S and M chromosomes were metacentrics, whilst among the L chromosomes 3 pairs were submetacentrics and 1 was metacentric. Although the maximum number of satellites observed was 4 (Fig. 2a), most cells exhibited only 2 satellites, generally with a distended secondary constriction. The meiotic analysis of the Deco cultivar always revealed 10 bivalents (Fig. 2b). Some binucleate tapetal cells exhibited tetraploid nuclei with c.40 chromosomes in each nucleus.

*In situ* hybridization revealed 6 sites of 18S–25S rDNA, located in the L chromosomes of all 4 cultivars studied. The New Jersey, Waltam Washington and G4×14 cultivars presented 2 chromosome pairs with large subterminal sites and 1 chromosome pair with smaller interstitial sites (Figs. 1, 2c). In the Deco cultivar, heteromorphism was verified in the size of the rDNA sites, which were larger and subterminal in 3 chromosomes, and small and interstitial in 3 other chromosomes (Fig. 2d). Tetraploid cells, with  $2n=40$  and up to 8 rDNA sites, were also sometimes found.

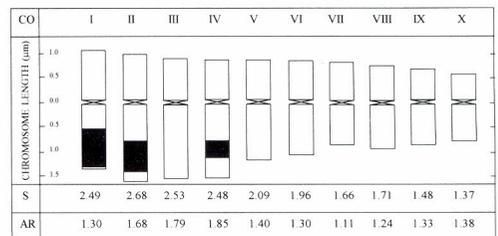


Fig 1. Idiogram of *Asparagus officinalis* Deco cultivar with 18S-25S rDNA sites (black regions) CO, chromosome ordering, S, chromosome size, AR, arm ratio.

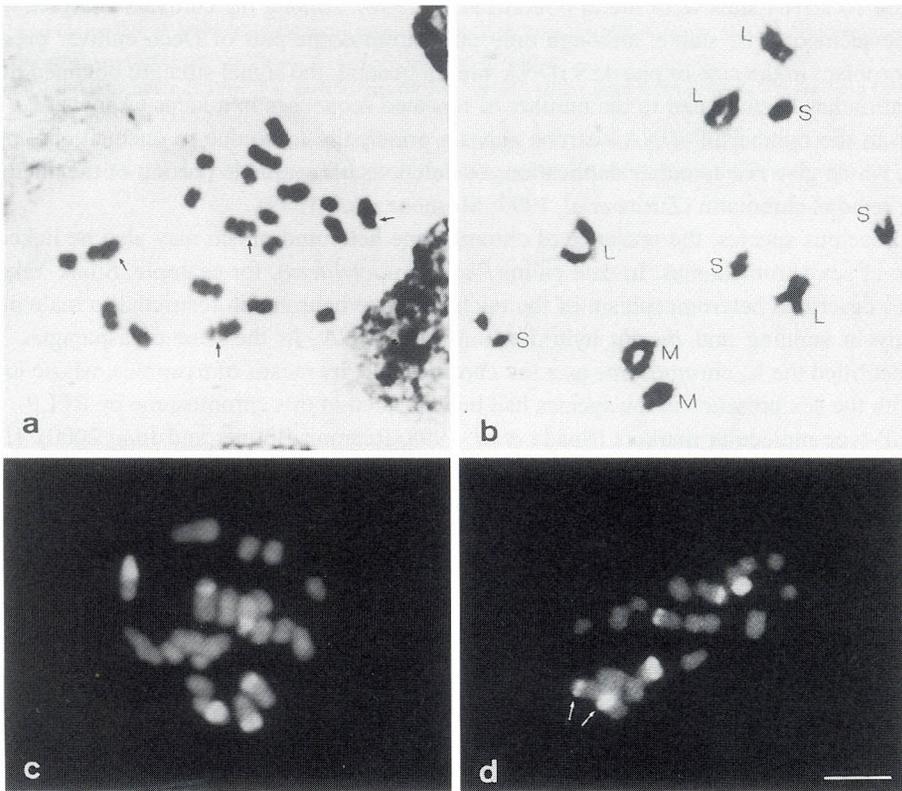


Fig. 2. Mitotic and meiotic cells of *Asparagus officinalis* L. a) Metaphase stained with Giemsa showing satellited chromosomes (arrows), b) diakinesis displaying 10 bivalents indicated by S (smaller), M (medium) and L (longer), c,d) *in situ* hybridization with probes SK 18S-25S showing 6 rDNA sites in New Jersey and Deco cultivars, respectively. Note the signal length heteromorphism in 1 chromosome pair in d (arrows). The bar in d corresponds to 5  $\mu$ m.

### Discussion

The chromosome number, size and morphology observed in the present work on 4 *Asparagus officinalis* cultivars were similar to those reported by Löptien (1979), except for 1 chromosome pair identified as L by the last author and M in the present work. In the cultivars analysed here, the karyotypic formula was always  $4L+2M+4S$ . This kind of variation might be due to an intraspecific polymorphism or due to technical difficulties inherent to small sized chromosomes. Kar and Sen (1985), for example, observed differences in the chromosome size and in the number of secondary constrictions in 4 populations of *A. racemosus* ( $2n=20$ ). The small chromosome size hinders the location of the primary and secondary constrictions of *A. officinalis*, preventing the detection of small variations in karyotypes that might exist between different cultivars. Meiotic analysis did not presented any indication of structural heteromorphisms or alterations in pairing and chromosome segregation. Endopolyploid metaphases were found in the anther tapetum, as they have been described in several other genera.

*In situ* hybridization with 45S rDNA probes showed 6 different loci for rRNA genes. The distribution of some sites coincided with the position of secondary constrictions reported in the present work and by Löptien (1979). The number of secondary constrictions observed was smaller than the number of rDNA sites, as reported in many other species. In *Vigna unguiculata*, for instance, the maximum number of secondary constrictions known was 2, whereas by *in situ* hy-

bridization 10 rDNA sites were found (Guerra *et al.* 1996). Among the cultivars analysed here, the karyotype seemed to be stable, although only one chromosome pair of Deco cultivar presented a heteromorphism in the size of one 45S rDNA site. In general, the signal strength obtained by *in situ* hybridization has been related to the number of repeated sequences in a locus (Appels *et al.* 1980). Variation in the number of rDNA cistrons appears principally to be due to unequal chromatic exchanges, which give rise to either duplications or deletions of a variable portion of the nucleolar organizing regions chromatin (Zurita *et al.* 1997, Moscone *et al.* 1999).

In dioecious species, the presence of chromosome heteromorphism may also be linked to the existence of sex chromosomes. In date palm (*Phoenix dactylifera*), for example, Siljak-Yakovlev *et al.* (1996) described heteromorphism of the nucleolar heterochromatin restricted to male plants by chromomycin staining and *in situ* hybridization with rDNA. In the case of asparagus, Löptien (1979) identified the L<sub>5</sub> chromosome as a sex chromosome, by means of trisomics, whose loci associated with the sex presence in the species had been located in this chromosome by RFLP-, RAPD- and AFLP-type molecular markers (Spada *et al.* 1998, Reamon-Büttner and Jung 2000). However, the heteromorphism observed here in a single plant of Deco cultivar was not detected in individuals from other cultivars and, therefore, it does not seem to be related to the sex determination.

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