CARYOLOGIA Vol. 58, no. 4: 374-379, 2005

Functionality of major and minor 45S rDNA sites in different diploid wild species and varieties of sunflowers

De Paula Wilson B.M., Juca A.B. San-Martin, Priscila Boneventi, José Marcelo D. Torezan and André Luís L. Vanzela *

Laboratório de Biodiversidade e Restauração de Ecossistemas, Departamento de Biologia Geral, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Campus Universitário, 86051-990, Londrina, Paraná, Brazil. Phone and fax number: +55 43 3371 4417.

Abstract — The genus *Helianthus* includes diploid (2n = 2x = 34), tetraploid (2n = 4x = 68) and hexaploid (2n = 6x = 102) species, and genome evolution based on extensive diversity due to free hybridization, introgression and polyploidy. The wild diploid species *H. debilis*, *H. praecox* and *H. nuttallii* showed 2n=34 with four 45S rDNA sites. The cultivated sunflower (*H. annuus*), also diploid, showed six and eight 45S rDNA sites, being six major and two minor terminal rDNA sites. Samples were studied using C-CMA$_3$ and NOR banding to determinate the functionality of minor 45S rDNA sites. Additionally, FISH with 45S and 5S rDNA probes were performed. Results showed that the number and the position of 45S and 5S rDNA sites maintenance in cultivated karyotypes, but provide evidence of a possible increasing in number of rDNA 45S sites when compared with the wild diploid species. Besides, results of C-CMA$_3$ and NOR banding showed that the minor sites are not associated with GC-rich heterochromatin but, even thus, are functional.

Key words: Ag-NOR, C-CMA$_3$ banding, FISH, *Helianthus*, rDNA.

INTRODUCTION

The genus *Helianthus* belongs to the Asteraceae family and includes some 50 native species of North America (Schilling and Heiser 1981), being *H. annuus* L. the most important cultivated species. Chromosome counting show diploid species with 2n = 2x = 34, tetraploid species with 2n = 4x = 68 and hexaploid ones with 2n = 6x = 102 (Jackson and Murray 1983; Chandler *et al.* 1986). *Helianthus annuus* possesses 2n=34, with a karyotype arranged in 13 meta- to submetacentric pairs and four acrocentric ones (Schrader *et al.* 1997). Cuéllar *et al.* (1996, 1999) detected six NOR-bearing chromosomes by FISH and silver nitrate impregnation in *H. annuus* var. *macrocarpus*. However, Schrader *et al.* (1997) and Messina and Biagetti (1999) reported six major terminal NORs, plus two minor terminal NORs. In a subsequent study, Vanzela *et al.* (2002) found diploid wild species of *Helianthus* with four 45S rDNA sites and diploid *H. annuus* with six and eight hybridization signals, two of them being minor ones. Besides, these authors found eight terminal sites in the tetraploid *H. simulans* and 12 sites in the hexaploids *H. tuberosus*, *H. laetiflorus* and *H. pauciflorus*.

Ribosomal genes have been considered excellent chromosome markers because they are organized in hundreds to thousands tandem repetitions, occupying one or more chromosome regions per haploid complement, which can vary intra- and interspecies in number, location and size. Minor rDNA sites have been described in few plant species, and have been sometimes associated to nonfunctional genes or pseudogenes. A very illustrative example is that of *Hordeum vulgare*, which presents several minor rDNA sites (Pedersen and Linde-Laursen 1994). However, it is widely accepted that when rRNA genes are functional in interphase, these regions are responsible for nucleolus formation, with size and number of nucleoli varying according to activation level and tissue metabolism. Thus, when these NORs are transcriptionally active in the preceding interphase, they appear as secondary constrictions on the subsequent metaphase chromosomes (Jiménez *et al.* 1988). Similarly, the maximum number of nucleoli found in cells can represent the maximum number of functional rDNA sites of

* Corresponding author: e-mail: andrevanzela@uel.br.

an organism. But, when two species are hybridized, genes of one complement can remain functional while those of the other one may be inactivated (PONTES *et al.* 2003). Thus, the silencing could happen due to combination of two genomes in the hybrids, associated with some change in the rDNA region such as extensive rDNA methylation (VIEIRA *et al.* 1990) or controlled by methylation in the intergenic spacers (SAKOWICZ and OLSZEWSKA 1997). The genome evolution in the genus *Helianthus* is associated with an extensive diversity, due to free hybridization among species, besides introgression and polyploidy (HEISER 1965; DORADO *et al.* 1992; RIESEBERG *et al.* 2000). Thus, the present study was carried out to investigate the functionality of major and minor 45S rDNA sites in three wild species of *Helianthus* and in seven varieties of *H. annuus* and additionally, to determine the association between rDNA functional sites to GC-rich heterochromatin segments.

