Saponaria officinalis Karyology and Karyotype by Means of Image Analyzer and Atomic Force Microscopy

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KEY WORDS *Saponaria officinalis*; karyology; karyotype; flow cytometry; chromosomal volumes; atomic force microscopy

ABSTRACT The aim of this work was to offer a contribution to the characterization of taxonomic entity of *Saponaria officinalis* ($2n = 28$; an herbaceous perennial species; saporin, a type 1 Ribosome Inactivating Protein, is present in leaves and seeds) by a cytogenetic and karyomorphological approach. We investigated the karyotype's morphometry correlated with Stebbin's symmetric index; the same information has been used for computing the indices resemblance between chromosomes (REC), symmetric indices (SYI), and total form (TF%) which allow the comparison between species and evaluation of karyological evolution. Fluorescence intensities of the stained nuclei were measured by a flow cytometer and, for the first time, values for nuclear DNA content were estimated by comparing nuclei fluorescence intensities of the test population with those of appropriate internal DNA standards. Our study is also aimed to introduce chromosomal volumes, which were determined by atomic force microscopy (AFM), as novel karyomorphological parameter which could allow for chromosome discrimination especially when tiny ones are present. *Microsc. Res. Tech. 71:730–736, 2008.*

INTRODUCTION

From a cytogenetical point of view, *Saponaria officinalis* L. has not been the subject of many studies because of its lack of perceived commercial value and the extremely small size of its chromosomes. *Saponaria officinalis* belongs to the *Caryophyllaceae* family. It is a perennial, herbaceous plant, with a dark reddish crawling branched rhizome. The trunks are ascendant, some times woody at the base. *Caryophyllaceae* are found everywhere, but prefer temperate and cold climates. *Saponaria officinalis*' habitat is heterogeneous varying from ditches to meadows, from shady humid places to rocky ones and it grows on mountains up to 1,200 m elevation. It grows spontaneously along the margins of the streets, in the hedges, and close to the water. The scientific interest in this plant is mainly because of its production of saporins, enzymes better known with the name of RIPs, ribosome-inactivating proteins. The main activity of RIPs is to irreversibly damage the ribosomes; in particular, the major subunit that is no longer capable to bind the elongating factors, with subsequent arrest of the proteins synthesis (Stirpe, 2004). The karyotype of this species has not yet been studied. In this work, an image analysis system was applied to *Saponaria officinalis* karyotype to allow a chromosome classification. Because of the homogeneus morphology of chromosomes in the whole complement of *Saponaria officinalis*, it is important to define some symmetric indexes (Levitzky, 1931) that allow the characterization of the phyletic relations between *Saponaria officinalis* and other species of the same genus that produce saporines. The evolution of the karyotype of the *Caryophyllaceae* will be useful to delineate the evolutionary history of the genes that codify the saporines. The karyomorphometric parameters and the indexes TF% (Huziwara, 1962) resemblance between chromosomes (REC) and symmetric indices (SYI) (Greihuber and Speta, 1976) were determined. On the basis of these parameters and the Stebbins classifications (Stebbins, 1971), it should be possible to shade some light on the karyotype evolution of the species, within the genus (Venora et al., 2000). Accurate estimations of nuclear genome sizes are important for mapping of plant genomes and development of strategies for isolation of plant genes (Arumuganathann and Earle, 1991). To evaluate the nuclear DNA content in *Saponaria officinalis* fluorescence intensities of the stained nuclei were measured by a flow cytometer, values for nuclear DNA content were estimated by comparing nuclei fluorescence intensities of the test population with those of appropriate internal DNA standards. In this study following successful work on high-resolution imaging of chromosomes (reviewed in Ohtani et al., 2002; Thalhammer and Heckl, 2004), the volumes of complete metaphase sets of *Saponaria officinalis* have been calculated from three-dimensional atomic force microscopy data. This study demonstrates a novel application of atomic force microscopy as a high-resolution measuring instrument for analyzing

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Received 20 November 2007; accepted in revised form 16 April 2008
Contract grant sponsor: RIA grants of University of L'Aquila.
DOI 10.1002/jemt.20613
Published online 10 July 2008 in Wiley InterScience (www.interscience.wiley.com).

