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Flow cytometry and chromosome numbers variation in argan tree *Argania spinosa* (L.) Skeels

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Abstract

Argania spinosa L. Skeels is an endemic species of west-central Morocco, which is characterized by a high diversity of morphological and genetic traits. It constitutes a natural resource for oleo-agro-sylvo-pastoral uses. All conservation and genetic breeding strategies aimed to domesticate argan require a good knowledge of the plant material. However, several studies focused on agronomical, morphological, phytochemical, and molecular characterization, while the cytogenetic aspects were less investigated. The objective of this work is to identify the chromosome number and ploidy level on the national argan collection at the Agadir Regional Agronomic Research Center, Morocco. The determination of the chromosome number was carried out on root tips of germinated seeds collected from five trees genotypes selected on various morphological aspects. As a result, chromosome count on active root tip cells showed variation in the number ($2n = 20$; $2n = 22$; $2n = 24$) with a stable ploidy level ($2n = 2x$) that is confirmed by flow cytometry. These results combine two previous findings ($2n=20$, $2n=24$) and reveal a third existence of twenty-two chromosome. As a conclusion, *A. spinosa* has three chromosomal numbers which represent the genetic diversity of the chromosomal number that this species exhibits. More studies are required to explain this variation on chromosome numbers for future breeding programs and to avoid incompatibilities.

Keywords: *Argane* tree; chromosome number; cytogenetic; flow cytometry; genetic diversity

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Introduction

Argania spinosa (L.) Skeels (Sapotaceae), the only representative of the family in Morocco, is an allogamous monoecious tree with entomophilic pollination (Nerd *et al*., 1998; Ajerrar *et al*., 2020). It is an endemic species of west-central Morocco; its forest area is estimated to 800,000 hectares (Msanda et *al.*, 2021). The Arganereae offers several ecosystem services, namely the storage and sequestration of carbon, habitats of several endemic species, contributes to the conservation of biodiversity, improves soil fertility, prevents soil erosion, regulates and creates microclimates (Karmaoui, 2016). The Biosphere Reserve of *Argania* covers an area of 2,560,000 ha (Msanda et *al.*, 2021), in which the local population (3 million people, including 2.2 million in rural areas) exploits this natural resource for energy purposes (wood and shell) as well as a fodder resource (leaves, and fruit pulp). Therefore, they are dependent on the exploitation of argan forests (Laaribya et *al.*, 2017).

The economic value of the argan tree is based mainly on its almonds, from which highly marketable oil is extracted (M'hirit, 1987). Argan oil is very known for its quality, with an interesting saponifiable composition, excellent effects, and several benefits (Berrougui et *al*., 2003; Drissi et *al*., 2006; Koufan *et al*., 2020a). Argane's shells are used in combustion for heating and even as a bio-composite base. Biochar from argan shells enriches the soil by improving nutrient and water retention (Bouqbis et *al*., 2016). In addition, the pulp, bark, leaf, root, and even press cake have interesting medicinal uses (Moukal, 2004). Because of its importance and for its protection, UNESCO declared the Arganereae on December 8th, 1998, as the first Biosphere Reserve in Morocco. In 2021, UNESCO declared May 10th the international day of the argane tree. Well known for its high genetic diversity, efforts of scientific research achieved detection and characterization of specie's genetic diversity on the morphological, agronomical, biochemical and molecular level and marked its exploitable genetic potential (El Mousadik *et al*., 1996; Ait Aabd *et al*., 2012; Ait Aabd *et al*., 2013; Ait Aabd *et al*., 2015; Mouhaddab *et al*., 2017; Koufan *et al*., 2020b). Unlike the cytogenetic approach, that is less studied.