MATERIALS AND METHODS

Samples of wild species and varieties of sunflowers tested for NOR banding, C-CMA$_3$ banding and FISH, were obtained from the germplasm bank of Empresa Brasileira de Pesquisa Agropecuária, (Embrapa Soja), Londrina-PR, Brazil (Table 1). For chromosome preparations root tips were pretreated with 2mM 8-hydroxyquinoline for 24 h, fixed in ethanol: acetic acid (3:1, v:v) for 24 h, and stored at –20^{0}C or immediately used. Root tips were washed in distilled water, softened in 4% cellulase plus 40% pectinase at 37^{0}C for 1-2 hr, and squashed in a drop of 45% acetic acid. Slides were frozen in liquid nitrogen for cover slip removing. Preparations were immediately used for NOR banding or FISH with rDNA probes, or aged for later C-CMA$_3$ banding.

For NOR banding, slides were air-dried and immediately treated with 50% $AgNO_3$ at 60ºC in a moist chamber (HOWELL and BLACK 1980, with modifications). Afterward, material was washed in distilled water, air-dried, and mounted in Entellan. Nucleolus counting was carried out in slides of at least five different individuals of each variety, and only non-overlapping cells were considered. Photographs were taken with Kodak Proimage color film 100 ISO.

As FISH with the 45S rDNA probe had been previously done in *H. debilis*, *H. praecox* and *H. nuttallii* (VANZELA *et al.* 2002), the FISH was carried out only for the *Helianthus annuus* varieties. The p*Ta*71 probe containing 45S rDNA and p*Ta*794 probe containing 5S rDNA sequences, both isolated from wheat (GERLACH and BEDBROOK 1979; GERLACH and DYER 1980), were used for FISH. Probes were labeled with biotin-14-dATP by nick translation. Material was incubated in RNase (100µg/ml), post-fixed in 4% (w/v) paraformaldehyde, dehydrated in a 70%-100% graded ethanol series and air-dried. The probe (20-100ng) mixture was denatured at 70^{0}C for 10 min and immediately chilled on ice. Chromosome denaturation/hybridization was done at 90^{0}C for 10 min, 50^{0}C for 10 min and 38^{0}C for 5min using a thermal cycler (MJ Research), and kept at 37^{0}C overnight in a moist chamber. Post-hybridization washes were performed at 80% stringency in 2xSSC, 0.1xSSC/20% formamide, 0.1xSSC, 2xSSC, and 4xSSC/0.2% Tween 20, at 42ºC for 5 min each. Biotin-labeled probe was detected with avidin FITC-conjugate, and slides were mounted in a solution composed of 50% Antifade (Vector Labs), 50% glycerol in McIlvaine buffer pH 7.0 and 4% propidium iodide (2.5µg/mL^{-1}). Photographs were taken with Kodak Proimage color film 100 ISO.

For C-CMA$_3$ banding, preparations were treated according to SCHWARZACHER *et al.* (1980),

Table 1 — Wild species and varieties of sunflowers studied and frequency of nucleoli in the root tips cells.