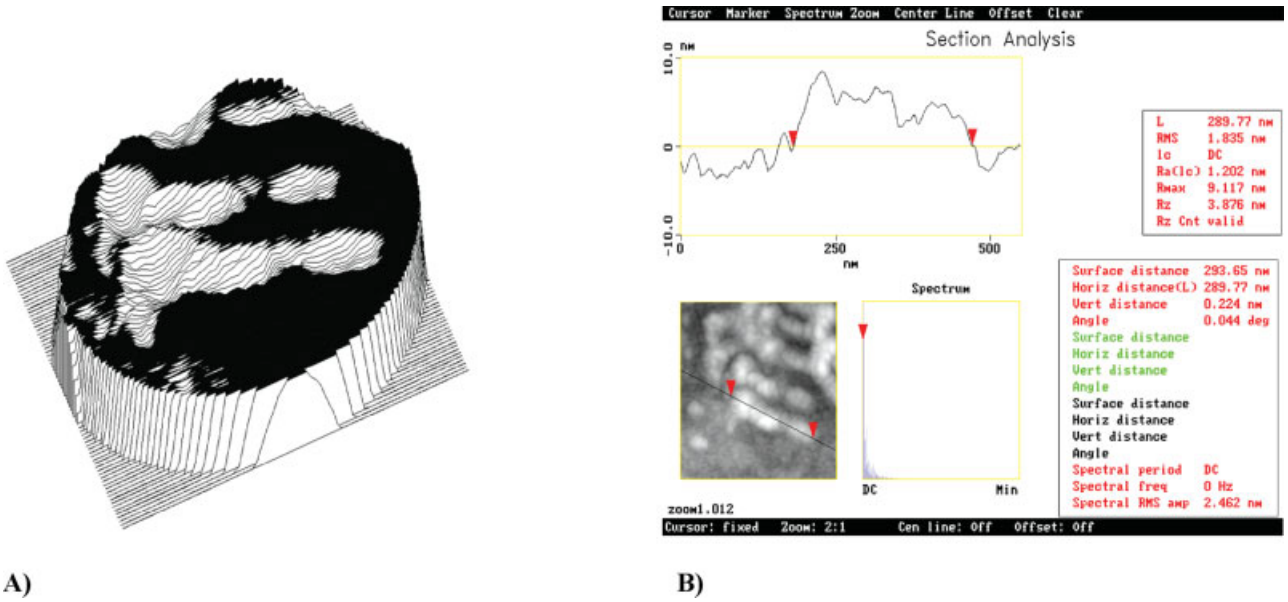

Fig. 1. Volume calculation. (**A**) Selected area at the base of the chromosome in elaboration. (**B**) Section Analysis of the profile of the heights of the selected chromosome. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

volumes of biological structures (Lee et al., 1997). This approach will permit chromosomes identification without the use of destructive cytochemical treatments (Winfield et al., 1995).

MATERIALS AND METHODS

Karyomorphometry

For the karyotype analysis root tips were obtained from *Saponaria officinalis* hydroponic seedlings; mitotic metaphase chromosomes were obtained by the squash method as described in previous works (Conicella and Errico, 1990; Khokhar et al., 1989). In brief, root meristems were pretreated with a 0.1% colchicine aqueous solution for 3 h at room temperature followed by an overnight fixation in 3:1 ethanol-acetic acid (v:v). The roots were hydrolyzed in a solution of 1N HCl for 10 min at 60°C and stained with Carmineo-acetic with 10% of 1N HCl. Root tips meristems were squashed under a coverslip in a drop of 45% acetic acid. Microscopic investigation was carried out with a Zeiss Axioplan 2 microscope (Carl Zeiss Jena GmbH, Jena, Federal Republic of Germany) connected to a KS400 Zeiss image analysis system, with the dedicated karyotyping Software Ikaros 3.4 (Metasystem GmbH, Altlussheim, Federal Republic of Germany). The automated image analysis system allowed us to obtain karyomorphometric data (total lengths of short arms, long arms, and satellites) with a high degree of accuracy, measurements being taken directly in the first image of the chromosomes (Venora et al., 1991). The classification of Stebbins (1971) and the karyotype symmetry of *Saponaria officinalis* with Rec, Syi (Greilhuber and Speta, 1976), and TF% (Huziwara, 1962) indices were determined.

Nuclear DNA Content by Flow Cytometry

The nuclei were isolated from 30 mg of *Saponaria officinalis* leaf tissue, obtained from young leaves deprived of the nerving. The tissue was chopped inside a glass Petri dish in 1 mL of cold lysis buffer (15 mM TRIS, 2 mM Na_2EDTA, 0.5 mM Spermine, 80 mM KCl, 20 mM NaCl, 0.1% Triton X-100 dissolved in deionized water, adjusted to pH to 7.5 using 1N NaOH; the solution was filtered with a 0.22 μm filter to eliminate unsolubilized particles, and then 220 μL of mercaptoethanol with a final concentration of 15 mM were added; Dolezel et al., 1989) together with 75 μg/mL PI and an equal amount of RNase. The sample was filtrated with nylon net with ϕ 21 μm pores and incubated for 30 min in polystyrene test tubes at room temperature before the analysis. The internal standard, *Zea mays*, was added before the coloration, during the chopping phase, in order to obtain fluorescent signals so the standard and the sample could be compared (Dolezel et al., 1998).