The breeding program from wild types seems to be the straighter method for genetic improvement and before conception of argane orchards. Genetic diversity can be found in genome sequence and the number of chromosomes (De Vicente *et al*., 2004). Polyploidy means increasing the number of chromosomes in an animal or plant cell (multiple of the number of haploids). It can be an auto-polyploidy (duplication without cytokinesis) or an allopolyploidy (fusion of two unreduced gametes). Also, the genetic variation can be at the origin of the anomalies of the chromosomal number like aneuploidy. It is defined as the presence of an abnormal number of chromosomes. In general, chromosome number variation can be a speciation factor. It plays a fundamental role in plants evolution, diversification, and ecological adaptation. Karyotype assessments by counting the number of metaphase chromosomes is time consuming and laborious. Highly skilled manipulators are needed to accomplish it, in addition, tissues containing few numbers of dividing cells, which may not be readily available. However, flow cytometry has arisen as a far more reliable methodology for the determination of genome size and ploidy level. It permits measurement of the fluorescence of large numbers of stained nuclei within seconds, more samples within minutes (Seker *et al*., 2003). In fact, with the enormous biodiversity at the biometric, phenological, agro-morphological, and molecular level, the allogamous pollination system, and pollen incompatibility (Ait Aabd et *al*., 2022), the existence of chromosomal variation remains to be checked in the argan tree species. Indeed, this study aims to investigate the chromosome number on a collection of genotypes morphologically and phenologically differentiated.

Materials and Methods

Plant material

The study was undertaken on five genotypes selected previously based on their morpho-biometry and phenology (tree shape, height, number of trunks, flowering period, fruit shape and fruit ripening duration). The trees were planted at Melk-Zhar experimental farm, Regional Agronomic Research Center, Agadir, Morocco.

Chromosome counting

The protocol of Majourhat et *al*. (2007) was adopted with minor modification. Mature fruits were collected from each tree and kept until drying. After pulping and crushing seeds, they were disinfected using Sodium Hypochlorite, rinsed with sterile water, and then germinated on Petri dishes with moist filter paper at 26 °C. Root tips were cut out when the radicles were about 2-3 cm long. They were pre-cooled four hours in ice water. The samples are then fixed in glacial ethanol-acetic acid (3: 1) for 16 h at 4 °C, then transferred to 70% ethanol solution and kept at 4 °C. The root tips were hydrolysed in 5 N HCl for 10 min. Finally, the samples were stained in Orcein Acetic solution for two hours then squashed under coverslips. Slides were observed under different objectives to find well spread cells and show the best meta-phasic plates. Mitotic chromosomes were photographed under 1000X magnification using an optical microscope. Each photographed cell was drawn to count it accurately. To prevent experiment artefacts, more than 3 counts were made per each preparation and plant.

Flow cytometry

Cells were isolated from specimens collected directly from each genotype. Leaf samples (5 mm^2) were chopped with razor blade in presence of 400 µL nuclei extraction buffer in Petri dishes. The samples were filtered directly into a sample tube of 50 µm and stained with 1,5 mL of 4',6-diamidino-2-phenylindole buffer stain. Following 5 min incubation at room temperature, stained samples were run in flow cytometer. Analysis was repeated twice for each genotype.

Results

Chromosome numbers

Chromosomal counting is carried out on well spread, drawn, and photographed plates. It was difficult to count the chromosomes given their small size (0.59 to 1.69 µm) (Majourhat et *al*., 2007). Despite, three chromosome numbers were counted: 20, 22 and 24 (Figure 1), which means three base numbers (n=10, n=11, n=12). The number of chromosomes varied between mother trees. Indeed, three genotypes with 22, one with 24, and one with 20 were observed.