Species/ Varieties	2n	Scored	Frequency of cells with different number of nucleoli (%)							
			1	2	3	4	5	6	7	8
H. praecox	34	547	31.44	41.86	20.66	6.03	-	-	-	-
H. debilis	34	1121	37.20	34.79	20.79	7.22	-	-	-	-
H. nuttallii	34	239	37.24	33.05	20.50	9.20	-	-	-	-
HA 413	34	1881	44.92	22.00	18.71	8.61	2.55	0.95	1.22	1.01
RHA 274	34	2211	58.75	21.80	11.53	4.38	2.30	0.76	0.31	0.13
RHA 282	34	1769	63.82	21.93	11.13	2.26	0.39	0.11	0.22	0.11
RHA 364	34	2065	54.28	24.40	13.22	5.03	1.98	0.63	0.14	0.29
RHA 396	34	872	60.21	23.51	11.81	2.98	0.69	0.23	0.23	0.34
HACMS 413	34	1011	41.54	28.68	22.55	6.53	0.29	0.10	0.19	0.10
RHA 345	34	1064	35.71	34.30	21.24	7.89	0.65	0	0.18	0

with modifications. Slides were incubated in 45% acetic acid at 60ºC for 10 min, 5% $Ba(OH)_2$ at 25ºC for 10 min and 2xSSC pH 7.0 at 60ºC for 1h and 30 min, and air-dried. After three days, samples were stained with a drop of 0.5mg/ml CMA_3 in McIlvaine buffer, pH 7.0, and distilled water (1:1), containing 2.5mM $MgCl_2$ for 1h and 30 min and mounted in a solution of glycerol:McIlvaine buffer, pH 7.0. After three days, slides were observed and photographed with Kodak Tmax 100 ISO.

RESULTS AND DISCUSSION

The three wild species of *Helianthus* (*H. debilis*, *H. praecox* and *H. nuttallii*) and the seven varieties of *Helianthus annuus*, showed a chromosome number of 2n=34 and a karyotype composed mostly by meta-submetacentric chromosomes and few acrocentric pairs (Table 1), in agreement with JACKSON and MURRAY (1983), CHANDLER *et al.* (1986) and SCHRADER *et al.* (1997). Great differences in karyotype and chromosome morphology among the varieties were not observed, indicating that there were no visible structural rearrangements through conventional analysis. FISH with the 45S rDNA probe located eight terminal hybridization signals on the short arms of acro- and submetacentric pairs in every varieties (Figure 1B), of which six are major and two are minor sites, similar to that reported by SCHRADER *et al.* (1997), MESSINA and BIAGETTI (1999) and VANZELA *et al.* (2002). However, these last authors showed only four hybridization signals in *H. debilis*, *H. praecox* and *H. nuttallii*, besides six hybridization signals in a variety of *H. annuus.* No interstitial hybridization signal with 45S rDNA probe was located in the species and nor in the varieties. On the other hand, FISH with 5S rDNA probe realized only in the *H. annuus* varieties, showed hybridizations signals always in the proximal region of the long arm of a NOR-bearing submetacentric pair and a non NOR-bearing metacentric pair (Figure 1C). In addition, VANZELA *et al.* (2002), using FISH with 45S rDNA and subsequent C-CMA_3 banding in some *Helianthus* species, showed that all four 45S rDNA hybridization sites are also associated to GC-rich heterochromatin. However, the C-CMA_3 banding performed in the present study demonstrated six instead of eight GC-rich terminal blocks, as expected for the occurrence of rDNA terminal regions (Figure 1A). Besides of a small pericentromeric GC-rich block in the NOR-bearing submetacentric pair close to the 5S rDNA sites. Despite that, some authors suggest that nucleolus-associated heterochromatin can be responsible for low rDNA expression (SAKOWICZ and OLSZEWSKA 1997), functional NOR associated with heterochromatin seems to be very common in most plant and animal genomes, which is usually associated by both C-banding and CMA_3-banding. However, in some cases, NORs cannot react positively after C-banding or fluorochrome staining (GUERRA 2000). This could explain the fact of the minor 45S rDNA sites have not been evidenced by C-CMA_3-banding.