Chromosome Preparation for Atomic Force Microscopy Investigation

The roots tips of *Saponaria officinalis* were placed in distilled water at 0°C for 24 h (Liu et al., 2003) and then fixed with ethanol/acetic acid glacial 3:1 at 4°C for 24 h (Martin et al., 1996). Root tips were washed with Citrate buffer (Citric acid 100 mM and tri-sodium citrate 100 mM, pH 4.8) to remove fixative for 10 min. After washing, 1-mm long root tips were cut off and digested with enzyme solution (2% Cellulase Onozuka R-10 from Trichoderma viride, Merck, 1% Pectinase from Rhizopus sp, Fluka BioChemika, 7.5 mM KCl and 7.5 mM EDTA, pH 4.0) at 37°C for 60 min. The meristem cells were collected and resuspended in distilled water for 30 min and placed again in Citrate buffer. Root tips and meristems were squashed under coverslips in a drop of 30% acetic acid (Sugiyama et al., 2004). The sample was washed with distilled water, heated above a warm plate and dehydrated in a series of Etha-

nol (50–70–80–90–95%), and then placed in a drier (with salts), for at least 12 h, before being observed.

Volume Calculation

The atomic force microscopy (AFM) images were collected with a Nanoscope III (Digital Instruments). The scanning was performed in tapping mode and the scan speeds were typically ~1 Hz. The elaboration of the images was made with Digital Instruments software and with SXM image, a program for image processing, free shareware from the NIH (National Institutes of Health, USA; free download at http://www.liv.ac.uk/~sdb/ImageSXM/#what). In a three-dimensional visual field of chromosomes spreads (see Fig. 1) the area (Fig. 1A) was selected at the base of the chromosome which was determined by analyzing the heights profile as shown in Figure 1B. We calculate the volume of the chromosomes by multiplication the average of the chromosome heights (nm) (Fig. 1B) with the selected area at the base of the chromosome (nm^2).

RESULTS AND DISCUSSION

Optical Microscope Karyology

A metaphase plate of *Saponaria officinalis* at the transmission light microscope is shown in Figure 2 where the somatic chromosome number $2n = 28$ was observed; the general characteristics of the chromosomes are summarized in Table 1 and ideograms are shown in Figure 3. In *Saponaria officinalis*, individual chromosome length ranged from 0.77 to 1.35 μm. Twelve pairs were metacentric and two were submetacentric, according to the classification of Levan (Levan et al., 1964). The haploid chromatin length was 14.91 μm. No satellites were shown. The indices REC, SYI, and TF% and the Stebbins' categories are summarized in Table 2. The application of an image analyzer allows a detailed karyotyping of the *Saponaria officinalis* where the chromosome number is known (Garbari et al., 1973).

Flow Cytometry

The cytofluorimetric analysis shows the Height (Fig. 4A) and Area (Fig. 4B) related to the fluorescent emission of *Saponaria officinalis* nuclei. These derive from the measurement of the maximum instantaneous value of fluorescent intensity emitted by a nucleus (Height) and from the total evaluation of the fluorescent emission, from the moment when the nucleus passes through the laser beam (minimum excitation). The integral measurement of the emitted fluorescence corresponds to the Area; the ploidy and the DNA content are measured with the Area. If there are no noticeable morphologic alterations, the two values should coincide. In Figure 5 is presented a dot plot of a suspension of nuclei isolated from *Saponaria officinalis* and *Zea mays*. On the *x*-axis is shown the integral intensity of the fluorescent signal emitted by the nuclei, fluorescence pulse area (FPA). The measure of their size is on the *y*-axis, evaluated as the duration of the fluorescent signal fluorescence pulse width (FPW) emitted by nuclei during their passage through the area where they are irradiated with the excitation laser. This measurement was done with a pulse processing module (PPM) to measure, the area together with the height of the signal. It is therefore possible to measure the total amount of fluorescence emitted from the nuclei (FPA)

