

In vitro-induced tetraploidy in sugarcane (*Saccharum officinarum* L. cv. CP57) using colchicine: protocol optimization and cytomorphological characterization

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Research Article

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Abstract

An efficient *in vitro* protocol was developed for inducing stable tetraploidy in sugarcane (*Saccharum officinarum* L. cv. CP57), a cultivar with restricted flowering that hinders conventional breeding. *In vitro*-grown plantlets were treated with colchicine (0, 50, 100, and 200 mg L⁻¹) for 24, 48, or 72 h. The 72 h treatments resulted in complete mortality. The optimal treatment for polyploidy induction was 50 mg L⁻¹ colchicine for 24 h, which yielded the highest survival rate (83.3 ± 4.4%) and successfully produced tetraploids. Morpho-physiological analysis 60 days post-acclimatization revealed that this treatment significantly reduced tiller number (by 58%) and plant height (by 18%) while increasing chlorophyll content (by 18%) compared to diploid controls. Stomatal density decreased by 76%, while stomatal length and width increased by 150% and 120%, respectively, serving as reliable indicators of polyploidy. Cytological analysis confirmed tetraploidy (2n = ~230 chromosomes) in 68% of cells from plantlets treated with 50 mg L⁻¹ colchicine for 24 h. Karyotype analysis showed a 58% increase in total chromosome length and enhanced karyotype symmetry in tetraploids. The established protocol effectively induces tetraploidy in sugarcane cv. CP57 and provides a valuable biotechnological tool for breeding programs aimed at enhancing genetic diversity and improving agronomic traits.

Introduction

Saccharum officinarum L., commonly known as sugarcane, is a perennial C₄ grass within the Poaceae family (tribe Andropogoneae) and ranks among the world's ten most strategically important crops. It is widely cultivated across tropical and subtropical regions (Verma et al. 2024). Sugarcane contributes approximately 70% of global sugar production (FAO 2023) and yields a range of value-added co-products, including bioethanol, pulp and paper, animal feed, and various chemical derivatives, highlighting its critical role in both food and energy security (Cardona et al. 2010; Dias et al. 2012). Consequently, sugarcane breeding programs focus on developing high-yielding, genetically diverse cultivars to meet escalating global demand.

In numerous sugarcane-growing regions, including Iran, conventional breeding is hindered by the species' limited flowering and seed production under local environmental conditions (Piperidis and D'Hont 2020). This necessitates the exploration of biotechnological approaches, such as *in vitro* culture and mutagenesis, to create novel genetic variation and overcome the narrow genetic base among commercial cultivars (Dhooghe et al. 2011). Among these strategies, artificial chromosome doubling via induced polyploidy is a powerful technique to enhance genetic diversity and agronomic performance (Sattler et al. 2016). Artificial polyploidy modifies genome size, influencing morphological, physiological, and cytological characteristics, thereby facilitating both evolutionary research and crop improvement (Van de Peer et al. 2017). Induced polyploids often exhibit superior attributes compared to diploids, such as increased biomass, higher levels of metabolites, and greater resilience to biotic and abiotic stresses, although these outcomes vary by genotype and species (Salma et al. 2017).

In vitro polyploidy induction is particularly advantageous as it allows for precise control of antimitotic agent concentration and exposure time on a large number of uniform explants under aseptic conditions. This is typically achieved using agents such as colchicine, trifluralin, or oryzalin, with colchicine being the most commonly used due to its effectiveness in inhibiting spindle formation during mitosis (Blakeslee and Avery 1937). Various explant types including apical meristems, microshoots, and callus cultures have been successfully treated with colchicine to produce polyploid plants (Nhut et al. 2022). However, optimal colchicine concentrations and exposure durations differ widely depending on species, cultivar, and explant type, typically ranging from 0.006% to 0.3% (w/v) (Dhooghe et al. 2011).

To date, no research has specifically investigated the Iranian sugarcane cultivar CP57 (*S. officinarum* L. cv. CP57) under local conditions. Like many cultivated *Saccharum* lines in Iran, CP57 exhibits poor flowering, restricting traditional breeding efforts. This study aimed to establish an optimized *in vitro* protocol for inducing polyploidy in CP57 and to assess its effects on morphological, physiological, and cytological properties. We hypothesized that colchicine treatment would reliably induce tetraploid individuals, identifiable through changes in chromosome number, stomatal traits, plant morphology, and chlorophyll content. Our specific objectives were to:

1. Establish and optimize an efficient *in vitro* protocol for inducing stable tetraploid plants of sugarcane cv. CP57.
2. Characterize the morphological effects of chromosome doubling (tiller number, plant height).
3. Evaluate stomatal density and dimensions as indicators of ploidy level.
4. Measure changes in chlorophyll content following colchicine treatment.
5. Conduct detailed karyotype analyses to verify ploidy status and assess genomic changes.