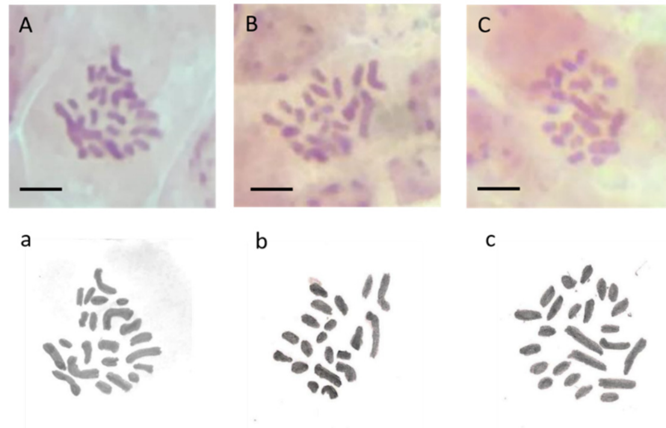

Figure 1. Chromosomes count on *A. spinosa*: microscopic photography (A, B, C) and draw (a, b, c) of meta-phasic plate showing 22 chromosomes, 20 chromosomes and 24 chromosomes. Scale bars represent 5 μm.

Flow cytometry profiles

Figures 2, 3 and 4 show different raw flow cytometer profiles. First peak (around 50) is background noise and the second peak is cells fluorescence intensity emission. Profile 1 represent the maximal fluorescence intensity measured (around 230), profile 2 with intermediate intensity (around 200) while profile 3 represent the minimal fluorescence intensity detected (around 150). It was observed that fluorescence intensity of DAPI stain varied according to genotype. Cells intensity detected is correlated to relative nuclei DNA content.

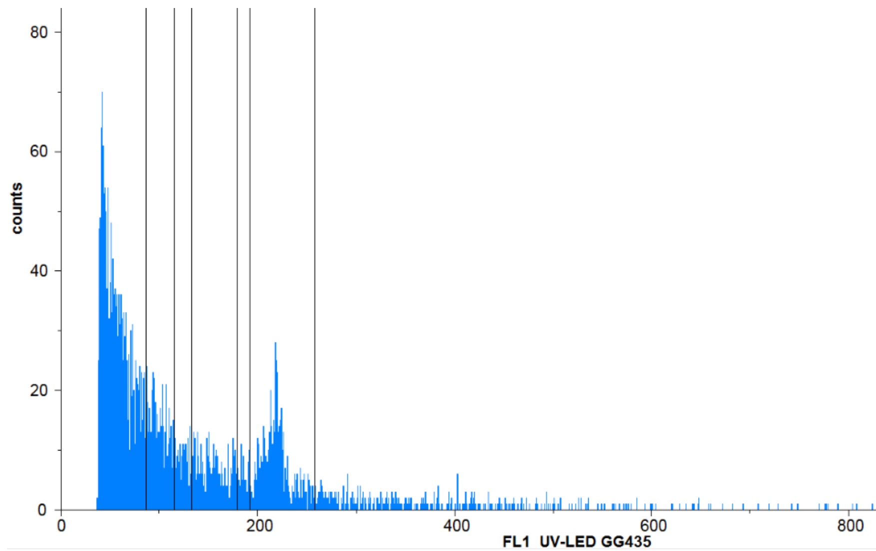

Figure 2. *Argania spinosa* flow cytometry profile 1, cells count represented on the Y axis while fluorescence intensity is represented on the X axis