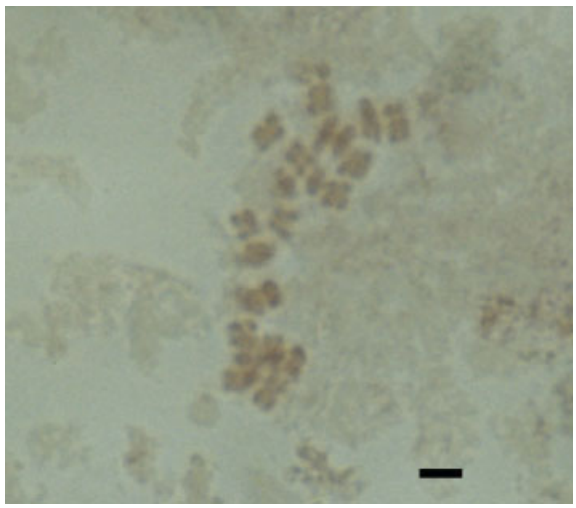

Fig. 2. *Saponaria officinalis* metaphase spread stained with Carmineo-acetic. Bar = 1 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE 1. Chromosome morphometric data of Saponaria officinalis

Chromosome number	Relative length (%)	Long arm (μm)	Short arm (μm)	Total length (μm)	Arm ratio	REC
1	9.05 ± 0.08	0.75 ± 0.02	0.60 ± 0.02	1.35 ± 0.03	1.25	
2	8.92 ± 0.10	0.67 ± 0.02	0.66 ± 0.03	1.33 ± 0.02	1.02	98.52
3	8.45 ± 0.06	0.68 ± 0.03	0.58 ± 0.03	1.26 ± 0.03	1.17	93.33
4	7.91 ± 0.08	0.70 ± 0.02	0.48 ± 0.02	1.18 ± 0.02	1.46	87.41
5	7.65 ± 0.05	0.58 ± 0.02	0.56 ± 0.02	1.14 ± 0.02	1.04	84.44
6	7.38 ± 0.06	0.70 ± 0.02	0.40 ± 0.02	1.10 ± 0.02	1.75	81.48
7	7.04 ± 0.06	0.58 ± 0.03	0.47 ± 0.02	1.05 ± 0.02	1.23	77.78
8	6.84 ± 0.08	0.62 ± 0.03	0.40 ± 0.03	1.02 ± 0.03	1.55	75.56
9	6.77 ± 0.06	0.53 ± 0.02	0.48 ± 0.02	1.01 ± 0.02	1.10	74.81
10	6.44 ± 0.05	0.51 ± 0.03	0.45 ± 0.02	0.96 ± 0.02	1.13	71.11
11	6.24 ± 0.05	0.60 ± 0.03	0.33 ± 0.02	0.93 ± 0.02	1.82	68.89
12	6.17 ± 0.03	0.50 ± 0.02	0.42 ± 0.02	0.92 ± 0.02	1.19	68.15
13	5.97 ± 0.03	0.45 ± 0.02	0.44 ± 0.03	0.89 ± 0.02	1.02	65.93
14	5.16 ± 0.02	0.43 ± 0.02	0.34 ± 0.02	0.77 ± 0.02	1.26	57.04
Total length (μm) 14.91 ± 0.16	σ_x	0.47 ± 0.02	0.47 ± 0.02			

REC, Resemblance between chromosomes.

and the duration of the emission (FPW). Through the sorting it was possible to verify that the signals produced by the nuclei of *Saponaria officinalis* were gathered in R1, and that in R2 were present the signals derived from the *Zea mays* standard nuclei. Since the content of DNA in *Zea mays* is 5.46 pg DNA/nucleus it was possible to establish the DNA content in *Saponaria officinalis* as 4.65 pg DNA/nucleus.

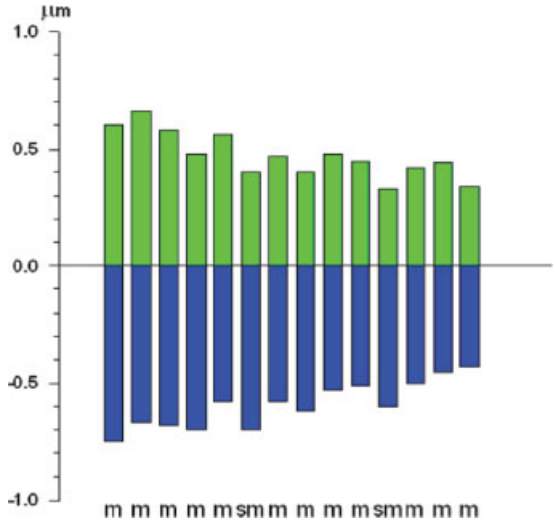

Fig. 3. Idiogrammatic representation of the haploid karyotype of *Saponaria officinalis*. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE 2. Indexes of symmetry, REC (Resemblance between Chromosomes), SYI (Symmetric Indices), TF% (Total Form), and Stebbin's category

