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Estimation of Nuclear DNA Content and Determination of Ploidy Level in Tunisian Populations of *Atriplex halimus* L. by Flow Cytometry

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1. Introduction

The genus *Atriplex* (Chenopodiaceae) contains various species distinguishable by different morphology, biological cycles and ecological adaptations (Le Houérou, 1992). Because of their favorable crude protein content, many species of *Atriplex* are excellent livestock fodder during of season periods when grasses are low in feed value. *Atriplex*, as well as other shrub species, are important components of arid land vegetation (Haddioui & Baaziz, 2001). Chenopodiaceae are known as a plant family with many special ecological adaptations, enabling them to grow on very special stands (Freitas, 1993).Among the species of *Atriplex* in North Africa, *Atriplex halimus* L. (Chenopodiaceae) is an important high-protein livestock forage plant in arid and semi-arid zones of North Africa. It is particularly well-adapted to arid and salt-affected areas (Le Houérou, 1992). This species is interesting because of its tolerance to environmental stresses, its use as a fodder shrub for livestock in low rainfall Mediterranean areas (Le Houérou 1992; Cibilis et al. 1998; Zervoudakis et al. 1998; Haddioui & Baaziz, 2001) and its value as a promising forage plant for large-scale plantings (Valderrábano et al. 1996). Considerable variability has been described within *A. halimus* L., at both the morphological and isozyme polymorphism levels (Franclet & Le Houérou, 1971; Le Houérou 1992; Haddioui & Baaziz, 2001). Abbad et al. (2003) reported that the differences in leaf morphology among populations from geographically-distant sites were apparently under genetic control. Based on differences in habit, plant size, leaf shape and fruit morphology, *A. halimus* has been divided into two subspecies: *halimus* and *schweinfurthii* (Franclet & Le Houérou, 1971; Le Houérou, 1992). The two sub-species show relatively large levels of morphological variability. The base chromosome number in the

genus *Atriplex* is x = 9 (Nobs, 1975; McArthur & Sanderson, 1984) with variable ploidy levels occurring in several species. The subspecies are based on differences in morphology, with respect to habit, size, leaf shape and fruit morphology. However the existence of intermediate morphotypes complicates the designation of plants as one or the other subspecies (David et al. 2005).

Description and Conservation of *A. halimus* L. genetic resources seem particularly important for the rehabilitation of disturbed areas by salt and low rainfall. There is little literature concerning nuclear DNA content and ploidy levels in *A. halimus* L. The evaluation of genomic size and ploidy levels while determining its nuclear DNA content by flow cytometry is necessary.

In nature, considerable variation in nuclear DNA content occurs both within and among plant species. Manipulation of ploidy level is an important tool for plant breeding in a number of crops. Flow cytometry is increasingly employed as the method of choice for determination of nuclear DNA content and ploidy level in plants (Galbraith et al. 1997). Flow cytometry is a technique which permits rapid estimation of nuclear DNA content (Doležel, 1991) and has been already found very useful in plant taxonomy to screen ploidy levels and to determine genome size (Doležel, 1997).

The method is based on the isolation of single cells or nuclei in suspension and on the staining of nuclei with DNA fluorochromes. The fluorescence emitted from each nucleus is then quantified using a flow cytometer. Although the method was originally developed for the analysis of humain and animal cells, it is now widely used also for plants (Galbraith et al. 1989; Doležel, 1991). Fluorochromes currently used for flow cytometric estimation of DNA content can be broadly classified into two groups: stains that intercalate with double stranded nucleic acids and include ethidium bromide (EB) and propidium iodide (PI); and dyes and drugs that show a base preference and include Hoechst 33258 (H33258), 4′,6-diamidino-2-phenylindole (DAPI), mithramycin (MI), chromomycin A3 (CH), and olivomycin (OL). As flow cytometry provides only relative values, comparison with a reference standard having a known DNA content is necessary to determine picogram quantities of DNA. To make such a comparison valid, emitted fluorescence must be proportional to nuclear DNA content both in a reference standard and in the sample (Doležel et al. 1992).

Flow cytometry is used widely for determining amounts of nuclear DNA content. It can also be used to determine (DNA) ploidy (Lysák & Doležel, 1998; Emshwiller, 2002), although cytological studies are required for confirmation (Bennett et al. 2000). This protocol showed to be convenient (sample preparation is easy), rapid (several hundreds of samples can be analysed in one working day), it does not require dividing cells, it is non-destructive (one sample can be prepared, e.g., from a few milligrams of leaf tissue), and can detect mixoploidy. Therefore the method is used in different areas ranging from basic research to plant breeding and production.

The aim of this work was to evaluate the use of flow cytometry as a quick, reliable tool to determine ploidy level and estimated nuclear DNA content of populations of *A. halimus* L. from different sites in Tunisia. This would allow elucidation of the relationships between ploidy, subspecies, morphology and edapho-climatic conditions for this important shrub.