When ribosomal genes are active in the previous interphase they can appear more decondensed and visualized as satellites or secondary constriction in the subsequent metaphases. In fact, secondary constrictions of *H. annuus* varieties were always observed in the terminal regions of the short arms of acro- and submetacentric chromosomes, as previously established with conventional staining (not presented here) and FISH. However, the number of visible satellites varied among samples and it was never more than six (see the NOR banding at Figure 1D). Absence of metaphases with seven and/or eight satellites or NORs could be explained for at least three ways: (i) the size of the minor 45S rDNA sites is very reduced to generate visible satellites; (ii) differences in chromosome condensation could reduce visualization under conventional staining; and (iii) these minor sites could not be functional in preceding interphase nuclei. Thus, based on the assumption that the maximum number of nucleoli per nucleus should correspond to the number of rRNA-synthesizing sites, the functionality of minor 45S rDNA sites was tested for silver nitrate impregnation. Number of nucleolus was determined to the seven varieties and compared to the three wild species, each with four 45S rDNA sites. *Helianthus praecox*, *H. debilis* and *H. nuttallii*, showed a maximum number of four nucleoli (Table 1), corresponding to the expected number of four 45S rDNA sites previously described by VANZELA *et al.* (2002). The seven varieties of *H. annuus* showed a maximum number of eight nucleoli (Figures 1E-1K), also corresponding to the number of 45S rDNA sites, as described above (Table 1).

Even silver nitrate staining confirms that minor blocks are potentially active, this technique does not allow the quantification and comparison of functionality between major and minor 45S rDNA sites. Our finding of a low frequency of cells with eight nucleoli (Table 1) does not mean that such regions are functional in just a few cells.