Stebbin's CAT	REC	SYI	TF%
1A	62.56	79.64	44.33

AFM Karyology

A typical AFM metaphasic expansion is shown in Figures 6 and 7. Roots tips chromosomes of *Saponaria officinalis* are localized in an area of 5 μm^2. In barley they develop up to 100 μm^2, in corn up to 40 μm^2 (McMaster et al., 1996). The metaphase chromosomes present two important requirements that allow the measurement of their volume: they are separated from each other; therefore, they do not overlap and present few cellular debris and cytoplasmic material that could jeopardize accurate measurement. In Table 3, the chromosome volumes measures determined from the analysis of 10 metaphasic plates and their relative Arm Ratio (L/s) are shown. Applying this technique to the classification of *Saponaria* chromosomes, it is important to underline a dimensional coherence. There is a discrete overlapping of different measure classes because of the high degree of symmetry of the chromosomes. The individual identification of a chromosome is therefore validated only with a high number of samples. The isolation of a chromosomic suspension could further improve the quality of the images obtained, and therefore make the statistic validation more reliable to the detection of possible structural modifications of the sample due to the different preparation procedure used. The measure of the volumes obtained were coherent with the ones determined measuring the Arm ratio and the lengths. Previous studies have demonstrated that dehydratation technique of preparing samples is associated with a variable amount of specimen shrinkage (Kozubek et al., 2000). The shrinkage represents a miniaturization of the sample in which all structural components retain their usual relationships. In order to evaluate chromosome shrinkage we compare the length measures of the chromosomes obtained by the optical microscope with those ones by AFM. The chromosomes lengths determined by optical microscopy range from 0.80 to 1.35 µm while those ones as revealed by the AFM vary from 0.30 to 0.80 µm. We can evaluate then that alcohol dehydratation can shrink structures by as much as 50 to 60%.

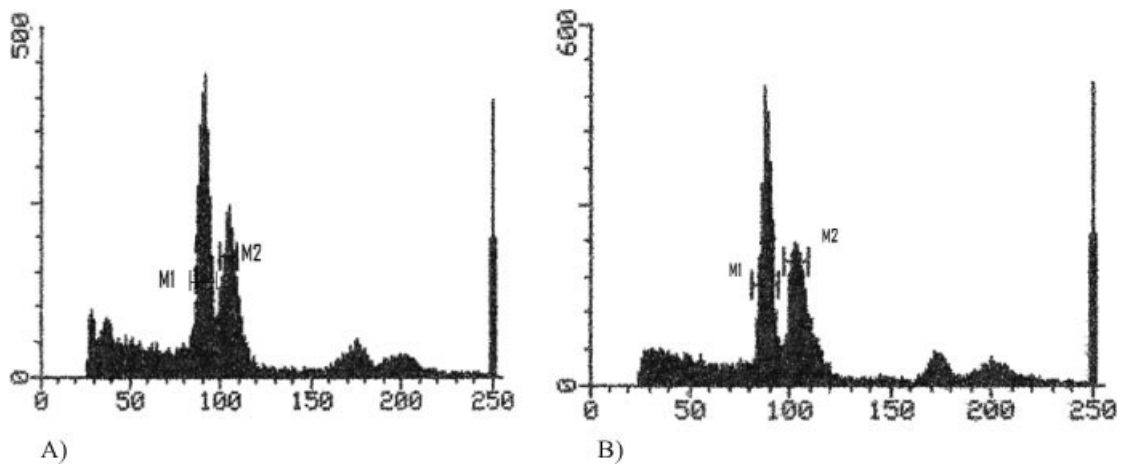

Fig. 4. Flow cytometry fluorescence analysis of PI stained leaf nuclei in suspension. On the *x*-axis, different fluorescence intensities (arbitrary units) are shown, corresponding to *Saponaria officinalis* (M1) and *Zea mays* (M2). (**A**) Histogram height derive from the measurement of the maximum instantaneous value of fluorescent intensity emitted by a nucleus. (**B**) Frequency histogram of numbers of nuclei (*y*-axis) per channel as a function of relative fluorescence intensity.