By establishing this optimized protocol, this research seeks to provide a reliable biotechnological tool to broaden the genetic foundation of sugarcane, enhancing key agronomic traits and stress tolerance.

Materials and Methods

Plant material and *in vitro* culture establishment

Shoot tips excised from lateral buds of sugarcane (*Saccharum officinarum* L. cv. CP57) were surface-sterilized and cultured on Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose, 2 mg L⁻¹ 6-benzylaminopurine (BAP), and 0.5 mg L⁻¹ kinetin. The pH was adjusted to 5.8 prior to adding 0.8% (w/v) agar and autoclaving at 121°C for 20 min. Cultures were maintained in a growth chamber at 25 ± 1°C under a 16-h photoperiod with a light intensity of 45–75 µmol m⁻² s⁻¹ provided by cool white fluorescent lamps. After four weeks, uniform plantlets approximately 4–5 cm in height were selected for colchicine treatment.

Experimental design and colchicine treatment

The experiment was arranged in a completely randomized design (CRD) with a 4 × 3 factorial arrangement. The factors were colchicine concentration (0, 50, 100, and 200 mg L⁻¹) and exposure duration (24, 48, and 72 h). Each treatment consisted of six replicates, with five plantlets per replicate.

For treatment, uniform plantlets were placed in 250 mL culture vessels containing 100 mL of liquid MS medium with the corresponding colchicine concentration. The vessels were placed on a rotary shaker at 30 rpm and maintained in complete darkness at 25°C for the designated durations. Following exposure, plantlets were rinsed three times with sterile distilled water and transferred to solid MS rooting medium containing 3 mg L⁻¹ naphthaleneacetic acid (NAA).

Acclimatization of plantlets

After two weeks on rooting medium, plantlets with well-developed roots were transplanted into 10-cm pots filled with a sterilized 1:1 (v/v) mixture of cocopeat and perlite. The plantlets were acclimatized in a greenhouse at 25 ± 2°C and 60–70% relative humidity. They were irrigated as needed and fertilized weekly with half-strength Hoagland's solution.

Morphological and stomatal analysis

Sixty days post-acclimatization, five plants per replicate (n = 30 per treatment) were evaluated for the following traits:

Tiller number: The total number of new tillers per plant was counted.

Plant height: Measured from the substrate surface to the tip of the youngest fully expanded leaf (cm).

Stem diameter: Measured 5 cm above the substrate surface using a digital caliper (mm).

For stomatal analysis, impressions were taken from the abaxial surface of the third fully expanded leaf between 09:00 and 11:00 h using clear nail varnish. The dried impressions were mounted on glass slides and observed under a light microscope (Olympus CX43).

Stomatal density was determined by counting the number of stomata per mm² in three randomly selected fields of view at 400x magnification.

Stomatal length and width were measured for ten stomata per field across three fields per plant using image analysis software (ImageJ, NIH, USA).

Cytological examination and karyotype analysis

Root tips (~ 1 cm) were collected from control and treated plants, pre-treated with 0.002 M 8-hydroxyquinoline for 4 h at 4°C, and fixed in Carnoy's solution (3:1 ethanol:glacial acetic acid) for 24 h. The root tips were hydrolyzed in 1 N HCl at 60°C for 10 min, stained with Schiff's reagent for 1 h, and

squashed in a drop of 45% acetic acid. Metaphase spreads were examined under a light microscope (Nikon Eclipse E200).

Chromosome counts were performed on fifty well-spread metaphase cells per treatment. Karyotype analysis was conducted on five complete metaphase plates per ploidy level using KaryoType software (v2.0). The following parameters were determined: long arm (L) and short arm (S) lengths, total chromosome length (TCL), arm ratio (AR), centromeric index (CI), intrachromosomal asymmetry index (A_1), interchromosomal asymmetry index (A_2), and total form percentage (TF%). Chromosomes were classified based on arm ratio, and karyotype symmetry was assessed according to Stebbins (1971).

Statistical analysis

Data were subjected to two-way analysis of variance (ANOVA) using R software (v4.3.1), with colchicine concentration and exposure duration as fixed factors. The assumptions of homogeneity of variances were verified using Levene's test. Mean comparisons were performed using Tukey's Honestly Significant Difference (HSD) test at a 5% probability level ($p \leq 0.05$). Data are presented as mean $\pm$ standard error (SE).

