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# Integrated Optimization of Seed Germination, Cytogenetic Techniques, and Digital Image Analysis for Karyotype construction in Selected Plant Species from North Cyrenaica, Libya

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**Keywords:**
Seed Germination.
Cytogenetic.
Karyotype Analysis.
Chromosome Visualization.
Digital Image Analysis.
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**ABSTRACT**

Seed germination and chromosome preparation are fundamental steps in cytogenetic studies, particularly in plant species with seed dormancy and specific germination requirements. In North Cyrenaica, Libya, cytogenetic information remains limited for many native and endemic plant species. The aim of this study was to optimize seed germination, chromosome preparation, and digital image analysis for karyotype construction in three plant species from North Cyrenaica, Libya: Arum cyrenaicum, Arbutus pavarii, and Origanum cyrenaicum. Different germination treatments were evaluated to obtain suitable root tips for chromosome analysis. Species-specific procedures were developed through the optimization of pretreatment, fixation, hydrolysis, staining, and slide preparation steps. Several image processing software tools were also assessed to improve chromosome visualization and karyogram construction. The three species exhibited different germination responses, reflecting variation in dormancy mechanisms and environmental requiremnets. The optimized procedures produced clear metaphase chromosomes suitable for chromosome counting and karyological analysis. Digital image analysis inhanced chromosome visualization and facilitated individual chromosome identification, homologous chromosome pairing, and karyogram construction. The developed workflow offers a reliable approach for seed germination and cytogenetic studies of native and endemic plant species, and provides valuable baseline data for future taxonomic and evolutionary research on the Libyan flora.

# التحسين المتكامل لإنبات البذوروالتقنيات السيتوجينية وتحليل الصور الرقمية لإعداد النمط النووي في انواع نباتية مُختارة من شمال برقة، ليبيا

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**الكلمات المفتاحية:**
انبات البذور.
علم الوراثة الخلوية.
تحليل الهيئة الصبغية.
اظهار الكروموسومات.
تحليل الصور الرقمية.
نباتات ليبيا.

**الملخص**

يُعد انبات البذور وتحضير الصبغيات من الخطوات الأساسية في الدراسات السيتوجينية، وخاصةً في الأنواع النباتية التي تتميز بسكون البذور واحتياجات انبات خاصة. و لا تزال المعلومات السيتوجينية المتعلقة بالعديد من الأنواع النباتية المحلية والمتوطنة في شمال برقة، ليبيا، محدودة. هدفت هذه الدراسة إلى تطوير وتحسين بروتوكولات انبات البذور واعداد الصبغيات وتحليل الصور الرقمية لإعداد النمط النووي (الهيئة الصبغية) لثلاثة انواع نباتية من شمال برقة، ليبيا، وهي: Arum cyrenaicum وArbutus pavarii وOriganum cyrenaicum. تم تقييم معاملات مختلفة للإنبات بهدف الحصول على قمم جذرية مناسبة للتحليل الصبغي. كما جرى تطوير اجراءات خاصة بكل نوع نباتي من خلال

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تحسين خطوات المعالجة المسبقة والتثبيت والتحلل الحمضي والصبغ واعداد الشرائح المجهرية. بالإضافة إلى ذلك، تم تقييم عدة برامج لمعالجة الصور الرقمية بهدف تحسين اظهار الصبغيات واعداد الكاريوغرام (المخطط النووي). اظهرت الأنواع النباتية الثلاثة استجابات مختلفة للإنبات، مما يعكس اختلاف آليات سكون البذور والمتطلبات البيئية. وقد اسفرت الإجراءات المحسنة عن الحصول على صبغيات الطور الاستوائي بصورة واضحة ومناسبة لعد الصبغيات واجراء التحليل الكاريولوجي. كما ساهم تحليل الصور الرقمية في تحسين اظهار الصبغيات، وسهل التعرف على الصبغيات الفردية، وتجميع الصبغيات المتماثلة في ازواج وبناء الكاريوغرام. تُوفر المنهجية المُطورة في هذه الدراسة إطاراً عملياً موثوقاً لدراسات انبات البذور والتحليلات السيتوجينية للنباتات المحلية والمتوطنة، كما تقدم بيانات أساسية مهمة يمكن الاستفادة منها في الدراسات التصنيفية والتطورية المستقبلية للنباتات الليبية.

## 1. Introduction

The flora of North Cyrenaica, although not highly diverse (about 1300 species), is of remarkable importance due to the presence of numerous Cyrenaican and Libyan endemic species, as well as several rare taxa with a southeastern Mediterranean distribution [1]. El-Jabal El-Akhdar (the Green Mountain) in Cyrenaica, Libya, is recognized as the region with the highest plant species diversity in the country, particularly within its depressions and valleys [2]. More than 50% of the endemic plant species are distributed in El-Jabel El-Akhdar [3].

Seed germination is a key process for forest regeneration, ecosystem restoration, and biodiversity conservation, particularly under changing environmental conditions [4]. In Libya, especially in El-Jabal El-Akhdar, understanding the germination requirements of native and endemic species is essential for sustaining vegetation cover, restoring degraded habitats, and supporting the long-term conservation of the region's unique biodiversity.

Several local studies have attempted to understand the germination requirements of plant species from El-Jabal El-Akhdar in order to support biodiversity conservation and forest restoration efforts. For example, [3] investigated the germination of *Arbutus pavarii* seeds, highlighting the environmental and thermal factors affecting germination success. Similarly, [5] and [6] studied the germination of *Ceratonia siliqua* seeds and identified specific treatments and stratification methods required to stimulate germination.

Successful seed germination is a prerequisite for cytogenetic investigations because germinated seeds provide actively growing root tips, which are the most commonly used source of dividing cells for chromosome visualization and karyotype analysis in higher plants [7]. Therefore, understanding germination requirements is essential for obtaining suitable material for chromosome studies.

Chromosome number and morphology are important characteristics of a species and its evolution. However, researchers often face significant challenges in this process, including seed dormancy, irregular germination rates, and specific environmental requirements such as temperature, light, and moisture [8]. In many plant species, dormancy mechanisms are complex and require stratification or pre-treatments to overcome physiological barriers to germination, which can delay experiments and reduce sample availability [9].

There are also several difficulties in obtaining good somatic metaphase spreads and observing chromosomes, including rigid cell walls, the tendency of chromosomes to resist spreading on microscope slides, and difficulty in obtaining a large number of dividing cells, as well as challenges in chromosome spreading and staining [10, 11, 12, 13]. Various methods have been developed to improve the preparation of microscopic slides and facilitate the controlled squashing of root tips, thereby enhancing chromosome dispersion, reducing mechanical damage, and increasing the reliability of cytogenetic observations [14, 11, 13, 15, 7].

Another issue is that many cytogenetic tools developed for chromosome measurements were unable to calculate karyotype asymmetry [16, 17, 18, 19, 20, 21, 22, 23, 24]. More recently, several software tools have been developed to facilitate chromosomal measurements and calculate karyotype asymmetry, such as MicroMeasure 3.3 [25], IdeoKar [26], and KaryoType [27], later updated and released as MATO [28]. These tools have been tested for years and successfully applied in previous studies [29, 30, 31, 32, 33], However, none of these programs can automatically recognize homologous chromosomes. Therefore, image editing software such as Adobe Photoshop (CS5 and CS6), Inkscape 0.92, and ImageJ have been used in many studies to manually cut and arrange homologous chromosomes [34, 35, 30, 36, 37, 38].