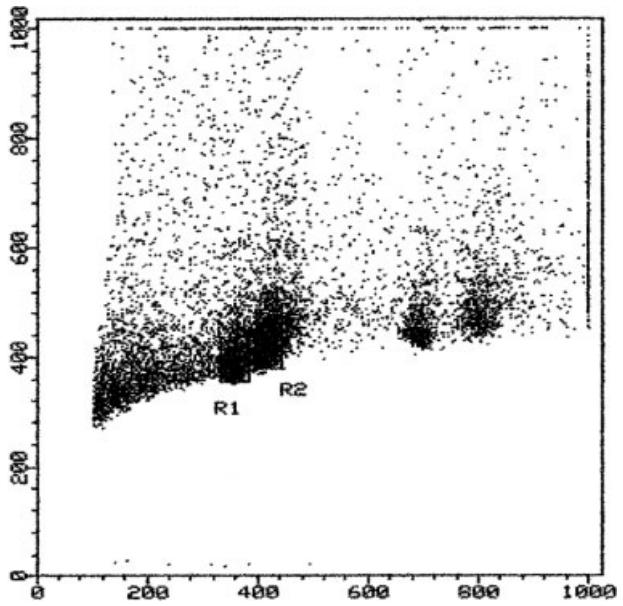

Fig. 5. Two-parameter histogram of Fluorescence Pulse Area (FPA) versus Fluorescence Pulse Width (FPW). FPA measures the integral intensity of the signal of fluorescence of nuclei; FPW measures their greatness, valued as the time of the signal of fluorescence. *Saponaria officinalis* (R1) and *Zea mays* (R2).

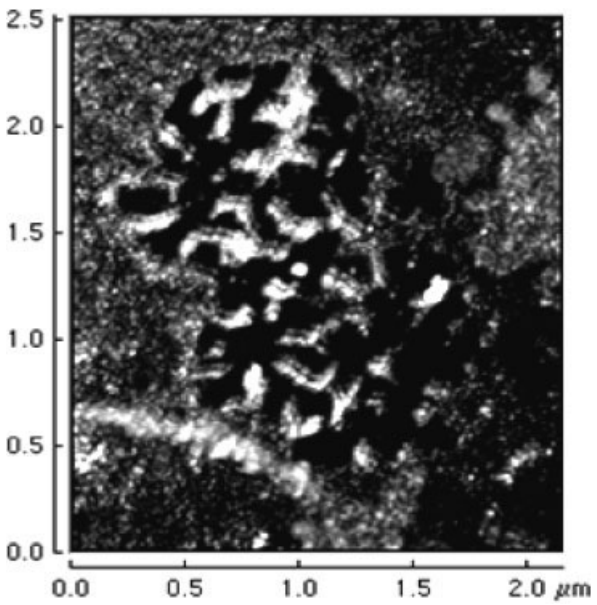

Fig. 6. AFM image of metaphase spread of *Saponaria officinalis.*

The usage of the combination of symmetry and volumetric measures revealed itself as a very efficient method for the chromosomic identification of an homogeneous kariotype, such as the kariotype of *Saponaria officinalis*. In Figures 8 and 9 are presented interphasic nuclei, where two nucleoli can be recognized, since they have a different height and diameter. Considering the ratio between the nucleoli and the nucleus area, it seems they are in regression. Some granular structures are recognizable in the area around the nucleus, organized within a diameter of 30–70 nm. Their dimensions, (better visualization in Fig. 10) make us hypothesize they are ribosomes. Further studies are needed for a confirmation. In Figure 10, it is possible to observe an anaphasic nucleus, and two partially overlapping telophasic nuclei can be seen in Figure 11.

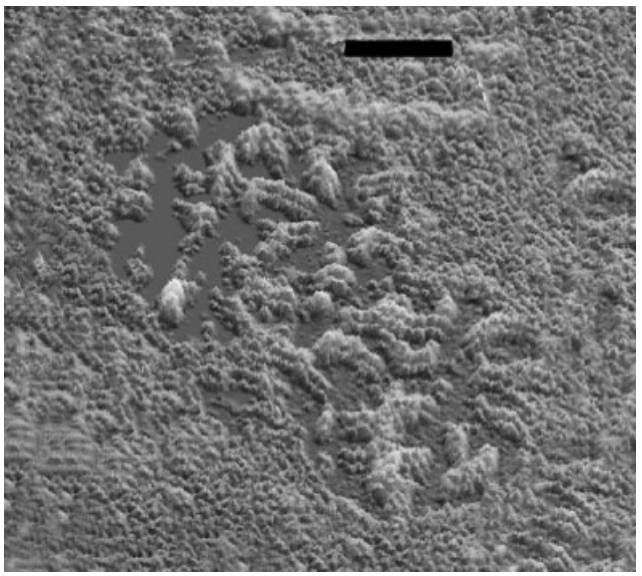

Fig. 7. Three-dimensional visual field of metaphase spread; bar = 1 μm.