Results

Survival and growth post-treatment

Exposure to colchicine for 72 h at all concentrations (50, 100, and 200 mg L⁻¹) resulted in 100% mortality of *Saccharum officinarum* cv. CP57 plantlets, and these treatments were excluded from further analysis. Survival rates for 24 h and 48 h treatments are presented in Table 1. After 24 h, plantlets treated with 50 mg L⁻¹ colchicine exhibited the highest survival rate ($83.3 \pm 4.4\%$), decreasing to $50.0 \pm 6.5\%$ at 100 mg L⁻¹ and $16.7 \pm 3.2\%$ at 200 mg L⁻¹ ($p \leq 0.05$). Extending exposure to 48 h further reduced survival: $66.7 \pm 5.3\%$ at 50 mg L⁻¹, $33.3 \pm 4.4\%$ at 100 mg L⁻¹, and 0% at 200 mg L⁻¹. Control plantlets (0 mg L⁻¹) maintained 100% survival across both durations.

Morphological Changes

A two-way ANOVA revealed highly significant main effects of colchicine concentration and exposure duration ($p < 0.001$) on tiller number and plant height, with significant interaction effects ($p < 0.01$) for both traits (Table 1).

Control plantlets yielded a mean tiller count of 12.0 ± 1.0 per plant. Following 24 hours of colchicine treatment, tiller numbers exhibited a concentration-dependent reduction: 5.0 ± 0.8 (−58%) at 50 mg L⁻¹, 3.5 ± 0.6 (−71%) at 100 mg L⁻¹, and 1.8 ± 0.5 (−85%) at 200 mg L⁻¹. Prolonging the exposure to 48 hours resulted in a further decrease in tiller counts (Fig. 1).

The mean height of control plants was 30.5 ± 1.2 cm. Following 24 hours of colchicine treatment, plant heights decreased in a concentration-dependent manner: 25.0 ± 1.0 cm (−18%) at 50 mg L⁻¹, 20.0 ± 1.0

cm (–34%) at 100 mg L⁻¹, and 15.2 ± 0.8 cm (–50%) at 200 mg L⁻¹. A 48-hour exposure further reduced plant height (Fig. 2).

Table 1

Two-way ANOVA summary (MS values) for morphological, stomatal, and physiological traits.

Source of Variation	df	Tiller Number	Plant Height (cm)	Chlorophyll Content (SPAD)	Stomatal Density	Stomatal Width*	Stomatal Length*
Colchicine (C)	3	232.38**	441.08**	294.76**	180.18**	1496.36**	1186.26**
Duration (D)	1	0.74 (ns)	108.79**	70.67*	134.32**	401.57**	339.38**
C × D	3	10.44**	28.96**	35.11**	85.38**	224.59**	360.60**
Error	35	0.42	3.10	1.69	1.57	11.87	15.45
CV (%)		13.68	8.05	7.31	9.43	7.79	10.72

Chlorophyll Content

The highest chlorophyll content (SPAD value) was recorded in plants treated with 50 mg L⁻¹ colchicine for 24 hours (45.8 ± 1.3), representing an 18% increase compared to the 24-hour untreated control group (38.9 ± 1.1). Conversely, the lowest chlorophyll content was observed in plants treated with 200 mg L⁻¹ colchicine for 48 hours (8.7 ± 0.5). All treatments differed significantly from each other and the controls (Fig. 3).

Stomatal Traits

ANOVA indicated significant effects of colchicine concentration and exposure duration (p < 0.001) on all stomatal traits, with significant interaction effects (p < 0.05).

In control leaves, stomatal density averaged 22.5 ± 1.2 stomata mm⁻². Following 24 h of colchicine treatment, density dropped to 5.4 ± 0.8 stomata mm⁻² (–76%) at 50 mg L⁻¹, 4.1 ± 0.7 mm⁻² (–82%) at 100 mg L⁻¹, and 2.0 ± 0.5 mm⁻² (–91%) at 200 mg L⁻¹. Forty-eight-hour treatments further reduced density (Fig. 4).

Control stomata measured 19.8 ± 0.6 µm (length) and 24.2 ± 0.7 µm (width). The 50 mg L⁻¹/24 h treatment enlarged stomata to 49.5 ± 1.0 µm (+ 150%) in length and 53.2 ± 1.2 µm (+ 120%) in width. The 200 mg L⁻¹/24 h treatment produced the largest stomata (60.2 ± 1.5 µm length, + 204%; 65.3 ± 1.5 µm width, + 170%) (Fig. 5, 6, 7).