2. Materials and methods

2.1 Plant material

Plants from nine populations of *A. halimus* were analysed: seven populations from Tunisia (Gabès, Médenine, Tataouine, Monastir, Tunis, Sidi Bouzid and Kairouan) and two populations which preliminary analyses had shown to be diploid (2n = 2x = 18) (Cala Tarida Spain) and tetraploid (2n = 4x =36) (Eraclea, Italy), respectively. Details of the original locations of these populations are given in Table 1.

Population	Position	Altitude (m asl)	Mean temperature (°C)		Annual precipitation (minus evapotranspiration, for populations Cala Tarida and Eraclea) (mm)
			Max. in hottest month	Min. in coldest month	
Gabes	33°54´ N, 10°06´ E	4	32.5	5.8	193
Kairouain	35°41´ N, 10°06´ E	60	37.6	4.4	321
Médenine	33°21´ N, 10°29´ E	116	36.7	6.2	195
Monastir	35°47´ N, 10°50´ E	2	33.4	9.2	383
Sidi- Bouzid	35°04´ N, 09°37´ E	354	37.8	5.0	237
Tataouine	35°50´ N, 10°28´ E	215	37.9	4.7	107
Tunis	36°51´ N, 10°19´ E	3	32.5	5.6	473
CalaTarida	38°56´ N, 01°14´ E	4	30.2	8.9	493-849 = -356
Eraclea	37°24´ N, 13°21´ E	120	29.0	8.1	560-878 = -318

Table 1. Description of the original locations of the populations of *A. halimus* L

2.2 Flow cytometry

Plants were grown from seeds in a peat-soil mixture, in a greenhouse, for 4 weeks (Figure 1). For most populations, four plants were analysed. For Tataouine and Tunis, due to poor germination, only three and two plants, respectively, were analysed. For each plant, one measurement was conducted in each analysis; an analysis being performed on four different days to give 16 measurements per population (12 and 8 for Tataouine and Tunis, respectively).

Flow cytometric estimation of nuclear DNA content was performed with a Partec PA II flow cytometer, using Propidium Iodide (PI) as the fuorescent stain. Samples of growing leaf tissue of *A. halimus* and tomato (*Lycopersicon esculentum* Mill., cv. Stupicke polni) (20 mg

Fig. 1. Plants used for measurements

fresh weight each) were prepared together. This tomato cultivar was chosen as the internal standard because of the similarity of its 2C nuclear DNA content (1.96 pg; Doležel et al. 1992) to that of *A. halimus*. Leaf material was chopped with a razor blade for 30–60 s, in a plastic Petri dish containing 0.4 ml of extraction buffer (Partec CyStain PI Absolute P Nuclei Extraction Buffer; Partec GMBH, Münster, Germany). To arrive at the DNA histograms, the resulting extract was passed through a 30 µm filter into a 3.5 ml plastic tube, to which was then added 1.6 ml of Partec CyStain PI Absolute P Staining Buffer, to give final PI and RNase concentrations of 6.3 µg ml^{-1} and 5.0 µg ml^{-1}, respectively. Samples were kept in the dark for 30 min before analysis by fow cytometry.

All stages of the extraction and staining were per formed at 4 °C. For cytometry, 20 mW argon ion laser light source (488 nm wavelength) (Model PS9600, LG-Laser Technologies GmbH, Kle-inosthein, Germany) and RG 590 long pass filter were employed (Figure 2). The precision and linearity of the flow cytometer were checked on a daily basis using 3 µm calibration beads (Partec). The gain of the instrument was adjusted so that the peak representing the G_0/G_1 nuclei of the internal standard was positioned on channel 100. At least 5000 nuclei were analysed in each sample. *A. halimus* nuclear DNA was estimated by the internal standard method, using the ratio of the *A. halimus*: tomato G_0/G_1 peak positions. The 2C nuclear DNA content of the unknown sample was calculated according to a formula: Sample 2C DNA content = (sample peak mean/standard peak mean) x 2C DNA content of standard (Doležel, 1997). The equivalent number of base pairs was calculated assuming that 1 pg DNA = 965 Mbp (Bennett et al. 2000).

Fig. 2. Flow cytometer used for estimation of nuclear DNA content

2.3 Chromosome counts

Seeds of the Tunisian populations Gabès, Tataouine, Monastir, Sidi Bouzid and Kairouan, and of the populations Cala Tarida and Eraclea, were sown in Petri dishes, on paper towels wetted with tap water. Root tips from 100 germinated seeds per plant (1–3 plants per population) were pre-treated with 8 hydroxyquinoline (2 mM) for 5 h at 4 °C, and fixed in 3:1 ethanol:acetic acid solution. The tissue was macerated for 45 min at 37 °C, with a mixture of cellulase and pectinase (10%) in a 10 mM sodium citrate buffer (pH 4.6). Feulgen stain was applied, followed by acetic-hematoxylin (Nuňez, 1968). At least five slides were observed for each seedling, using a Zeiss ST16 microscope.

2.4 Statistical analyses

A general Model analysis was used to determine the effect of population on nuclear DNA content. To determine whether mean values differed significantly ($p < 5$), the Student-Newman–Keuls test was used. These tests were performed using SPSS software, version 11.0. Log- transformation of values was not necessary since Cochran's C test ($p = 0.396$) and Bartlett's test ($p = 0.094$) showed that variance did not differ significantly between populations.