Digital image analysis has become an essential component in cytogenetic studies, as it significantly improves the accuracy and reproducibility of karyotype construction and chromosome measurements. Furthermore, image-based analysis facilitates the standardization of cytogenetic data and enables more reliable comparisons among species. Therefore, integrating digital image processing software with microscopic observations provides a more robust framework for karyomorphological investigations.

Karyomorphology is a powerful approach for obtaining useful baseline comparative information in systematic studies [39], including chromosome number, morphology, and chromosomal measurements [32]. It also helps evaluate genetic relationships among taxonomic categories and their divergence [40,41]. Karyological studies on Libyan flora are quite limited, especially in the El-Jabal El-Akhdar region in north Cyrenaica [42], recognized as an exceptional center of endemism within the country. This will provide valuable cytological information to support future taxonomic and evolutionary studies.

Therefore, the present study aimed to optimize seed germination protocols and cytogenetic procedures for chromosome visualization and karyotype construction in three plant species from North Cyrenaica, Libya.

## 2. Materials and Methods

### 2.1. Collection and Germination of Seeds

Plant materials were collected from their natural habitats in North Cyrenaica, Libya. Seeds were obtained from mature fruits and inflorescences, as collecting them before full ripening may result in incomplete embryo development and poor germination. Early collection may also reduce the amount of stored nutrients in the seeds, leading to lower germination rates. Several physical, chemical, and thermal treatments were tested sequentially to identify an effective protocol for breaking seed dormancy and obtaining actively growing root tips for karyotype analysis. Seeds were considered germinated when radicle emergence was visible.

#### 2.1.1 Arum *Cyrenaicum* Seed Germination

Mature fruits of *Arum cyrenaicum* (Araceae) were collected from the ancient ruins of Ptolemais, approximately 2 km from the Tolmitha region, on 17 May 2022. The seeds were carefully extracted from the fruits, thoroughly washed with distilled water to remove any pulp residues, and stored at 4°C until use (Fig. 1A).

A total of ten pre-germination treatments were applied to overcome seed dormancy and evaluate germination responses under different physical, chemical, and thermal conditions (Table 1). These treatments included soaking, scarification, chemical exposure, and temperature regimes, followed by incubation under the specified experimental conditions.

**Table 1:** Pre-germination treatments applied to *Arum cyrenaicum* seeds.

| Treat. | Pre-germination treatment | Conditions |
|---|---|---|
| T1 | Distilled water Soaking | 1-5days; incubation on moist filter paper at room temperature |
| T2 | Mechanical scarification + water soaking | 1-5 days; incubation at room temperature |
| T3 | Mechanical scarification + cold storage | 1 day; incubation at 4°C on moist paper towels |
| T4 | Sulfuric acid treatment | 50-90% $H_2SO_4$ for 5-20 min; rinsed for 30 min before incubation |
| T5 | Sandpaper abrasion + water soaking | 1 day; incubation at room temperature |
| T6 | Sandpaper abrasion + sulfuric acid treatment | 90% $H_2SO_4$ for 5-20 min; incubation at both room temperature and 4°C |
| T7 | Water soaking + scarification | 2 days; incubation in an air-conditioned room at 16-26°C |
| T8 | Water soaking + scarification + sulfuric acid treatment | 2 days soaking; 50- 90% $H_2SO_4$for 5-15 min with continuous stirring; rinsed with distilled water for 30 min |
| T9 | Water soaking+ scarification+ cold treatment | 1 day soaking; incubation at 4°C on moist paper towels |
| T10 | Hot water treatment | 100°C for 5-15 min; cooled at room temperature before incubation |

#### 2.1.2 *Arbutus Pavarii* Seed Germination

Fully fruits of *Arbutus pavarii* (Ericaceae) were collected from Wadi Emleka, approximately 19 km from the Tolmitha region, on 11 November 2021. Seeds were extracted manually from the fruits and thoroughly washed with distilled water to remove any remaining fruit pulp. Some seeds were stored at room temperature, while others were kept under refrigeration.

Prior to germination, seeds were soaked in distilled water for one day and then placed on moist filter paper in Petri dishes. Germination was conducted at room temperature for seven days (Fig. 1B). During periods of high ambient temperature, seeds were soaked in distilled water for one day and then placed on moist paper towels inside containers and stored at 4°C to enhance germination.

#### 2.1.3 *Origanum Cyrenaicum* Seed Germination

Mature inflorescences of *Origanum cyrenaicum* (Lamiaceae), containing fully developed seeds were collected from Wadi Bouhalfaya, approximately 3 km from the Al-Gubba region, on 6 November 2022. Seeds were extracted manually from the inflorescences, washed with distilled water, and stored at room temperature until use. Due to their very small size, the seeds were placed on moist filter paper in Petri dishes after soaking in distilled water for one day. They were then transferred to another moist filter paper in Petri dishes and incubated at room temperature for two days to germinate (Fig. 1C).

At high ambient temperatures, successful germination under normal conditions was difficult. Initial attempts using moist filter paper were unsuccessful due to rotting at higher temperatures, which negatively affected seed viability. Therefore, this method was avoided. Instead, seeds were placed in containers with a small amount of distilled water, sufficient to maintain moisture without full submersion. The containers were then stored in a refrigerator at temperatures ranging from 4 to 10°C for three to four days as a cold stratification treatment to break dormancy and enhance germination.