TABLE 3. Surface Area, Arm Ratio, and Volumes of *Saponaria* chromosomes

Chromosome	Area (nm^2)	Volume (nm^3)	Arm ratio (1/s)
1	30 ± 10	200 ± 80	1.66
2	15 ± 5	110 ± 40	1.56
3	13 ± 8	90 ± 30	1.35
4	11 ± 2	80 ± 20	1.93
5	11 ± 3	80 ± 20	1.08
6	10 ± 3	70 ± 20	2.14
7	9 ± 3	70 ± 40	1.59
8	8 ± 2	60 ± 10	1.25
9	7 ± 1	50 ± 10	1.63
10	7 ± 1	50 ± 10	1.05
11	6 ± 1	50 ± 10	1.42
12	5 ± 2	30 ± 20	1.67
13	4 ± 2	30 ± 20	1.38
14	2 ± 1	17 ± 2	1.25

CONCLUSIONS

The present work offers a contribution to the characterization of the taxonomic entity of *Saponaria officinalis* by means of a cytogenetical approach. *Saponaria officinalis* produces the toxin saporin, a ribosome-inactivating protein (RIP) which inhibits protein synthesis through a site-specific deadenylation of the large ribosomal RNA. RIPs have been of considerable interest and largely investigated in biomedical research, due to their employment in the construction of immunotoxins and other conjugates for use in therapeutic treatments (Bolognesi and Polito, 2004); on the contrary, the biological function and the physiological role of these proteins in plants remains unknown; moreover *Saponaria*

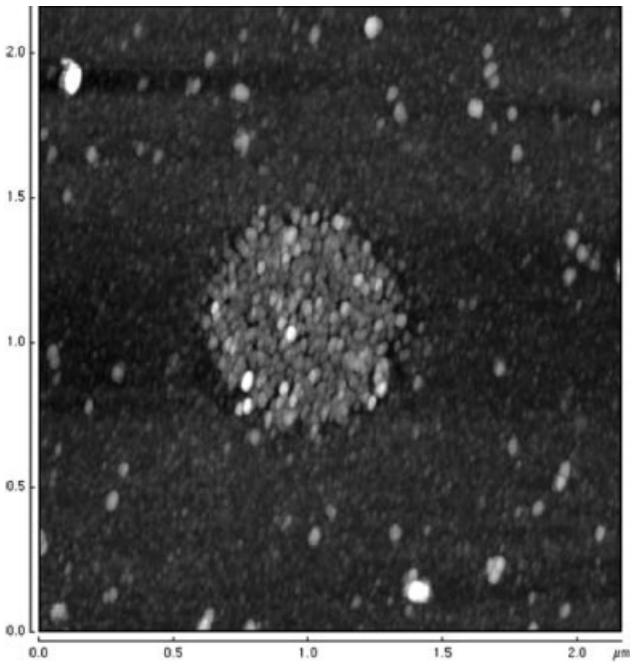

Fig. 8. AFM image of interphase nucleus.

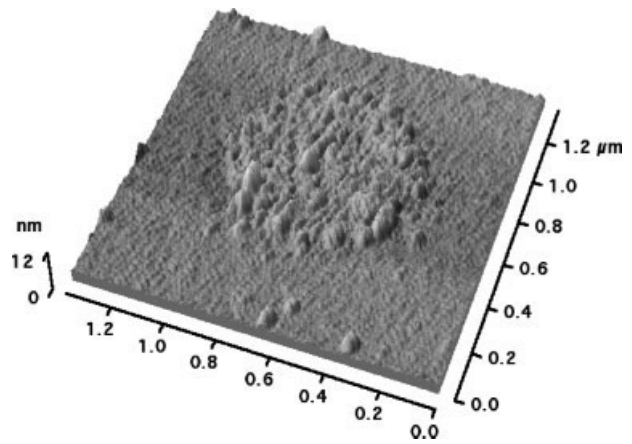

Fig. 9. Three-dimensional visual field of interphase nucleus.