3. Results

3.1 Flow cytometry

Using flow cytometry, the genome size of any species can be estimated after simultaneous measurements of the fluorescence of stained nuclei of the species and of the reference standard with known DNA content. Flow cytometry is a technique which permits rapid estimation of nuclear DNA content. Because of its speed, precision and convenience, this method of analysis of nuclear DNA content finds an enormous number of applications which cover basic research, breeding and production. The results obtained indicate that the technique might greatly simplify the analysis of plant genomes at the molecular level.We have estimated nuclear DNA content in nine populations of *A. halimus* L., two DNA ploidy levels were found: diploid and tetraploid. With respect to nuclear DNA, the 2C DNA content of population Cala Tarida was estimated to be 2.41pg. As expected, tetraploid populations had approximately two times higher DNA content, ranging from 4.918 pg in Tataouine to 4.972 pg in Gabes, but without statistically-significant differences among them ($p > 0.05$). The two populations which were not subjected to chromosome counting, Médenine and Tunis, had the mean nuclear DNA content of 4.950 and 4.970 pg, respectively, showing them to be tetraploid.

Representative histograms of the flow cytometric analyses of all populations are shown in Figures 3,4,5,6 and Figure 7. The coefficient of variation (= (100 standard deviation)/mean) values of *A. halimus* G_0/G_1 peaks ranged from 1.5 to 4.4%.

We can observe other peaks than the ones labeled G_0G_1, Because homogenization of the plant tissue produces debris that interferes with the detection and measurement of the isolated nuclei.

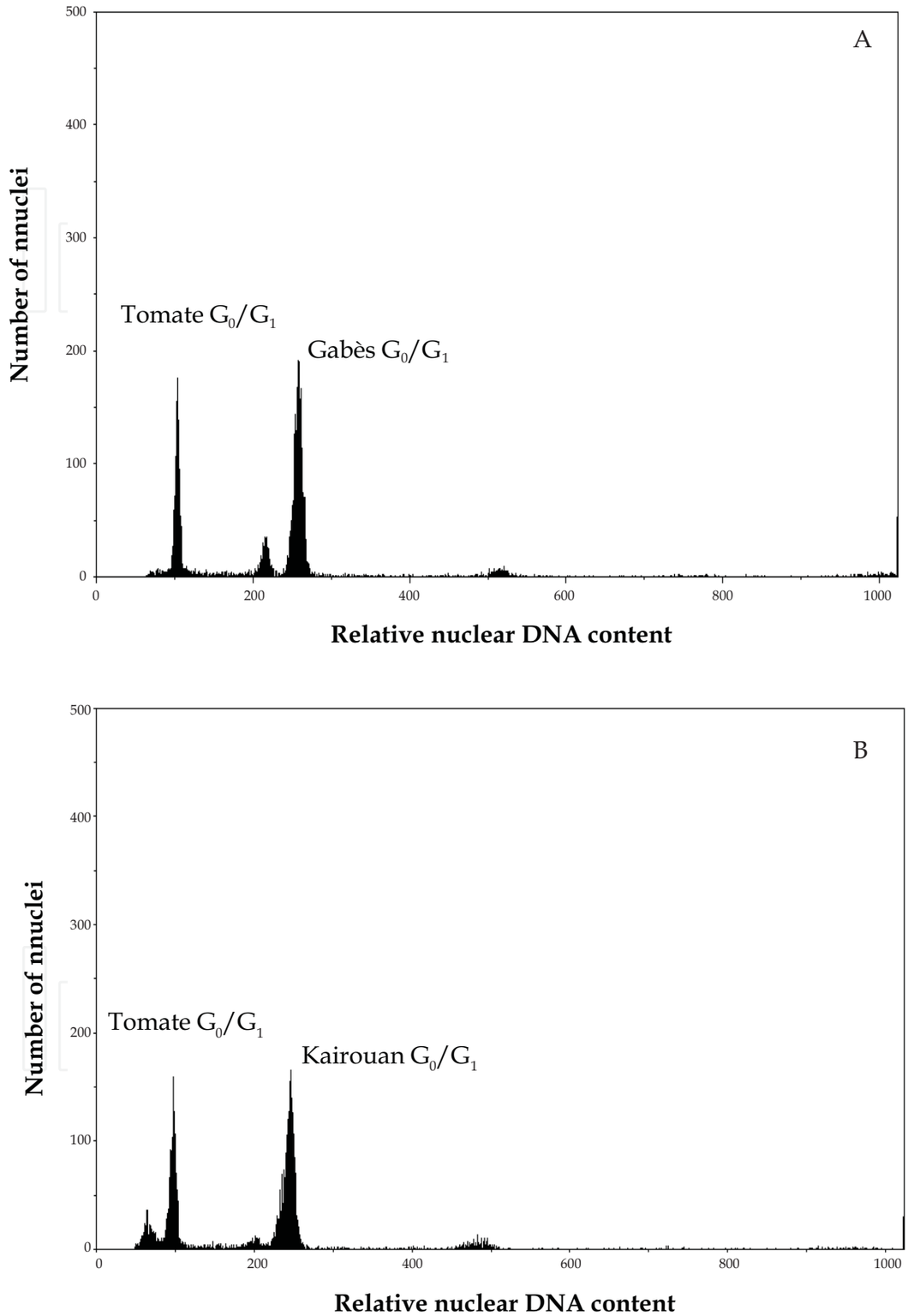

Fig. 3. Flow cytometric analyses of *A. halimus* L. : (A) Gabès, (B) Kairouan (Tunisia) populations