Chromosome Number and Karyotype Analysis

All control metaphase spreads consistently displayed a diploid chromosome count of 2n = 114 (Fig. 8). In the 50 mg L⁻¹/24 h treatment, 68% of metaphase cells (34/50) exhibited approximately 2n ≈ 230

chromosomes; the remainder retained the diploid number or showed minor aneuploidy. At 100 mg L⁻¹/24 h, only 42% of cells (21/50) reached the tetraploid count (Fig. 9).

Karyotype parameters for confirmed tetraploid plants (50 mg L⁻¹/24 h) versus diploid controls are summarized in Table 2. The detailed measurements for all individual chromosomes of the tetraploid are provided in Supplementary Tables. Total chromosome length (TCL) averaged $207.0 \pm 1.8 \mu\text{m}$ in diploids and $328.2 \pm 2.1 \mu\text{m}$ in tetraploids (+ 58%). The intrachromosomal asymmetry index (A_1) decreased significantly from 0.52 ± 0.01 to 0.48 ± 0.01 ($p < 0.05$), indicating a more symmetric karyotype. Stebbins' symmetry class shifted from B2 (diploid) to B1 (tetraploid).

Cytological analysis confirmed the ploidy level of both control and treated plants. Metaphase spreads from control plantlets consistently revealed a diploid chromosome count of $2n = 114$ (Fig. 10). In contrast, root tip cells from plantlets treated with 50 mg L⁻¹ colchicine for 24 h exhibited a tetraploid count of approximately $2n \approx 230$ chromosomes (Fig. 11). The karyogram of the control plant (Fig. 12) further illustrates the organized diploid set, while the karyogram of the treated plant (Fig. 13) visually demonstrates the successful chromosome doubling and the increased karyotype symmetry characteristic of the induced tetraploids.

Table 2

Karyotype parameters comparing diploid ($2n = 114$) and tetraploid ($2n \approx 230$) CP57 sugarcane.

Row	Index	Control	Treated
1	Number of Chromosomes ($2n$)	114	230
2	Total Genome Length (TL)	207.01	327.16
3	Coefficient of Variation (CV)	22.42	19.70
4	Asymmetry Index (AI)	5.65	7.68
5	Stebbins Symmetry Class (SC)	B2	B1
6	Relative Chromosome Length (RL)	1.74	0.87
7	Centromeric Index (CI%)	31.70	33.01
8	Total Form of Karyotype (TF%)	33.99	36.30
9	Chromosome Form Percentage (F%)	0.59	0.31
10	Relative Chromatin Content (VRS)	3.63	2.84
11	Average Chromosome Length (CL)	3.63	3.84
12	Relative Length of Shortest Chromosome (S%)	1.19	0.96
13	Range of Relative Chromosome Length (DRL)	2.37	1.88
14	Interchromosomal Asymmetry Index (A2)	0.16	0.21
15	Average Length of Long Chromosome Arms (L)	2.39	1.81
16	Average Length of Short Chromosome Arms (S)	1.23	1.03
17	Intrachromosomal Asymmetry Index (A1)	0.52	0.48
18	Karyotype Asymmetry Index (ASK)	66.02	63.88
19	Difference Index of Two Chromosome Arms (d-Value)	1.46	1.52
20	Ratio of Long to Short Chromosome Arms (AR)	1.94	1.76
21	Value of Short to Long Chromosome Arms (r-Value)	0.51	0.56

Cellular Abnormalities

Some colchicine-treated samples exhibited interphase nuclear abnormalities, including multinucleate cells (up to six nuclei per cell of varying sizes) and disrupted chromosome migration, reflecting colchicine-induced spindle inhibition (Fig. 14).

Discussion

This study successfully established an efficient *in vitro* protocol for tetraploid induction in sugarcane cv. CP57, with treatment using 50 mg L⁻¹ colchicine for 24 h proving highly effective in inducing stable tetraploidy. The 100% mortality observed at 72 h exposures corroborates earlier findings that prolonged colchicine exposure severely compromises cell viability (Touchell et al. 2020). Our results agree with previous studies in sugarcane hybrids, where moderate concentrations and shorter durations balanced survival and induction efficiency (Khan et al. 2008; D Hont, 2005).