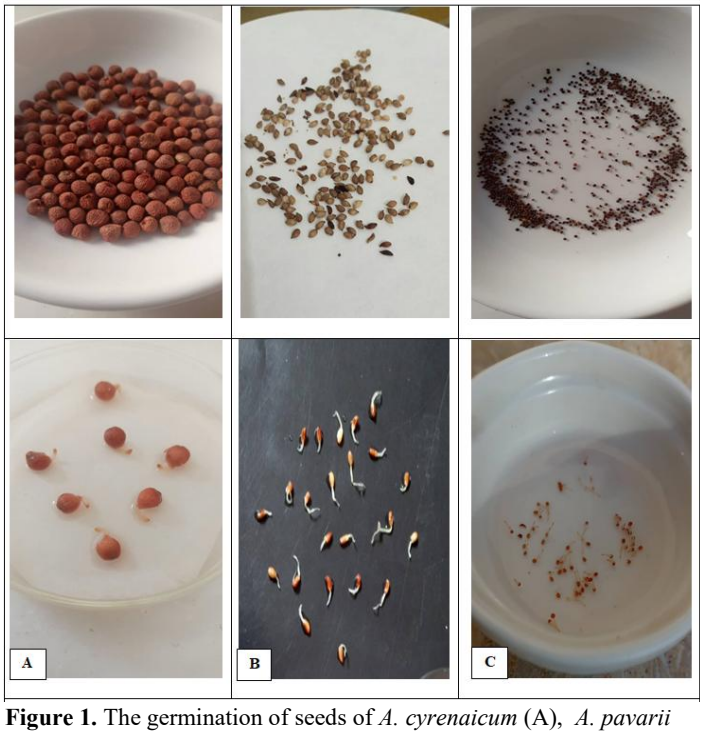


**Figure 1.** The germination of seeds of *A. cyrenaicum* (A), *A. pavarii* (B), and *O. cyrenaicum* (C).

### 2.2 Chromosome Preparation

#### 2.2.1 Chromosome Preparation of *Arum Cyrenaicum*, *Arbutus Pavarii*, and *Origanum Cyrenaicum*

Root tips obtained from germinated seeds were used for chromosome preparation (Fig. 2). Root tips at different developmental stages and lengths were collected and measured prior to cytological treatment to determine the most suitable stage for chromosome visualization. Root tips were pretreated with 0.1% colchicine solution (one tablet dissolved in 5 ml of distilled water) to arrest mitotic cells at metaphase. Different exposure periods were tested to determine the optimal conditions for metaphase chromosome accumulation and visualization. After colchicine treatment, root tips of *A. cyrenaicum* were washed with 45% acetic acid, whereas this step was omitted for *A. pavarii* and *O. cyrenaicum*. All samples were then fixed in Carnoy's solution I (ethanol: glacial acetic acid, 3:1, v/v) for 24 hours, followed by storage in 70% ethanol at 4°C until further use. Subsequently, the fixed root tips were hydrolyzed in 1N HCl at 60°C to soften the issues and facilitate chromosome spreading. The hydrolysis time was adjusted according to species: 20 minutes for *A. cyrenaicum*, and 30-32 minutes for both *A. pavarii* and *O. cyrenaicum*. After hydrolysis, samples of *A. cyrenaicum* and *O. cyrenaicum* were rinsed in cold distilled water for 20 minutes, while this step was optional for *A. pavarii*. Finally, root tips of *A. cyrenaicum* and *A. pavarii* were stained with 2% aceto-orcein solution (2g orcein dissolved in 100ml of 45% glacial acetic acid) for 17-20 minutes prior to slide preparation and microscopic observation.

#### 2.2.2 Slide Preparation

##### 2.2.2.1 *Arum Cyrenaicum* Slide Preparation

The growing root tip of *A. cyrenaicum* was excised using a sharp tool and placed on a clean glass slide. A drop of 45% acetic acid was added to the root tip, followed by placement of a coverslip (24mm × 40mm). The slide was then placed between folded filter paper and gently squashed using the thumb. Subsequently, the pointed end of a pencil was moved over the coverslip in a zigzag motion to eliminate air bubbles, facilitate cell separation, and enhance chromosome visibility.

##### 2.2.2.2 *Arbutus Pavarii* Slide Preparation

Due to the very small size and delicate nature of *A. pavarii* root tips, a modified preparation method was employed. The stained meristematic root tip was placed on a glass slide and carefully cut using a sharp tool. A small drop of distilled water was added, followed by placement of a small square piece of cellophane nylon over the sample. A coverslip (22mm × 22mm) was then gently placed on top of the cellophane.

To squash the sample, the coverslip was lightly tapped using the pointed end of a pencil. The slide was then placed between folded filter paper (or paper towels), and gentle pressure was applied using the thumb while moving the pencil tip in a zigzag motion across the coverslip. After squashing, the coverslip was removed, and the cellophane nylon containing the squashed material was carefully lifted and inverted onto a second clean slide. A new coverslip was placed on the second slide. Additionally, a coverslip was also placed on the original slide from which the cellophane had been removed. This modified technique proved highly effective for handling the delicate root tips of *A. pavarii*, allowing efficient elimination of air bubbles, improved cell spreading, and clear chromosome visualization, while minimizing the risk of coverslip breakage.

##### 2.2.2.3 *Origanum Cyrenaicum* Slide Preparation

Due to the extremely small size of *O. cyrenaicum* seeds, approximately the size of a grain of sand, special care was required during cytological slide preparation. Two to three germinated seeds were placed on a single slide, and the seed coat was carefully removed

using a sharp tool and gentle pressure to isolate the germinated root. A drop of 2% aceto-orcein stain (2g orcein in 100ml of 45% glacial acetic acid) was applied and left for 15-20 minutes. A small drop of distilled water was then added, followed by placement of a small square piece of cellophane nylon over the root tips. While the cellophane remained in place, it was quickly rinsed twice using Carnoy's solution or 70% ethanol. A coverslip (22mm × 22mm) was then gently placed over the cellophane. The slide was lightly tapped using the pointed end of a pencil to initiate squashing. It was then placed between two sheets of filter paper, and gentle zigzag pressure was applied using the pencil tip.

The coverslip was carefully removed, and the cellophane containing the squashed root tips was delicately transferred and inverted onto a second clean slide. A new coverslip was placed on the second slide, and another coverslip was also placed on the original slide. This modified method allowed delicate flattening of the root tips without damaging the cells, resulting in proper chromosome spreading and clear microscopic observation.