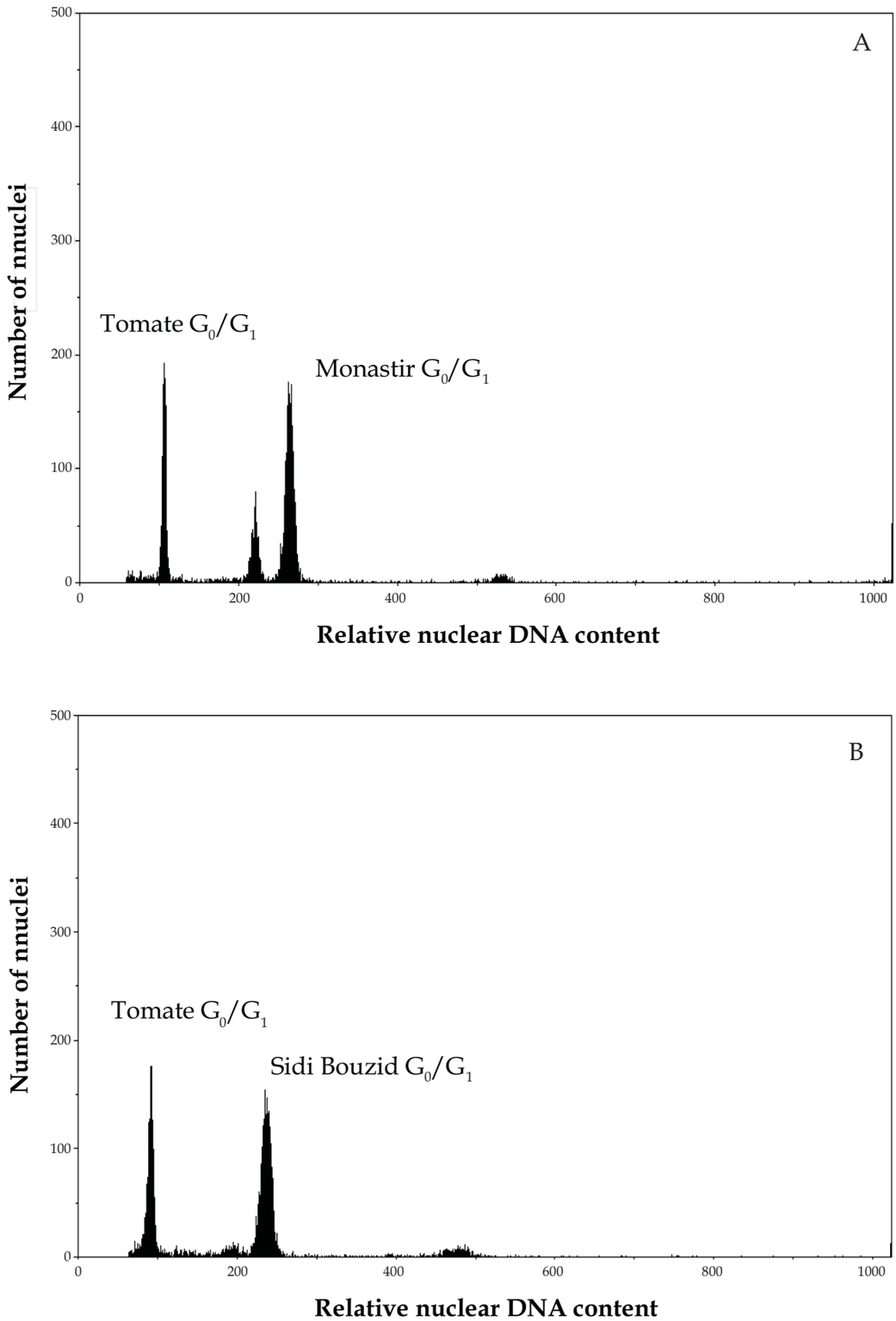

Fig. 4. Flow cytometric analyses of *A. halimus* L. : (A) Monastir, (B) Sidi Bouzid (Tunisia) populations