The morphological alterations—reduced tillering and plant height—are characteristic of artificial polyploids, often resulting from mitotic disruption and altered phytohormone transport (Chen et al. 2007). The dramatic changes in stomatal traits (increased size, decreased density) serve as reliable phenotypic markers for polyploidy in sugarcane, consistent with findings in other specie (Zhang et al. 2024).

Wang et al (2021) Reports that the chlorophyll content in tetraploid is significantly increased compared with the diploid variety, contributing to higher photosynthetic capacity. This enhancement suggests a potential for improved photosynthetic efficiency and biomass yield, though field validation is necessary.

Cytological analysis confirmed the protocol's efficacy, with a high rate (68%) of tetraploid cells achieved at the optimal treatment. The observed karyotype changes, including increased total chromosome length and enhanced symmetry, are consistent with genomic restructuring following polyploidization (Sattler et al. 2016). The presence of aneuploid cells and chimeras is a typical challenge in colchicine-induced polyploidy (Dhooghe et al. 2011), underscoring the need for careful selection or additional mitotic cycles to achieve stable, homogeneous tetraploid lines.

Conclusion

The optimized *in vitro* protocol employing 50 mg L⁻¹ colchicine for 24 h effectively induces tetraploidy in sugarcane cv. CP57 with high survival and induction rates. The tetraploid lines were unequivocally characterized through a combination of stomatal, morphological, physiological, and detailed cytological analyses. This protocol provides a valuable biotechnological tool for sugarcane breeding programs, enabling the creation of novel genetic diversity for the development of cultivars with enhanced yield potential and stress tolerance. Future work will focus on the agronomic evaluation of these tetraploid lines under field conditions and the use of flow cytometry for high-throughput ploidy screening.

Declarations

Funding:

This work was supported by the Agricultural Sciences and Natural Resources University of Khuzestan.

Conflict of interest:

The authors declare they have no financial or non-financial competing interests.

Ethics approval:

Not applicable.

Consent to participate:

Not applicable.

Consent for publication:

Not applicable.

Availability of data and materials:

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability:

Not applicable.

Authors' contributions:

MFZ: Investigation, Methodology, Formal analysis. PPM: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. KST: Methodology, Investigation (Cytogenetic analysis).