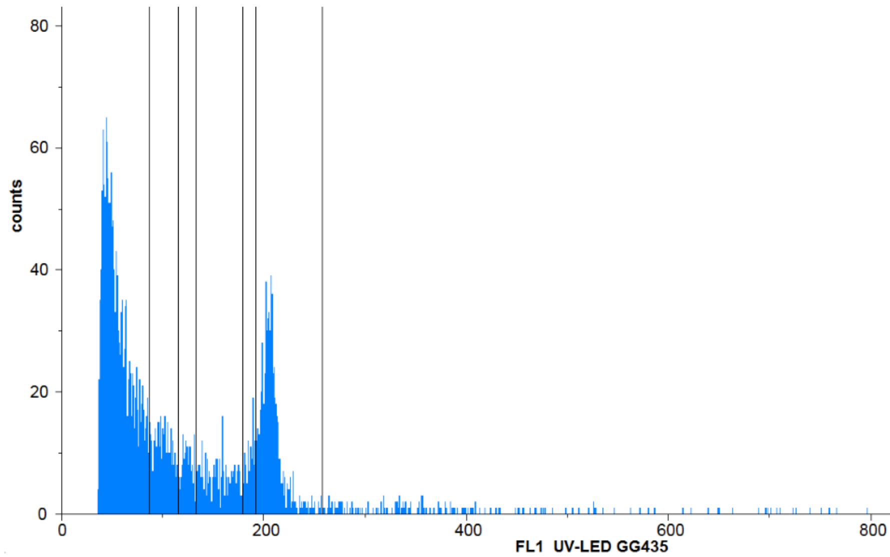

Figure 3. *Argania spinosa* flow cytometry profile 2, cells count represented on the Y axis while fluorescence intensity is represented on the X axis

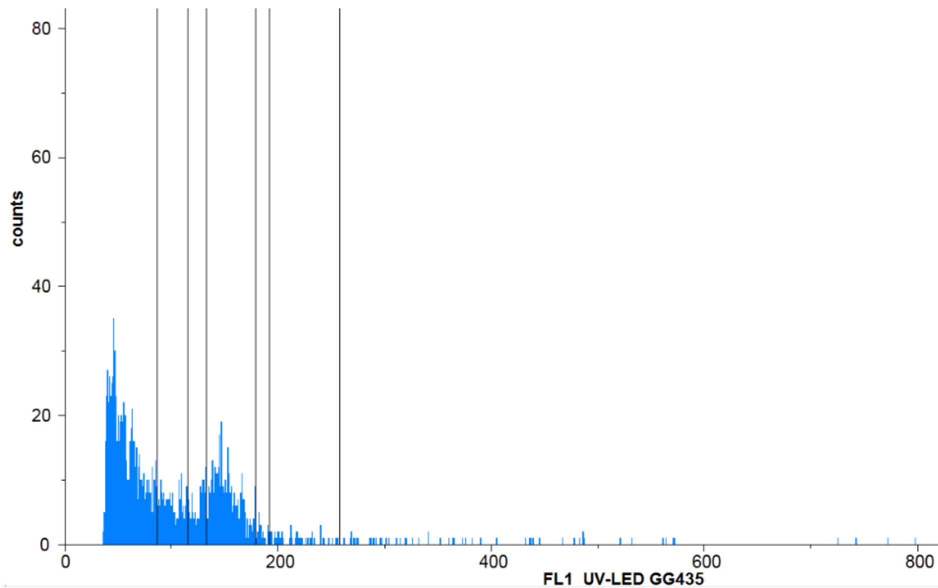

Figure 4. *Argania spinosa* flow cytometry profile 3, cells count represented on the Y axis while fluorescence intensity is represented on the X axis

Discussion

Miège (1954) reported 20 chromosomes on *A. spinosa* somatic cells, while Humphries *et al.* (1978) discovered 24 chromosomes on pollen mother cells. The current study on *A. spinosa* showed a new finding as the counting of 2n = 22 chromosomes which has never been reported. The highest diversity observed on argan tree forest could give an explanation of this variability of chromosome numbers. Based on flow cytometry analysis, the 3 chromosomes numbers (20, 22 and 24) were confirmed and showed none variation in ploidy level. In fact, the intensity dissimilarity observed between genotypes cannot be a score as a variation in ploidy level. Nevertheless, this slight variation may be due to the genome size or genome composition

variations (DAPI binds specifically to AT nucleotides). Understanding the genetic diversity of the argan tree is an essential basis that requires more scientific effort to achieve the essential objective of the genetic improvement of this species.