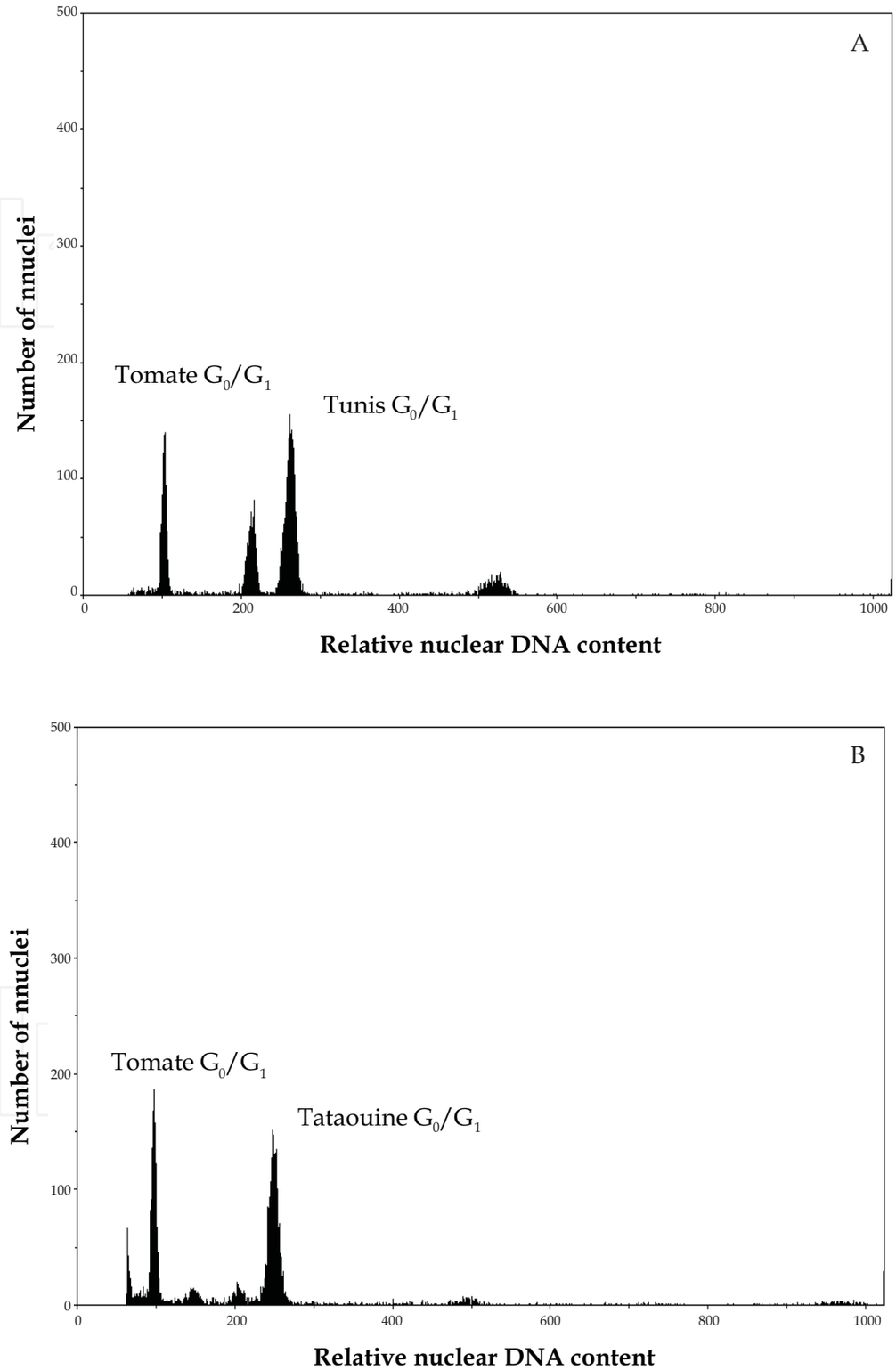

Fig. 5. Flow cytometric analyses of *A. halimus* L. : (A) Tunis, (B) Tataouine (Tunisia) populations