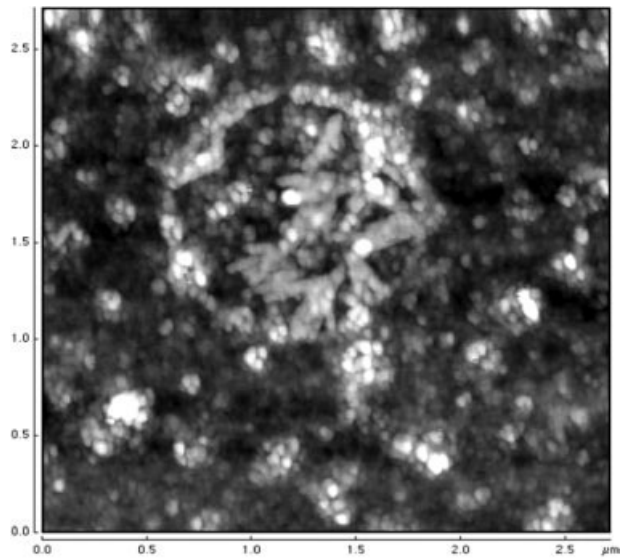

Fig. 10. AFM image of anaphase nucleus.

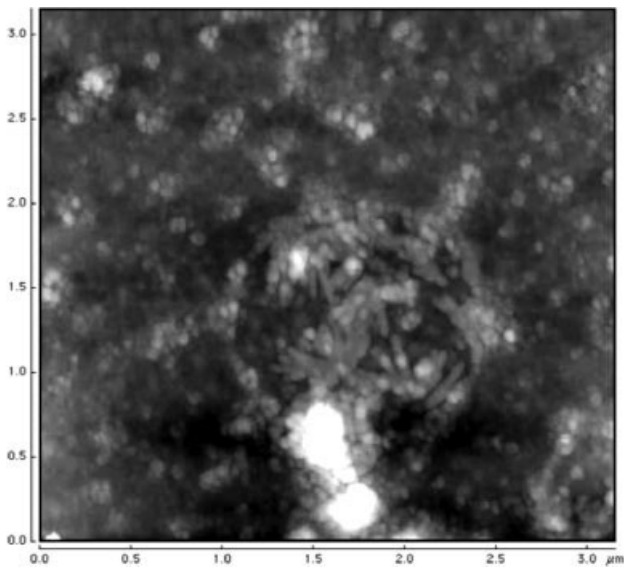

Fig. 11. AFM image of two telophasic nuclei partially overlapped.

officinalis has not been subjected to cytogenetic studies. In relationship to these interesting perspectives, the application of new techniques of chromosome preparation and staining as well as of improved methods of analysis, including image analysis has allowed us to evaluate the cytogenetical constitution of this species. The use of an automated image analysis system allowed the measurement of chromosomes with a precision which cannot be achieved with manual systems; moreover the measure is taken directly on the first image of the chromosomes projected by the objective, without serial treatments which could enhance error. The availability of such precise measures allows the determination of some indexes that are considered to be directly correlated with the evolution of the karyotype. The results confirm the number of chromosome ($2n = 28$), previously determinated by Garbari (1973), as well as a detailed karyomorphometry and a defined symmetry category, according to Stebbins; we also have determined the indexes of symmetry (TF%, SYI, and REC), essential for the evaluation of the evolutionary direction of the karyotype: these values range theoretically from 0 to 100 for REC and SYI and from 0 to 50 for TF%, therefore a karyotype with high values of these indices is considered less evolved (Venora et al., 1999). We describe a simple technique for direct visualization of plant chromosomes by means of AFM. Atomic force microscopy (AFM) is a relatively new technique (Binning et al., 1986), which has some applications in cytogenetics (Thalhammer and Heckl, 2004). While

scanning soft biological surfaces, the AFM can achieve a resolution of about 1 nm. The vertical resolution is mostly determined by the AFM scanner sensitivity, and typically is as precise as 0.01 nm (Wiechmann et al., 2001). The signal detected does not depend on any staining agent used moreover the imaging is nondestructive. There is no deterioration of the sample during the scanning like radiation damage in X-ray transmission microscope (XTM) and transmission electron microscopy (TEM) (Berdyyeva et al., 2005): this is an important advantage of AFM application to biological samples. An evaluation of the chromosomal volumes has been introduced as usefull measure for their identification; this approach assures minor modification of the native structure of the chromosome offering an important advantage before further treatments as, for instance, in situ hybridization. Fluorescence in situ hybridization (FISH) treatments, for example, cause severe alteration to the three-dimensional structural order of the chromosome while making it possible to observe intact nanostructures such as nucleosomes and chromatin fibers (Shichiri et al., 2003). In this study, ethanol-acetic acid was only used for the fixation of chromosomes by preserving the major structural details (Martin et al., 1996). This work proposes to introduce the determination of the chromosomal volumes by AFM as a new karyological parameter in order to demonstrate that a combined symmetry and volume approach may be more reliable than just arm ratio measurements alone.

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