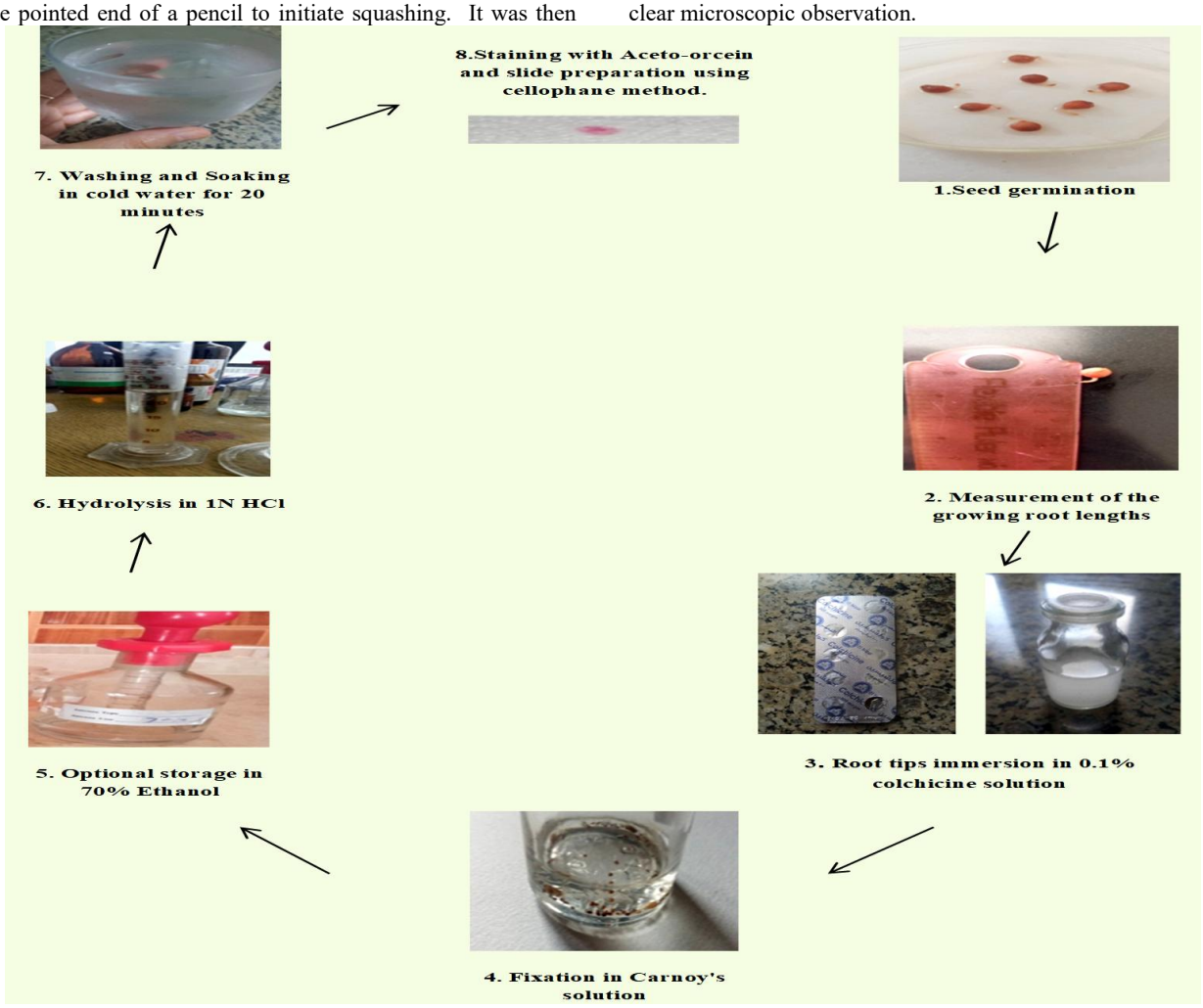


**Figure 2.** Cytogenetic procedure for karyotyping the studied plant species

**2.2.3 Chromosome Examination and Counting**

Chromosome examination and counting were performed using slides containing cells at the metaphase stage of mitosis (Fig.3). Metaphase spreads were observed and chromosomes were counted directly under an Olympus CX21 light microscope at 100x magnification with immersion oil. Images were captured using a YW5.0M digital camera. At least ten slides were prepared for each species, and a minimum of twenty well-spread metaphase cells were analyzed.

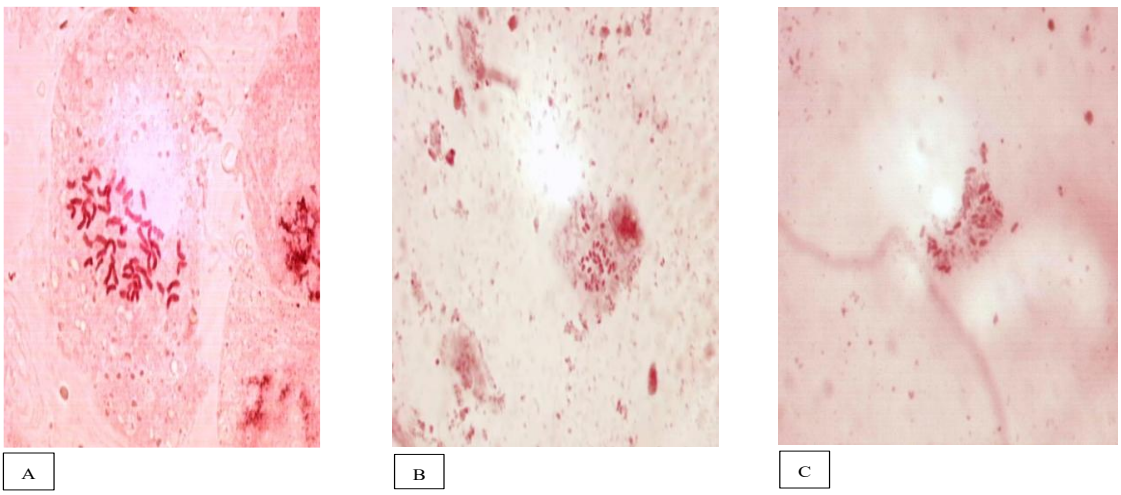


**Figure 3.** Metaphase chromosomes of the studied species observed under light microscopy (100×): (A). *Arum cyrenaicum*, (B). *Arbutus pavarii*, (C). *Origanum cyrenaicum*.

### 2.3 Image Processing and Software Analysis

#### 2.3.1 Overview

Due to the precise and complex nature of chromosome studies, it was essential to utilize digital and technical tools that enhance image quality and enable more objective and accurate analysis. Therefore, a set of software programs was used to assist in cropping and arranging chromosomes to construct the karyogram. These programs also supported the measurement of chromosome lengths and the calculation of karyotypic parameters, contributing to accurate karyotype analysis. This step is particularly important, as karyotype studies rely heavily on digital image processing and analytical tools, including software originally developed for engineering, scientific, and design purposes. Such tools provide flexible and precise image processing capabilities that support cytological research. The use of various software programs for chromosome image processing was incorporated gradually throughout the practical work. A single metaphase image (Fig. 4A) was initially used as a test case across multiple programs. The image was processed sequentially using AutoCAD 2022, Adobe Photoshop 2020, ImageJ, and PicsArt. Each program was evaluated based on image quality, ease of use, tool availability, and overall effectiveness in cropping and arranging chromosomes.

#### 2.3.2 AutoCAD 2022

Printed copies of the metaphase image (Fig. 4A) were first used to visually identify homologous chromosomes. Chromosomes with similar morphology were assigned matching numbers, as shown in (Fig. 4B). These chromosomes were then manually redrawn using carbon paper, cut out, and arranged into homologous pairs to produce (Fig. 4C).

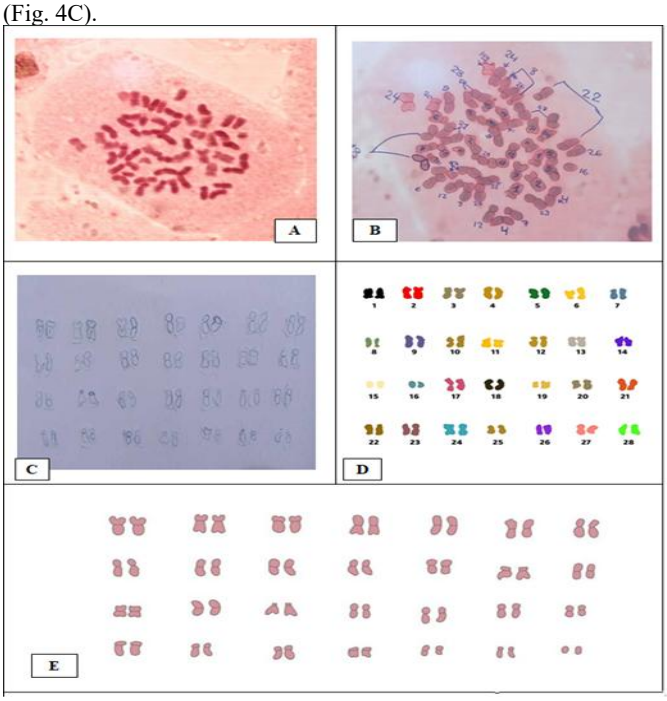


**Figure 4**. Original metaphase image (A), Numbering of homologous pairs (B), Manual drawing and arrangement (C), Initial AutoCAD 2020 output (D), Final AutoCAD drawing (E).