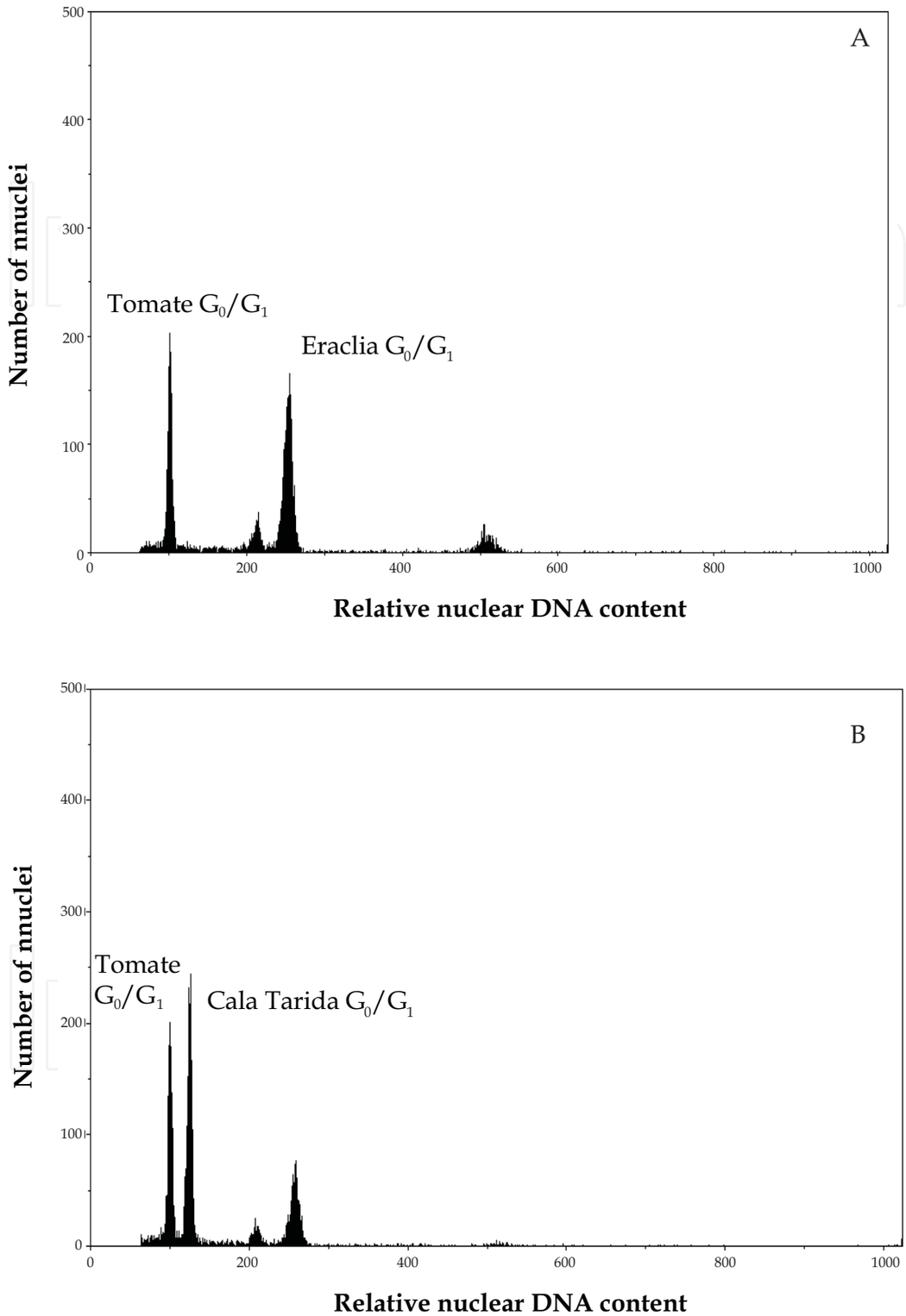

Fig. 6. Flow cytometric analyses of *A. halimus* L. : (A) Eraclia, (Italy) (B) (Cala tarida (Spain) populations

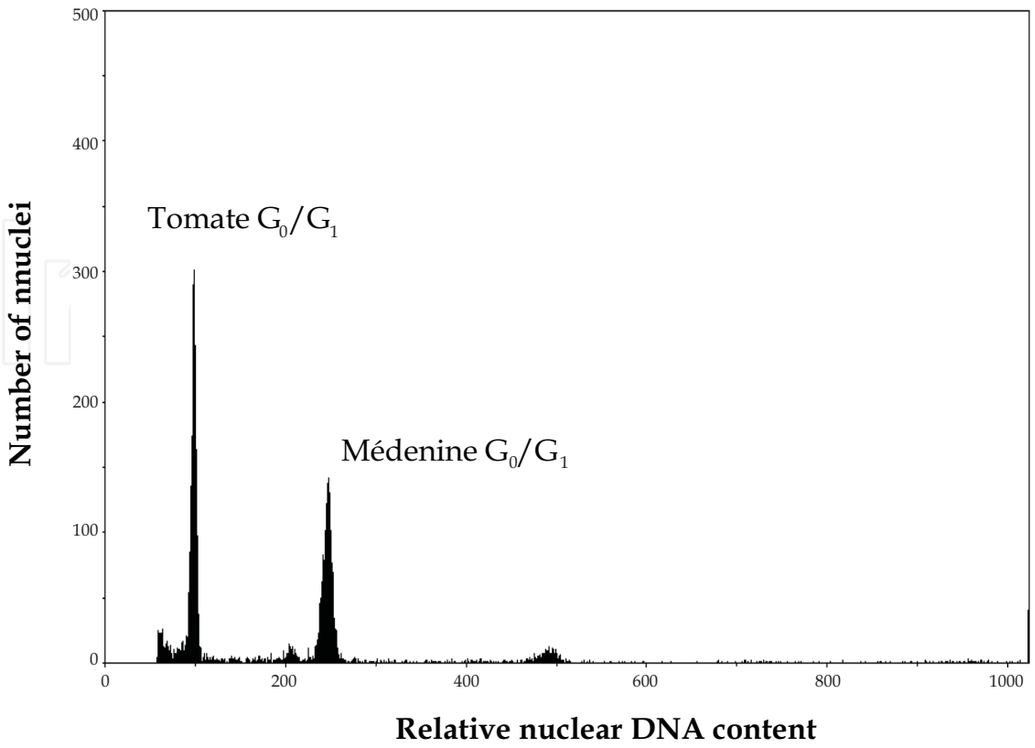

Fig. 7. Flow cytometric analyses of *A. halimus* L.: Médenine (Tunisia) population

The results of nuclear DNA content analysis are shown in Table 2. The mean 2C DNA content of the diploid population Cala Tarida was 2.412 pg. As expected, tetraploid populations had approximately two times higher DNA content, ranging from 4.918 pg in Tataouine to 4.972 pg in Gabès, but without statistically-significant differences among them ($p > 0.05$).