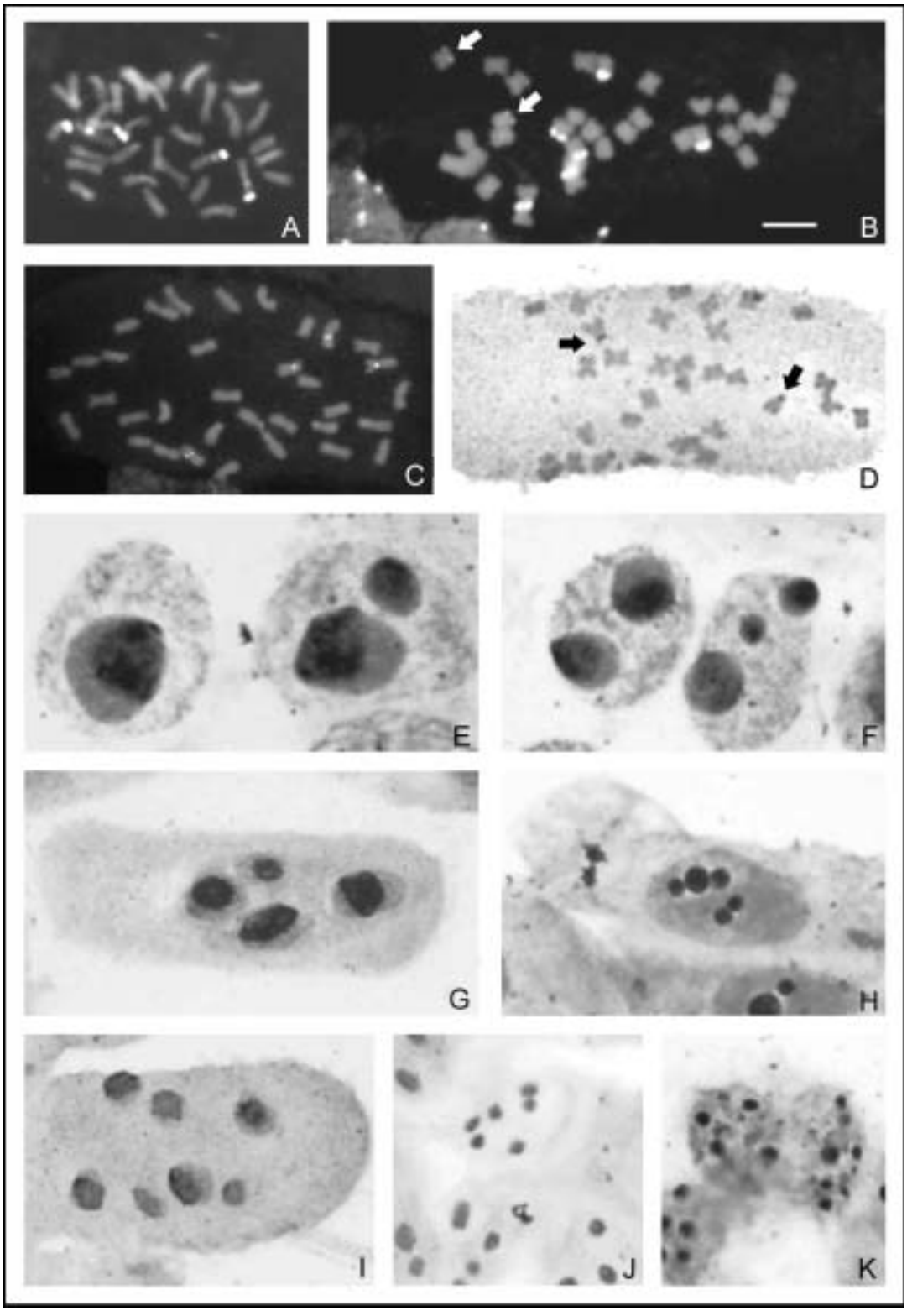

Fig. 1 — **A**) Metaphase C-CMA_3 banded of RHA 364 variety. **B**) FISH with 45S rDNA probe of HACMS 413. Arrows point to minor rDNA sites. **C**) FISH with 5S rDNA probe of HACMS 413. See intercalary sites. **D**) NOR banding in RHA 274 variety. Arrows point to NORs silver nitrate impregnated. **E-K**) Nucleoli numbers in the RHA 364 variety. One and two larger nucleoli **(E)**. Two and three nucleoli (**F**). Four nucleoli (**G**). Five nucleoli (**H**). Seven nucleoli (**I**). Six nucleoli (**J**). Eight nuclei (**K**). Bar = 5μm.

These segments may fuse to others to form one or few nucleoli. In other words, nucleoli number and size per cell can be varying according to the association rate during interphase, depending on each genome. Samples of *Helianthus* showed a tendency to possess a larger nucleolus or several smaller nucleoli, and intermediary forms as well (Figures 1E-1K). *Helianthus annuus* varieties exhibited the major 45S rDNA sites associated to GC-rich heterochromatin, as most of the organisms; however, in the minor 45S rDNA sites the GC-rich association was absent, what suggests that NOR activity does not depend completely of association with GC-rich repetitive DNA.

When karyotypes of *Helianthus* are analyzed conventionally, they indicate certain stability in chromosome size and shape. But, comparative genetic mapping studies in natural hybrids show a strong rearrangement incidence in the speciation process, as chromosomal breakages, fusions and duplications. Results of FISH with rDNA probes obtained here compared to those in the literature (Schrader *et al.* 1997; Messina and Biagetti 1999; Cuéllar *et al.* 1996, 1999; Vanzela *et al.* 2002) point out differences on the 45S rDNA sites number in diploid *Helianthus*, as expected for a group with intense genome changes (Rieseberg *et al.* 2000). Our results allow some suppositions: (i) the smallest chromosome number (2n=34) found in *Helianthus*, assumed here and in others studies as a diploid, could be polyploid, in accordance with the basic numbers x=4, 5 and 6 proposed for most Asteraceae; (ii) if the karyotypical evolution of *Helianthus* really involves frequent hybridization and introgression, as proposed by several authors (Rieseberg *et al.* 2000), these events probably did not affect the rDNA sites activity; (iii) there is probably an increase in number of 45S rDNA sites in sunflower varieties, with relationship to the wild species (Vanzela *et al.* 2002), based on the occurrence of wild karyotype with 2n=34 and four 45S rDNA sites, 2n=68 and eight sites, and 2n=102 with twelve rDNA sites. Thus, it is possible to propose that the 45S rDNA sites might have been amplified in a favorable way in *H. annuus* varieties, possibly due to the processes of genetic improvement, since stable numbers were observed in the wild species.

Acknowledgments — The authors are grateful to the Brazilian Agencies Fundação Araucária and CNPq for financial support, and Dr. Albert Leyva for the attendance with English.

REFERENCES

Chandler J.M., Jan C.C. and Beard B.H., 1986 — *Chromosomal differentiation among the annual Helianthus species*. Syst. Bot., 11: 354-371.

Cuéllar T., Belhassen E., Fernández-Calvín B., Orellana J. and Bella J.L., 1996 — *Chromosomal differentiation in Helianthus annuus var. macrocarpus: heterochromatin characterization and rDNA location*. Heredity, 76: 586-591.