The resulting image was imported into AutoCAD 2022, developed by Autodesk, a software widely used in engineering and architectural design. Although primarily intended for technical applications, AutoCAD includes several features that proved useful in this biological context, including precision drawing tools based on vector graphics, full control over dimensions and line structures, layer management, support for multiple file formats (such as images and PDFs), and a flexible interface for efficient tool access. Although AutoCAD is not specifically designed for biological research, it was effectively used here to practice drawing and organizing chromosomes into homologous pairs. The initial output (Fig. 4D) lacked accuracy and precision; however, with continued training, the quality of chromosome drawings improved significantly, resulting in Fig. 4E. Despite its primary application in engineering, AutoCAD supported the conceptual understanding of karyotypic arrangement, which is essential in cytogenetic analysis, as each species possesses a unique chromosomal set that must be organized into homologous pairs for proper study.

#### 2.3.3 Adobe Photoshop 2020

The same metaphase image (Fig. 4A) was then processed using Adobe Photoshop 2020. This software provides a wide range of image processing tools, including opacity control, layer management, cropping tools, and brushes for color blending and image smoothing. In order to effectively apply these tools for chromosome cropping, it was necessary to study their functions and gain practical experience, a process that typically required one to two months. Despite its advanced capabilities, the use of this program presented several challenges. Adjusting tool settings such as "opacity" and the "Crop Tool" required training to determine the most suitable values for accurately cropping chromosomes. Additionally, marking chromosome edges for the cropped (Fig.4A) led to various issues. In some cases, chromosomes lost their original shape after marking the edges, such as chromosome 1 (Fig. 5A and 5B). In other cases, portions of chromosomes were lost due to adhesion between adjacent chromosomes, which subsequently affected chromosome length measurements, as seen in chromosome 2 (Fig. 5B). Further difficulties were encountered when using tools such as the Brush Tool, Blur Tool, and Smudge Tool to remove banding or striation effects from the image, as shown in chromosome 3 (Fig. 5B). These smoothing tools negatively impacted image quality and led to the loss of important chromosomal details, such as the location of the centromere, secondary constriction, and satellite regions, when present. Overall, Adobe Photoshop 2020 is a powerful image-processing tool; however, it is not the most suitable option for cytological analysis due to its complexity, the time required for training, and its limited precision when cropping chromosomes, which are delicate structures that must retain their original morphology.

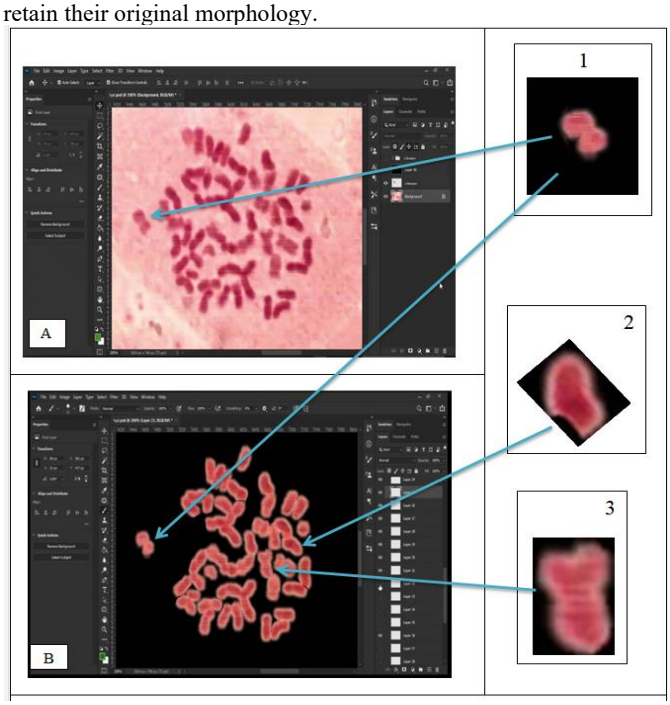


**Figure 5.** Chromosome cropping issues using Adobe Photoshop 2020: Altered shape of chromosome 1 (A & B), Loss of chromosome segments in chromosome 2 (B), and banding removal difficulties in chromosome 3 (B) (all indicated by arrows).

#### 2.3.4 ImageJ 1.46r

The same metaphase image (Fig. 4A) was processed using ImageJ 1.46r software (Java 1.6.0_20) [43], a widely used open-source program developed by the National Institutes of Health (NIH, USA). ImageJ enables the analysis and processing of scientific images, including the measurement of areas and pixel values, which can be converted into specific units based on the researcher's input. The software supports various image formats and is commonly applied in microscopy, cytology, and histological image analysis. In the current study, ImageJ was used for chromosome cropping; however, the process was relatively slow and time-consuming. The software does not provide a feature to collect and organize cropped chromosomes within a single file. In addition, it lacks efficient tools

for removing excess background or unwanted edges around each chromosome (Fig. 6A). Difficulties were also encountered in separating overlapping or closely adjacent chromosomes, and the overall image quality remained relatively low (Fig. 6B), making precise editing and cropping more challenging. The same image (Fig. 4A) was further processed using ImageJ to remove the lines caused by intense microscope lighting. This was done by selecting the "Process" option from the toolbar, then choosing the "Smooth" function. These lines made it difficult to identify the position of the centromere (primary constriction) for each chromosome, and also hindered the detection of secondary constrictions or satellites, if present.

To preserve the clarity of the image and provide accurate measurements, the image dimensions had to be consistent with the screen resolution. For example, the computer used in this study had a resolution of 720*1280 pixels, so the image had to be of the same or compatible size. The scale of the image was set by importing it into ImageJ, drawing a straight line, and selecting "Analyze" from the toolbar. A dropdown menu appeared, and "Set Scale" was chosen. The program then displayed the scale in pixels. The user would enter the image width (e.g.,720 pixels), a known distance (e.g.,10), and select micron (µm) as the unit. The program automatically calculated the scale as 72 pixels/ µm. Then, from the "Analyze" menu, "Scale Bar" was selected. A scale bar of 10µm was added, with font size 28, black or white color, and the position set to either the top or bottom of the image. To measure chromosome lengths, a straight line was drawn along each chromosome, then the "Analyze" > "Measure" tool was used, and the chromosome length appeared in micron. After measuring all chromosomes within the metaphase cell, the lengths were recorded manually on paper, focusing on the length of each chromosome. This was important because some chromosomes were very similar in shape, size, and centromere position. Therefore, arranging them into pairs was challenging and required precision, focus, and multiple measurements; relying on visual assessment alone was insufficient.

**2.3.5 PicsArt**

The same image, with the same scale, was then processed using the PicsArt program (26.0.5_lite). Multiple copies of the image were made while adjusting the percentage values of specific tools such as Size, Opacity, and Hardness.