Population	2C nuclear DNA content (pg)		1C genome size (Mpb)
	Mean	s.d.	
Tunis	4.970	0.095	2398
Monastir	4.958	0.104	2392
Kairouan	4.961	0.113	2394
Sidi Bouzid	4.950	0.106	2388
Gabes	4.972	0.135	2399
Medenine	4.950	0.116	2388
Tataouine	4.918	0.110	2373
Eraclea	4.967	0.098	2397
Cala Tarida	2.412	0.051	1164

Table 2. Estimated of nuclear DNA (2C) amounts (pg) ± s.d. (n = 8_16) for the studied populations of *A. halimus* L. General Linear Model analysis gave an F value of 1013.4 ($p < 0.001$) for the population effect on nuclear DNA content. Sub-groups not sharing a common letter differ significantly ($p < 0.001$), according to the Student-Newman–Keuls test

3.2 Chromosome counts

The chromosome numbers in the *A. halimus* L. populations studied are shown in table 3. For the Tunisian populations and the population from Eraclea (Italy), the chromosome number in a somatic metaphase nucleus was observed to be 36. Thus, since the base chromosome number in the genus Atriplex is x = 9 (Nobs 1975), these populations are tetraploid (2n = 4x = 36). The Cala Tarida population (Spain) was diploid (2n = 2x = 18).

Population	Chromosome number (2n)
Tunis	n.d.[a]
Monastir	36
Kairouan	36
Sidi Bouzid	36
Gabes	36
Medenine	n.d
Tataouine	36
Eraclea	36
Cala Tarida	18

[a]n.d., not determined.

Table 3. Estimated somatic chromosome number for the studied populations of *A. halimus* L

4. Discussion

An efficient method for the determination of the ploidy level is described, based on a measurement of the DNA content of interphase nuclei by flow cytometry. Both individual plants as well as plant populations can be used to obtain the desired DNA-histograms (De Laat et al. 1987).This can provide a diagnostic tool for separating the subspecies in cases where the morphological observation in inconclusive. Flow cytometry has been recognized as being superior to microscopic chromosome counts for a number of reasons. With recent technical improvements in modern flow cytometers, it is now a matter of days instead of months for researcher to become confident with the technique. Leaf material can collected at any growth stage, leaving tha plant alive, and only small amount of living material is necessary (Galbraith et al. 1997). The method was found to be reliable and highly sensitive for detecting small differences in DNA content (Lysák et al. 2000).

Ploidy determination using flow cytometry reduces the need for many morphological measurements and chromosome counts, and flow cytometry can be used on seedlings or mature plants with rapid, reliable results. The success of flow cytomery in distinguishing between species that differ in ploidy level is important for breedings programs (Stacy et al. 2002). Compared to conventional chromosome counting flow cytometry turned out to be highly competetive in terms of simplicity, accuracy and costs.

Polyploidy, which is known to occur in numerous dryland shrubs, is present in one third of the known *Atriplex* species (Stutz 1989). Polyploidy is one of the most important mechanisms in plant evolution. About 30-35% of phanerogamous species are polyploid. The ploidy levels frequently identified are tetraploid and hexaploid (Bouharmont, 1976). Within the genus *Atriplex*, the diploid state has been found in 26 species recorded in California

(Nobs, 1975) and 27 endemic species in Australia (Nobs, 1979). More recently, meiotic chromosome counts of n = 9 (2n = 18) have been determined from wild plants of *Atriplex* (Subgenus Theleophyton) *billardierei* gathered in New Zealand and on Chatham Island (De Lang et al., 1997). The existence of polyploidy has been found in *Atriplex canescens* (Stutz et al., 1975; McArthur, 1977; Sanderson and Stutz, 1994), in *A. tridentata* (Stutz et al., 1979), in *A. confertifolia* (Stutz et al., 1983; Sanderson et al., 1990). Considerable variability has been described within *A. halimus*, at both the morphological and isozyme polymorphism levels (Franclet & Le Houérou, 1971; Le Houérou, 1992; Abbad et al., 2003). Differences in floral sex ratio (male/female flowers), floral architecture and other vegetative and fruit morphological characteristics, related to population and growth conditions, have been reported recently (Abbad et al., 2003; Hcini et al., 2003; Talamali et al., 2003).