Cuéllar T., Orellana J., Belhassen E. and Bella J.L., 1999 — *Chromosomal characterization and physical mapping of the 5S and the 18S-5.8S-25S ribosomal DNA in Helianthus argophyllus, with new data from Helianthus annuus*. Genome, 42: 110-115.

Dorado O., Rieseberg L.H. and Arias D., 1992 — *Chloroplast DNA introgression in Southern California Sunflowers*. Evolution, 46: 566-572.

Gerlach W.L. and Bedbrook J.R., 1979 — *Cloning and characterization of ribosomal RNA genes from wheat and barley*. Nucleic Acids Res., 7: 1869-1885.

Gerlach W.L. and Dyer T.A., 1980 — *Sequence organization of the repeting units in the nucleus of wheat which contain 5S rDNA genes*. Nucleic Acids Res., 8: 4851-4865.

Guerra M., 2000 — *Patterns of heterochromatin distribution in plant chromosomes*. Genet. Mol. Biol., 24: 1029-1041.

Heiser C.B. Jr., 1965 — *Species crosses in Helianthus. III. Delimitation of sections*. Ann. Missouri Bot. Gard., 52: 364-370.

Howell W.M. and Black D.A., 1980 — *Controlled silver staining of nucleolus organizer regions with a protective colloidal developer; a one-step method*. Experientia, 36: 1014-1015.

Jackson R.C. and Murray B.G., 1983 — *Colchicine induced quadrivalent formation in the Helianthus: Evidence of ancient polyploidy*. Theor. Appl. Genet., 64: 219-222.

Jiménez R., Burgos M. and Diaz de la Guardia R., 1988 — *A study of the Ag-staining significance in mitotic NOR's*. Heredity, 60: 125-127.

Messina R. and Biagetti M., 1999 — *Chromosomal localization of rDNA in sunflower (Helianthus annuus L.) by fluorescent in situ hybridization.* J. Genet. Breed., 53: 259-261.

Pedersen C. and Linde-Laursen I., 1994 — *Chromosomal locations of four minor rDNA loci and a marker microsatellite sequence in barley*. Chrom. Res., 2: 65-71.

Pontes O., Lawrence R.J., Neves N., Silva M., Lee J.H., Chen Z.J., Viegas W. and Pikaard C.S., 2003 — *Natural variation in nucleolar dominance reveals the relationship between nucleolus organizer chromatin topology and RNA transcription in Arabidopsis*. Proc. Nat. Acad. Sci., 100: 11418-11423.

Rieseberg L.H., Baird S.J.E. and Gardner K.A., 2000 — *Hybridization, introgression, and linkage evolution*. Plant Mol. Biol., 42: 205-224.

SAKOWICZ T. and OLSZEWSKA M.J., 1997 — *DNA content, interphase AgNOR-area, number of HrDNA hybridization signals and the methylation level in coding rDNA sequence in different organs of Lupinus luteus L.* Genetica, 99: 67-72.

SCHILLING E.E. and HEISER C.B.JR., 1981 — *Infrageneric classification of Helianthus (Compositae).* Taxon, 30: 393-403.

SCHRADER O., AHNE R., FUCHUS J. and SCHUBERT I., 1997 — *Karyotype analysis of Helianthus annuus using Giemsa banding and fluorescence in situ hybridization*. Chrom. Res., 5: 451-456.

SCHWARZACHER T., AMBROS P. and SCHWEIZER D., 1980 — *Application of Giemsa banding to orchid karyotype analysis*. Pl. Syst. Evol., 134: 293-297.

VANZELA A.L.L., RUAS C.F., OLIVEIRA M.F. and RUAS P.M., 2002 — *Characterization of diploid, tetraploid and hexaploid Helianthus species by chromosome banding and FISH with 45S rDNA probe*. Genetica, 114: 105-111.

VIEIRA R., QUEIROZ A., MORAIS L., BARAO A., MELLO-SAMPAYO T. and VIEGAS W.S., 1990 — *1R chromosome nucleolus organizer region activation by 5-azacytidine in wheat x rye hybrids*. Genome, 33: 707-712.

Received IV.08.2005; accepted IX.06.2005