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Figures

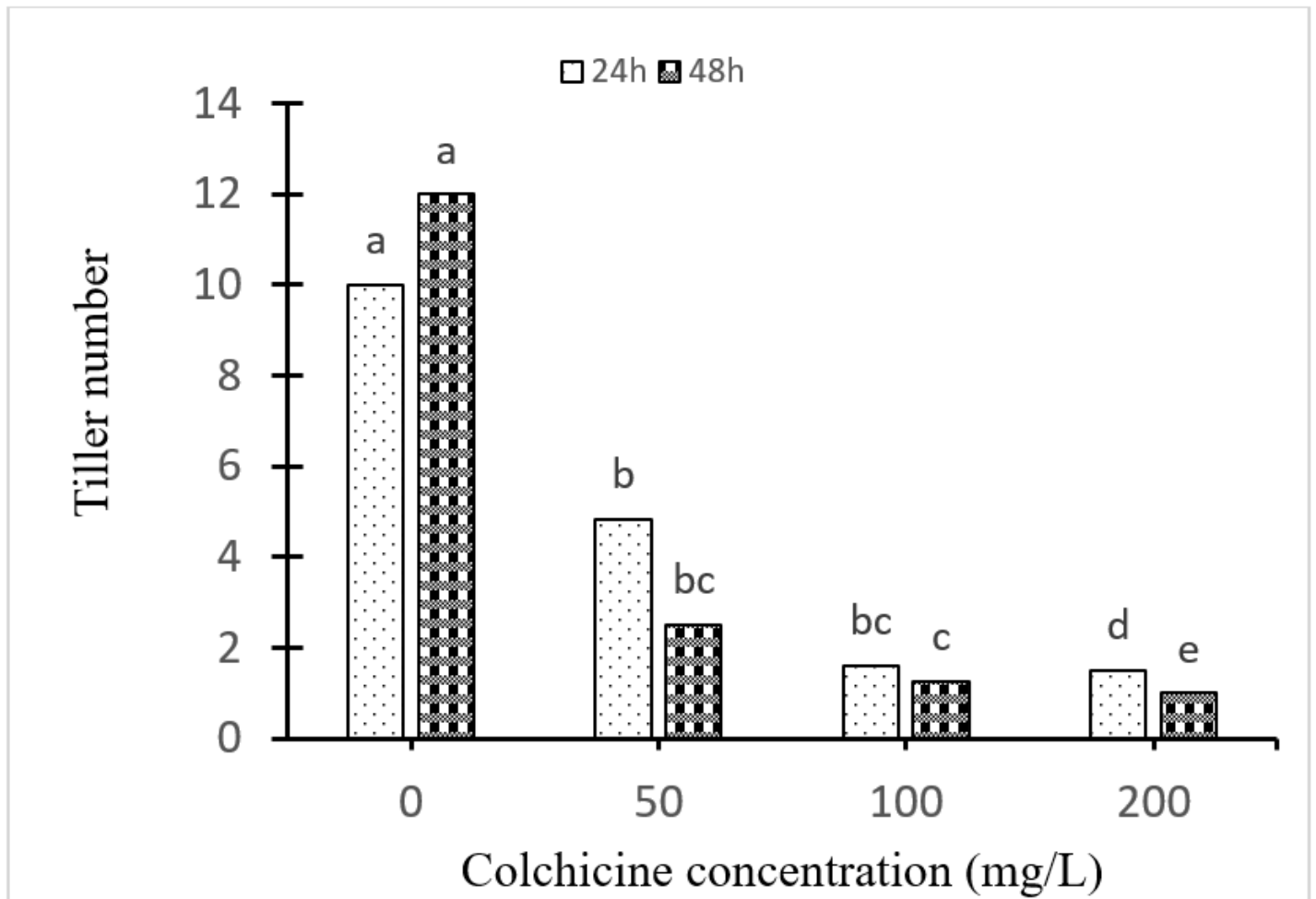


Figure 1

Effect of Different Concentrations and Application Durations of Colchicine Treatment on tiller number.