The basic data are chromosome numbers, but chromosome size, morphology, and staining characteristics may also be of important value (Stace *et al*., 2000). Systematic investigations are mainly based on molecular methods, while chromosome data provide essential and basic information, which may for example help in interpreting results from molecular studies (Baltisberger and Widmer, 2006). It is worth noting that the high genetic diversity revealed by molecular tools can be explained at chromosome's scale. Possession of more or fewer chromosome numbers influences the presence/absence of alleles on the whole genome. There is no report of intraspecific cytological variation in Sapotaceae until now. Johnson (1991) evaluated the number of chromosomes of 95 Sapotaceae species. All specimens showed n = 10, 11, 12, 13, or 14 and almost at the diploid level. The highest numbers (n = 13, 14) predominate in the tribes *Chrysophylleae* and *Omphalocarpeae*, whereas the numbers in *Mimusopeae*, *Isonandreae* and *Sideroxyleae* are almost on n = 10 or 11. Intraspecific cytological variation is frequent in plants, especially polyploidy. However, intraspecific variation in chromosome number is rare (Severns et *al*., 2008). Among species, genome size variation can occur as different ploidy levels (Terlević et *al.,* 2022) or chromosome number variation. It can vary or not at the same ploidy level (Bagheri et *al.,* 2022). Moreover, genome size variation can be correlated with morphological traits (Hoang et *al.,* 2019). In general, descending dysploidy and polyploidy played crucial roles in chromosome number evolution in angiosperm (Carta *et al.,* 2020) and may be the origin of this observed variation. In addition, abnormal *meiocytes* may be observed as a variation on chromosome numbers such as cytomixis, which is defined as the migration of chromatin between adjacent cells through cytoplasmic connection channels (Bellucci *et al*., 2003). This phenomenon detected in the meiocytes of several plants can cause variation in the chromosome number. However, this variation can be prevailing in populations of wild-type (Kaur and Singhal, 2015), having the form of different types of aneuploidy (trisomy, tetrasomy, double trisomy) or polyploidy (diploid, triploid, tetraploid, octaploid). Tetrasomy can be the origin that explains this variability (2n=20: no tetrasomy, 2n=22: one chromosome tetrasomy, 2n=24: two tetrasomy chromosomes). The union of two n+1 gametes can increase the number of chromosomes and restore euploidy in the offspring (2n+2) (Mayrose and Lysak, 2021). In addition, chromosome drive may be responsible for this diversity in chromosomal numbers (Camacho *et al*., 2000). Organisms can use a greater number of genes with an increase in the number of allelic variants. This is considered as interesting to plants in terms of synthesis rate and or the variability of the metabolic compounds produced (Pan et *al*., 2009). Duplicated genes can be maintained in copies (duplication divergence) which often undergo neo-functionalization or under-functionalization (Comai, 2005). A cytomorphologic study by counting chromosomes from the somatic cells (buds) on more genotypes is desirable to confirm these findings and achieve a genotype characterization associated with phenotypes. In addition, deepening in the study of the nature of chromosomes that makes the difference, their segregation, and their sequences is crucial. Furthermore, an analysis of genome size by a non-specific staining (like Iodide Propidium) to set standard genome size is important to facilitate the detection of cytotypes for rapid breeding program then avoid incompatibility during cross pollination process.

Conclusions

A. spinosa has three possible chromosome numbers representing the highest genetic diversity observed. This diversity reflects another side of the adaptability of this species to mitigate extreme climate conditions. These findings will make possible determination of high diversity and ecotypes identification belonging to this species based on chromosomal numbers.

Authors' Contributions

NAA Conceptualization; AEB, NAA, ST, HB, AT, RB, methodology; NAA, AEM, REB, MK, validation; AEB, NAA, HB, MK investigation; AEB, NAA, AEM, REB, RB, writing—original draft preparation; AEB, NAA, AT, RB writing—review and editing; NAA, AEM, REB, MK supervision; NAA, MK, RB funding acquisition.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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