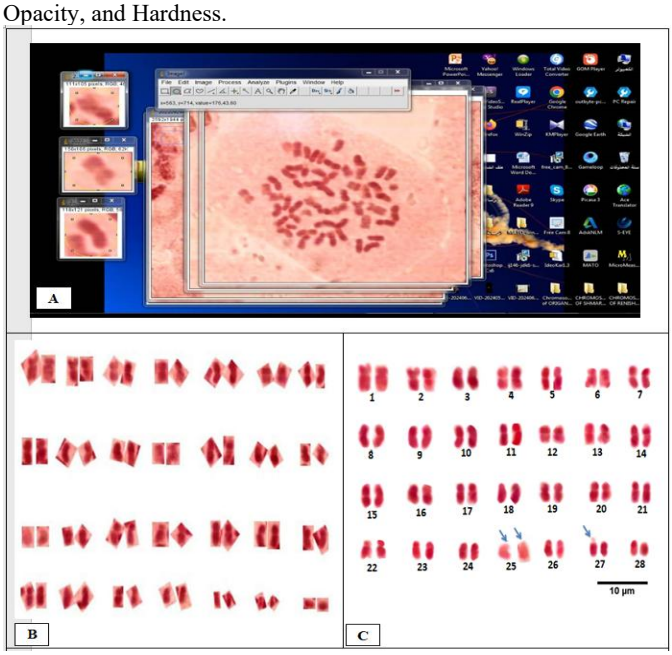


**Figure 6.** Chromosome trimming and separation issues in imageJ (A), Reduced image quality in ImageJ program (B), and PicsArt program (C).

Each chromosome was then cropped individually and placed on a white background, which was more suitable for editing. During cropping, attention was given to the presence or absence of secondary constrictions or satellite regions, as well as the clear visualization of centromere position (Fig. 6C).

**2.3.6 Ideokar**

Next, chromosomes that were similar in appearance were arranged into homologous pairs, from the longest to the shortest, according to the lengths measured earlier in imageJ, to construct the karyogram on the PicsArt (Fig. 6C). The karyogram image was then imported into the Ideokar software program [26]. The image scale was set to 10 µm using the ruler tool found in the main toolbar of the program. Each chromosome pair was measured accurately by pressing (Windows & "+") keys simultaneously to zoom in on the chromosome, making it appear clearly. This allowed the researcher to place markers accurately on both ends of the chromosome, determine the centromere position, and mark the presence of satellites or secondary constrictions, if visible. The software displayed live measurement readings. Moreover, higher image quality resulted in more accurate measurements and analyses Chromosomes are arranged from largest to smallest in the ideogram. Afterward, the chromosomal and karyological measurements were saved in an Excel file, and the mean and standard deviation of the chromosome and karyomorphological measurements were calculated using Microsoft Excel 2010.

## 3. Results and Discussion

### 3.1 Seed Germination

The germination responses of the studied species varied considerably depending on the applied treatments, reflecting differences in dormancy mechanisms and environmental requirements. The *Arum cyrenaicum* seeds required 15 days to germinate, with a germination percentage of 80%, whereas *Arbutus pavarii* seeds germinated within 7 days, showing a germination percentage of 85%. In contrast, *Origanum cyrenaicum* exhibited the highest germination percentage 92% and the fastest germination, occurring within 3-4 days under optimal conditions.

**3.1.1 *Arum Cyrenaicum***

Among the ten pre-germination treatments tested, most treatments including soaking, scarification, chemical scarification, and temperature variations, failed to induce germination in *A. cyrenaicum* seeds. Successful germination was observed only in one treatment, where seeds were soaked in distilled water for one day, followed by mechanical scarification using a sharp tool, and incubation on moist filter paper in a Petri dish at a temperature range of 12-15°C. Under these conditions, germination occurred within two weeks (Fig. 1A), indicating its effectiveness in breaking dormancy and promoting germination. This result is consistent with [6], which indicates that this treatment is among the most effective scarification methods for overcoming dormancy caused by seed coat hardness, as it increases the surface area available for water absorption and facilitates gas exchange required for germination[44]. Some seeds were also tested for germination over a period of nearly three months.

**3.1.2 *Arbutus Pavarii***

*Arbutus pavarii* seeds showed relatively better germination performance compared to *Arum cyrenaicum*. Successful germination was observed within seven days under the applied incubation conditions (Fig. 1B), indicating a lower degree of dormancy and a reduced requirement for complex pregermination treatments. However, high ambient temperatures reduced germination efficiency. In such conditions, transferring the seeds to a low temperature environment (4°C) after soaking improved germination success. This indicates that temperature plays a critical regulatory role in germination performance and may reflect adaptation to specific environmental conditions. Extended germination trials indicated that some seeds remained viable over longer periods, with germination observed up to approximately two months and three weeks.

**3.1.3 *Origanum Cyrenaicum***

Germination of *Origanum cyrenaicum* seeds was strongly influenced by temperature and moisture conditions (Fig. 1C). Initial attempts using moist filter paper under high temperature conditions resulted in seed decay due to rotting, which negatively affected seed viability. Successful germination was achieved by placing seeds in a small volume of distilled water (without full submersion) and incubating them at low temperatures (4-10°C). Germination occurred within 3-4 days. This response indicates that both temperature reduction and controlled moisture availability play a decisive role in overcoming seed dormancy. The improved germination under low temperature conditions may reflect ecological requirements necessary to overcome dormancy, indicating that such conditions mimic the species' natural habitat. Extended observations showed that some seeds could remain viable and germinate over a period of up to four months.

### 3.2 Cytogenetic Observations and Chromosome Preparation

Chromosome preparation is a key step in all cytogenetic techniques [13]. The applied protocol enabled successful preparation of metaphase chromosomes in all studied species, with species-specific responses to pre-treatment conditions. Colchicine is widely used in plant karyotype studies due to its ability to inhibit spindle fiber formation during mitosis, thereby arresting cells at metaphase where chromosomes are highly condensed and clearly visible. In the current study, a 0.1% colchicine solution was used for the pretreatment of root tip meristems in all investigated plant species and enabled the successful visualization of well-condensed and metaphase chromosomes. Chromosome preparation was strongly influenced by colchicine exposure time. The optimal pretreatment duration was 6h in *Arum cyrenaicum*, whereas 3h produced the best chromosome preparations in both *Arbutus pavarii* and *Origanum cyrenaicum*.