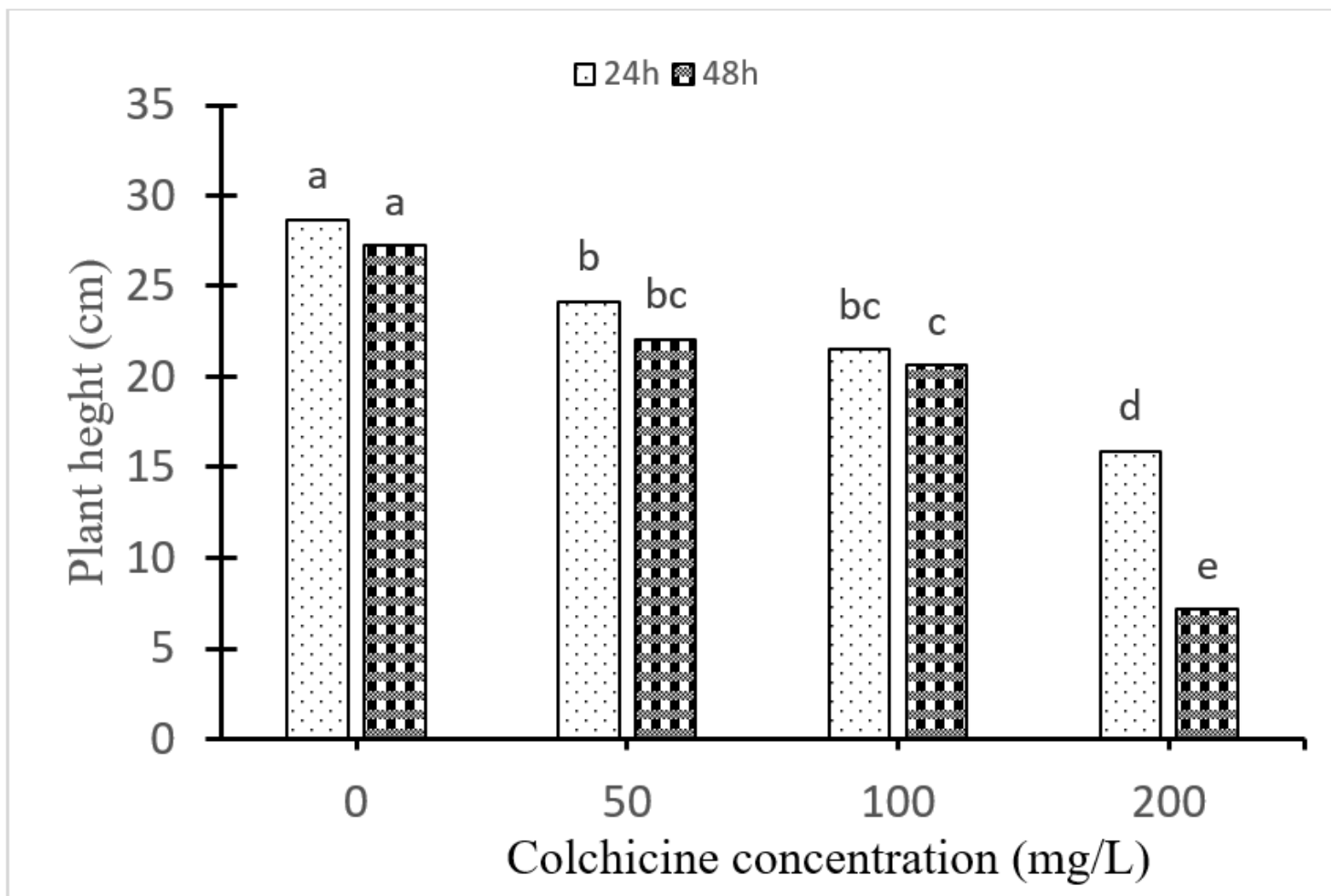


Figure 2

Effect of Different Concentrations and Application Durations of Colchicine Treatment on plant height

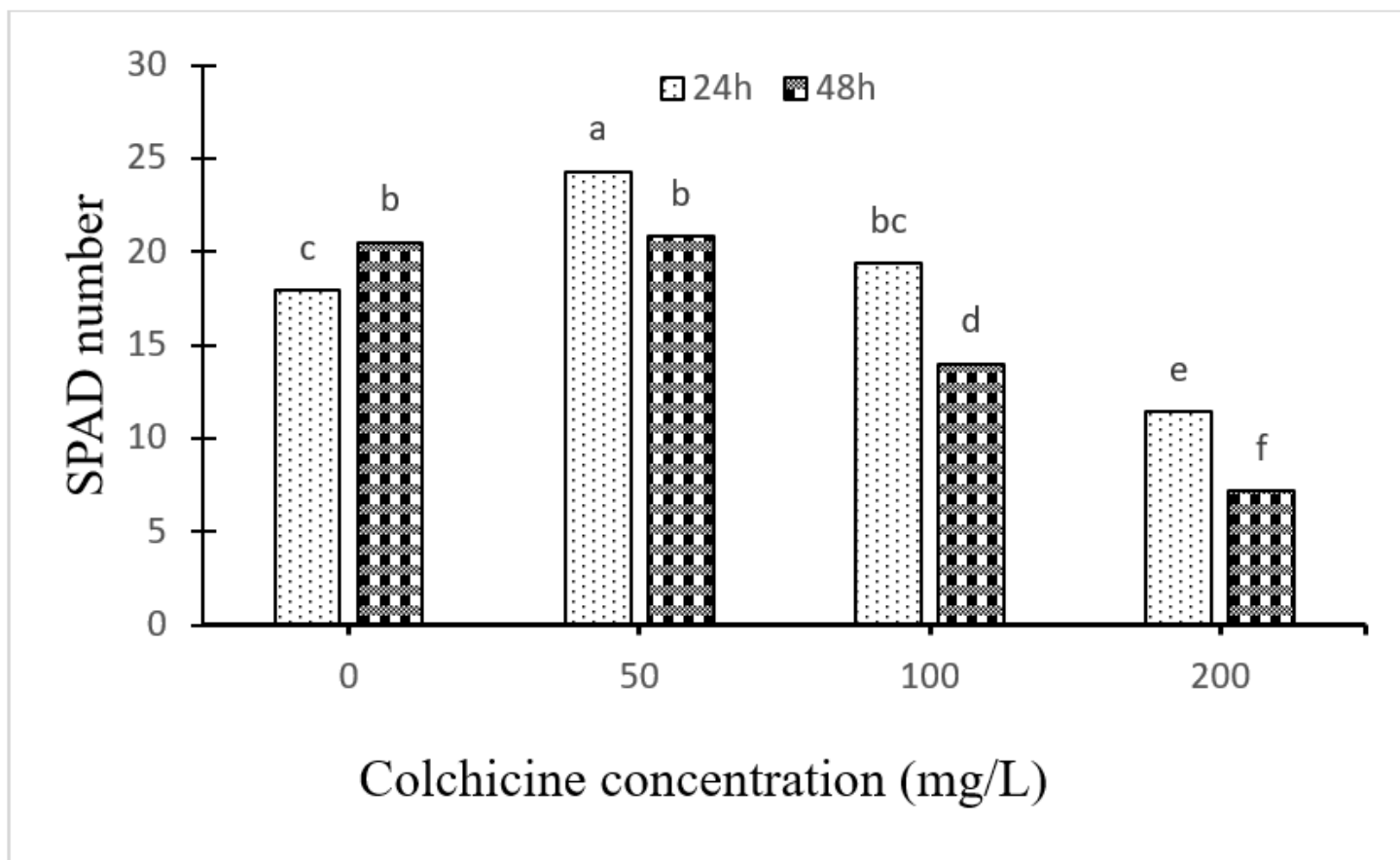


Figure 3

Effect of Different Concentrations and Application Durations of Colchicine Treatment on Chlorophyll Content