With respect to their morphology, the tetraploid populations from Tunisia and Italy studied here correspond to ssp. schweinfurthii, whilst the diploid Cala Tarida has a ssp. *halimus* morphology: ssp. *halimus* having a more erect habit, smaller in size (0.5–2.0 m height compared to 1.0– 3.0 m for schweinfurthii), shorter fruit-bearing branches (0.2–0.5 m compared to 0.5–1.0 m) and less-markedly-toothed fruit valves (Franclet & Le Houérou, 1971; Le Houérou, 1992). Franclet & Le Houérou (1971) & Le Houérou (1992) divided *A. halimus* into two subspecies: *halimus* and *schweinfurthii*. This was based on differences in morphology, with respect to habit, size, leaf shape and fruit morphology. Regarding distribution, ssp. *halimus* generally grows in higher-rainfall (>400 mm year_1) zones, in western Mediterranean areas such as France and Spain and on Atlantic coasts, whilst ssp. *schweinfurthii* is adapted to arid zones (100–400 mm rainfall period 1000–500 BC (Le Houérou, 1981); ssp. *schweinfurthii* may have subsequently populated such areas.

According to Le Houérou (2000), both subspecies are extremely heterogeneous in terms of their morphology, ecology, productivity and palatability to herbivores. However, ssp. *halimus* predominates in semi-arid to subhumid areas and has a higher leaf: shoot ratio than ssp. *schweinfurthii*, which is better adapted to arid environments but is less productive in terms of browsing biomass. The high levels of variability observed may be required to maintain plasticity in a highly fluctuating and diverse environment like Mediterranean Basin. Another reason for the higher intrapopulational variation of ssp *schweinfurthii* could be its polyploid character. According to Soltis & Soltis (2000), polyploids, both individuals and populations, maintain higher levels of heterozygosity than do their diploid progenitors. Moreover, most polyploids may have a much better adaptability to diverse ecosystems, which may contribute to their success in nature. This is illustrated in the case of ssp. *schweinfurthii*, by its much bigger distribution area than ssp. *halimus* and by its presence in very contrasting biotopes.

To our knowledge, this is the first report on genome size estimation in the Tunisian populations of *A. halimus* L. compared to a known range of genome size in plants (Bennet et al. 1997), the *Atriplex* specie should be considered taxa with a small size genome. Haddioui & Baaziz (2001), studying isozyme polymorphism in Moroccan populations of *A. halimus* (presumably tetraploid), found a relatively high degree of genetic diversity, predominantly due to within-population diversity, with between-population variation accounting for only 8%. These authors attributed this to the highly-outbreeding nature of *A. halimus*. The greater genetic heterozygosity of polyploids, at both individual and population levels, may give them a selective advantage in unstable environments (Sanderson et al., 1989; Soltis & Soltis,

2000). In the current work, we found no significant differences among plants within populations with respect to nuclear DNA content.

5. Conclusion

The Tunisian populations originated from widely-separated sites of contrasting climatic conditions plus a population from Eraclea, Sicily (Italy), were tetraploid (2n = 4x = 36) whereas a population from Cala Tarida, Ibiza (Spain) was diploid (2n = 2x = 18). With respect to nuclear DNA, the 2C DNA content of population Cala Tarida was estimated to be 2.41 pg. There was no significant difference among the tetraploid populations (or among plants within populations), whose 2C DNA content ranged from 4.92 to 4.97 pg. The present study clearly shows that the precision and rapidity of flow cytometric estimation of nuclear DNA content makes the method very attractive for estimation of genome size both in animal and plant species. This protocol showed to be convenient (sample preparation is easy), rapid (several hundreds of samples can be analysed in one working day), it does not require dividing cells, it is non-destructive (one sample can be prepared, e.g., from a few milligrams of leaf tissue), and can detected mixoploidy. Therefore the method is used in different areas ranging from basic research to plant breeding and production. On the other hand, determination of nuclear DNA content showed that certain populations with morphologies intermediate between those considered typical of ssp. *halimus* and *schweinfurthii* were tetraploid. This kind of approach, together with studies of morphology and isoenzyme polymorphism, as well as molecular techniques could be employed on a wider (and more detailed) geographical scale to ascertain the phylogenetic relationships within the species.

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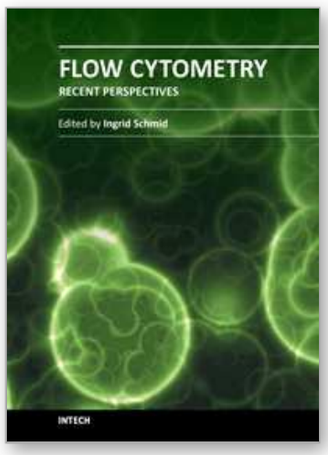

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"Flow Cytometry - Recent Perspectives" is a compendium of comprehensive reviews and original scientific papers. The contents illustrate the constantly evolving application of flow cytometry to a multitude of scientific fields and technologies as well as its broad use as demonstrated by the international composition of the contributing author group. The book focuses on the utilization of the technology in basic sciences and covers such diverse areas as marine and plant biology, microbiology, immunology, and biotechnology. It is hoped that it will give novices a valuable introduction to the field, but will also provide experienced flow cytometrists with novel insights and a better understanding of the subject.

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