Root tip length also had a marked influence on chromosome quality. The most suitable chromosome preparations were obtained from root tips measuring 5-8 mm in *A. cyrenaicum*, 5-6 mm in *A. pavarii*, and 3-4 mm in *O. cyrenaicum*. At these developmental stages, root meristems exhibited active mitotic activity and yielded well-condensed metaphase chromosomes suitable for accurate chromosome counting and karyological analysis. To illustrate how optimal chromosome preparation conditions were determined, *A. pavarii* is presented as a representative example. Root tips measuring 3-4 mm and treated with colchicine for 2 h produced poorly resolved metaphase plates, with only a limited number of chromosomes visible (Fig. 7A).These preparations were considered unsuitable for reliable chromosome counting, most likely due to inadequate metaphase accumulation and insufficient chromosome condensation. In contrast, root tips measuring 5-6 mm and treated for 3 h consistently produced clear, well-spread metaphase chromosomes, allowing unambiguous chromosome counts. Under these optimized conditions, the somatic chromosome number was repeatedly determined as $2n = 26$. The reliability of this count is further supported by its agreement with chromosome numbers reported for other species of the genus *Arbutus* [45,46,47,48]. These observations demonstrate that inadequate pretreatment conditions may lead to incomplete chromosome visualization and erroneous counts, whereas appropriate root developmental stage and pretreatment duration are essential for obtaining accurate and reproducible chromosome numbers.

Carnoy's solution is an important step for rapid fixation of the material, while hydrolysis with 1N HCl helps to disrupt the pectin layer and cell wall structure, facilitating cell dispersion and enabling the observation of individual chromosome morphology under a microscope after squashing [49]. In this study, three treatments were employed: (1) fixation of root tips in Carnoy's solution I at 4°C for 12 h, followed by hydrolysis in 1N HCl at 40-60°C for 5, 7, 10, 15, and 18 min; (2) fixation at 4°C for 24 h, followed by hydrolysis in 1N HCl at 60°C for 20 min; and (3) fixation at 4°C for 24 h, followed by hydrolysis in 1N HCl at 60 °C for 30-32 min. However, 12 h fixation combined with hydrolysis for less than 20 min resulted in unclear chromosome structures that were not suitable for observation (Fig.7B). In contrast, treatment 2 was optimal for observing chromosomes in *A. cyrenaicum*, while treatment 3 was optimal for obtaining well-spread chromosomes in *A. pavarii* and *O. cyrenaicum*.

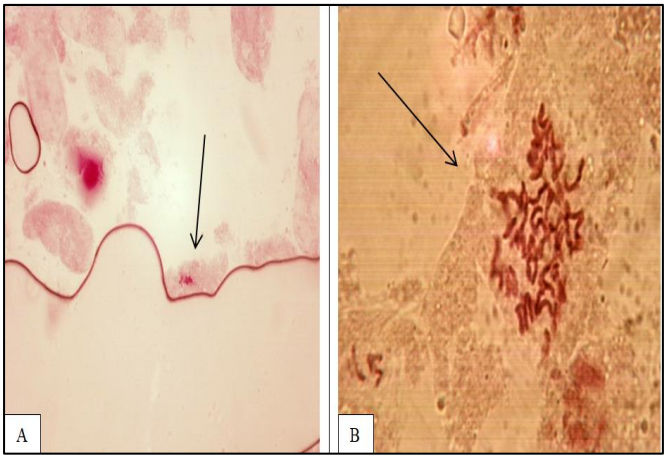


**Figure 7.** (A). Effect of root tip length and colchicine duration on chromosome visibility in *Arbutus pavarii*, (B). Effect of Carnoy's fixation solution and HCl hydrolysis on chromosome structures of *Arum cyrenaicum*.

Cold distilled water treatment played an important role in chromosome spreading by promoting cell separation and improving chromosome dispersion. In this study, cold distilled water at 4°C was used; a portion was applied to quickly rinse the root tips of *Arum cyrenaicum* and *Origanum cyrenaicum*, followed by immersion in the remaining cold water. Different soaking durations ranging from 2 to 18 min were tested; however, these durations were insufficient to achieve proper chromosome separation. The best chromosome spreads were obtained when the samples were soaked in cold water for 20 min, which improved chromosome clarity and separation. In contrast, the effect of this step was less pronounced in *Arbutus pavarii*. Well spread metaphase chromosomes could be obtained both with and without prolonged immersion in cold water, indicating that this treatment was not essential for chromosome separation in this species.

Staining quality also influenced chromosome visibility. Although 2% aceto-orcein produced satisfactory chromosome preparations in all investigated species, the use of a mixed stain consisting of 2% aceto-orcein and 1% aceto-carmine resulted in darker chromosome coloration and improved chromosome contrast in some preparations. This improvement facilitated chromosome visualization and karyological examination, particularly in preparations where chromosome boundaries were less distinct after staining with aceto-orcein alone.

Microscope configuration also affected chromosome visualization. During the initial stages of the study, chromosome observations performed using an Optika light microscope equipped with a YW5.0 M digital camera resulted in limited chromosome clarity and resolution (Fig. 8). These observations indicate that microscope optics can influence the quality of chromosome images and the accuracy of cytogenetic observations. Consequently, an Olympus CX21 microscope equipped with the same camera system was used for subsequent observations, providing clearer chromosome images and facilitating chromosome counting, morphological characterization, and karyotype analysis in all investigated species.

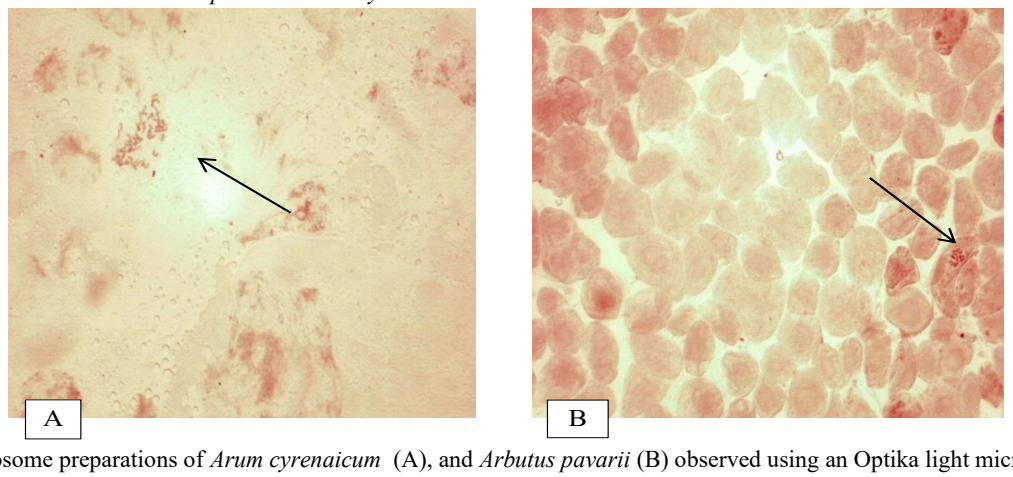


**Figure 8.** Chromosome preparations of *Arum cyrenaicum* (A), and *Arbutus pavarii* (B) observed using an Optika light microscope equipped with a Y.W 5.0 MP camera, showing limited chromosome clarity and resolution.

### 3.3 Slide Preparation Efficiency

Different slide preparation techniques were applied according to the physical characteristics of each species. For *A. cyrenaicum*, the conventional squashing method was sufficient to obtain clear metaphase spreads with good chromosome separation. In *A. pavarii*, the modified cellophane technique significantly improved slide quality by reducing mechanical damage and facilitating chromosome spreading, particularly due to the small and delicate root tips. Similarly, in *O. cyrenaicum*, the modified technique was essential due to the extremely small size of the germinated seeds and root tips. This approach enabled effective flattening of tissues and improved chromosome visibility. Overall, the modified preparation methods enhanced chromosome clarity, reduced air bubbles, and improved the reliability of cytological observations.

The somatic chromosome numbers of the studied species were successfully determined, confirming the reliability of the applied preparation methods. The chromosomes of *Arum cyrenaicum* ranged from 4.72±.21 to 2.88±.04 µm in length and were generally medium to small in size. In contrast, the chromosomes of *Arbutus pavarii* and *Origanum cyrenaicum* were smaller, ranging from 3.09 ±.03 to 1.98 ±.03µm and from 3.19 ±.01 to 1.98 ±.02 µm, respectively. The optimized chromosome and slide preparation, and image analysis procedures enabled the clear visualization of satellite chromosomes in all studied species, while secondary constrictions were observed only in *A. pavarii* and *O. cyrenaicum* (Fig. 9). These chromosomal features provided additional morphological characters that facilitated chromosome identification and karyotype interpretation. The successful detection of satellites and secondary constrictions demonstrates the quality of the developed cytogenetic protocol and the effectiveness of the image processing procedures used in this study. Such chromosomal markers are of considerable value in taxonomic and comparative cytogenetic studies, as they provide additional evidence for species characterization and the assessment of chromosomal relationships among taxa. The ecological, germination, and karyotype characteristics of the studied species are summarized in Table 2. The **ST** category and $\mathbf{M_{CA}}$ values indicated symmetrical karyotypes in all the selected plant species.

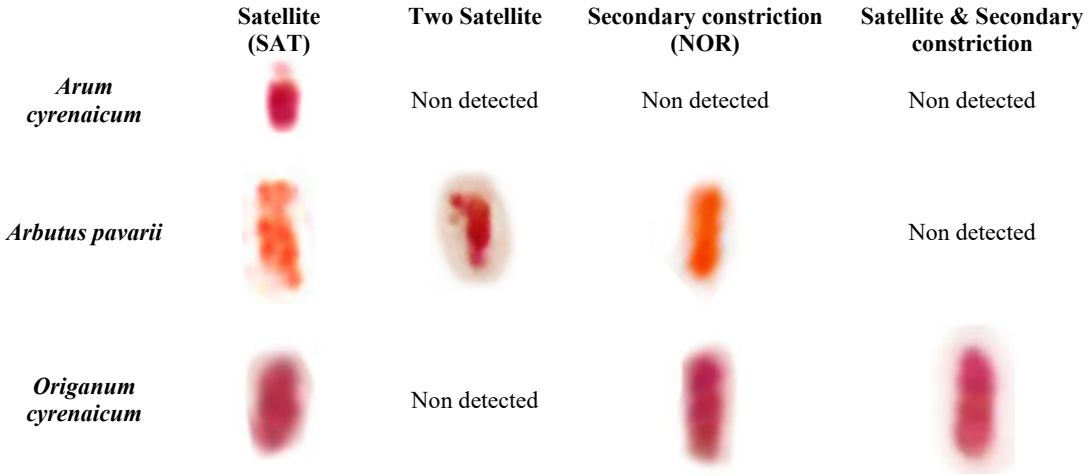


**Figure 9.** Satellite chromosomes and secondary constrictions observed in the studied plant species.

**Table 2:** Ecological, germination, and karyotype characteristics of the selected Cyrenaican species.

| Species | Site | Latitude (N) | Longitude (E) | Altitude (M) | Germination Period (Days) | Germination (%) | Somatic Chromosome Number (2*n*) & Karyotype Formula | ST | $\mathbf{M_{CA}}$ |
|---|---|---|---|---|---|---|---|---|---|
| *Arum cyrenaicum* | ancient ruins of Ptolemais | 32° 42′ 40″ | 20° 57′ 05″ | 12 | 15 | 80% | 2*n*=2*x*=56= 6M+38m+10sm+2st(2SAT) | 1A | 15.18 ±.72 |
| *Arbutus pavarii* | Wadi Emleka | 32° 43′ 51″ | 21° 01′ 24″ | 129 | 7 | 85% | 2*n*=2*x*=26= 4M+16m+6sm (4SAT) | 1A | 14.9 ±.18 |
| *Origanum cyrenaicum* | Wadi Bouhalfaya | 32° 76′ 50″ | 22° 24′ 29″ | 590 | 3-4 | 92% | 2*n*=2*x*=30= 6M+16m+8sm (3SAT) | 1A | 14.77 ±.66 |

**ST**= Stebbins' karyotype asymmetry classification (Stebbins, 1971), $\mathbf{M_{CA}}$=Mean centromeric asymmetry (Peruzzi & Eroğlu, 2013).

### 3.6 Comparative Evaluation of Image Processing Software

Following approximately three months and 17 days of practical training, several image processing software programs were evaluated using the same metaphase image (Fig. 4A), including AutoCAD 2022, ImageJ, Adobe Photoshop 2020, and PicsArt. The comparison revealed clear differences in performance among the tested programs. While ImageJ was effective for measurements, it showed limitations in chromosome isolation and organization. Adobe Photoshop required extensive training and presented challenges in maintaining chromosome morphology during cropping. AutoCAD was useful for conceptual arrangement, but lacked efficiency for detailed biological image processing. In contrast, PicsArt provided superior performance in chromosome visualization and editing. It enabled precise individual chromosome cropping, improved clarity, and allowed effective control over image properties such as size, contrast, brightness, and opacity. Additionally, it facilitated clearer identification of centromere positions, secondary constrictions, and satellite regions. Based on these advantages, PicsArt was considered the most suitable and reliable tool for chromosome image processing and karyogram construction in this study. These findings emphasize the importance of selecting appropriate digital tools to enhance the accuracy and efficiency of cytogenetic analysis.

## 4. Ideokar-Assisted Chromosome Measurements and Karyotype Analysis

IdeoKar was used for chromosome measurments and karyotype analysis in all investigated species. The software provided measurements of the short arm, long arm, centromere position, and total chromosome length, and automatically generated ideograms following chromosome measurement. In addition, IdeoKar automatically calculated several karyotype parameters, eliminating the need for separate statistical calculations. The software also included scale calibration tools that facilitated accurate chromosome measurement and karyological analysis.

## 5. Conclusion

This study demonstrated that successful cytogenetic investigation of plant species depends on the optimization of multiple interconnected

steps, beginning with seed germination and extending to chromosome preparation, visualization, and karyotype analysis. The developed protocols effectively addressed species-specific differences in germination behavior and chromosome preparation requirements, resulting in clear metaphase chromosomes suitable for cytogenetic characterization. The modified cellophane slide preparation technique and the application of digital image analysis further improved chromosome visualization, identification, and karyogram construction. In addition, the successful detection of chromosomal features such as satellite chromosomes and secondary constrictions highlights the quality and reliability of the optimized procedures. Overall, this study provides an integrated and reproducible methodological framework for seed germination, chromosome analysis, and karyotype construction, offering valuable tools and baseline cytogenetic data for future taxonomic, systematic, and evolutionary studies of the Libyan flora.

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