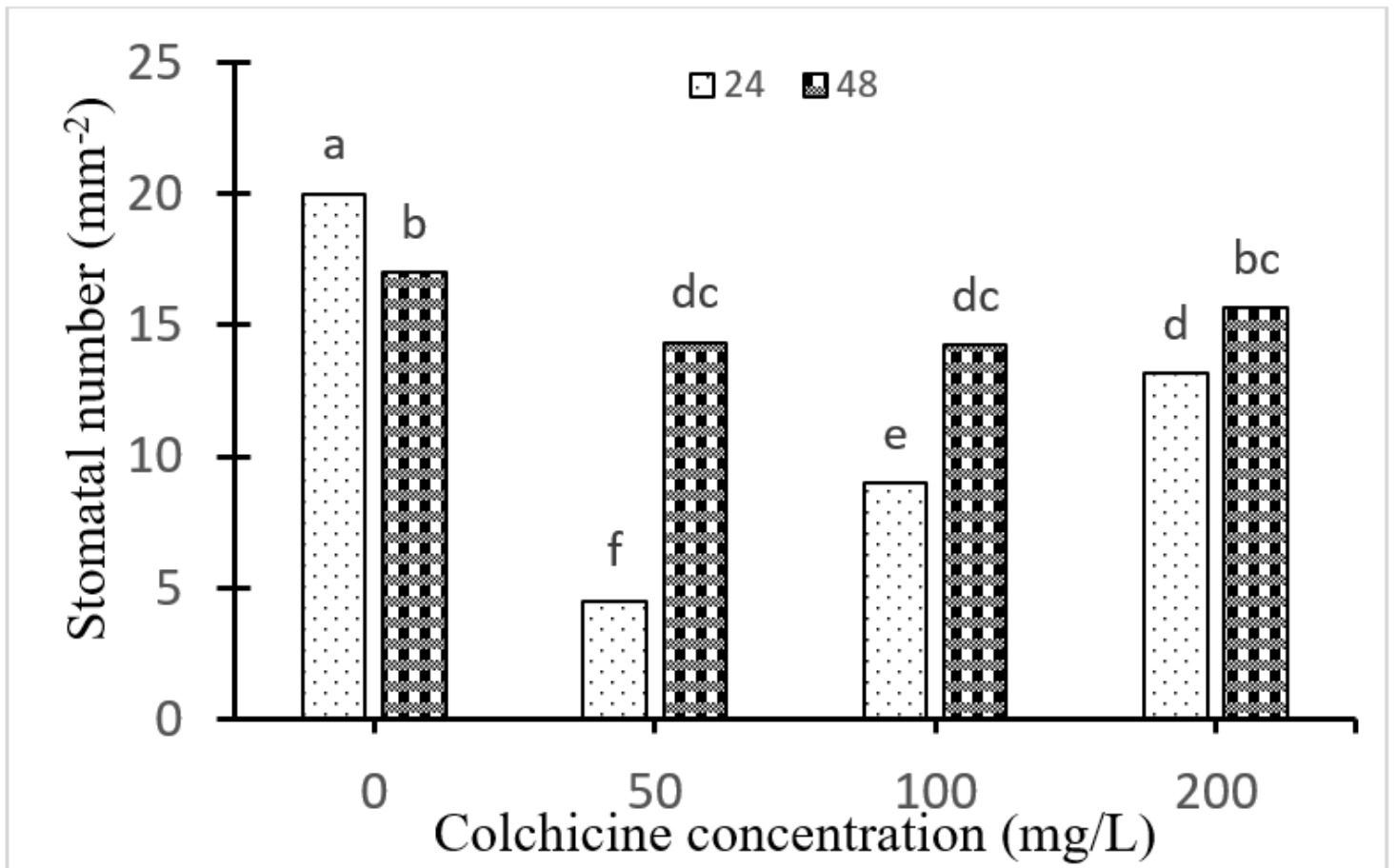


Figure 4

Stomatal density variations under different colchicine concentrations and durations.

Chromosome Number and Karyotype Analysis

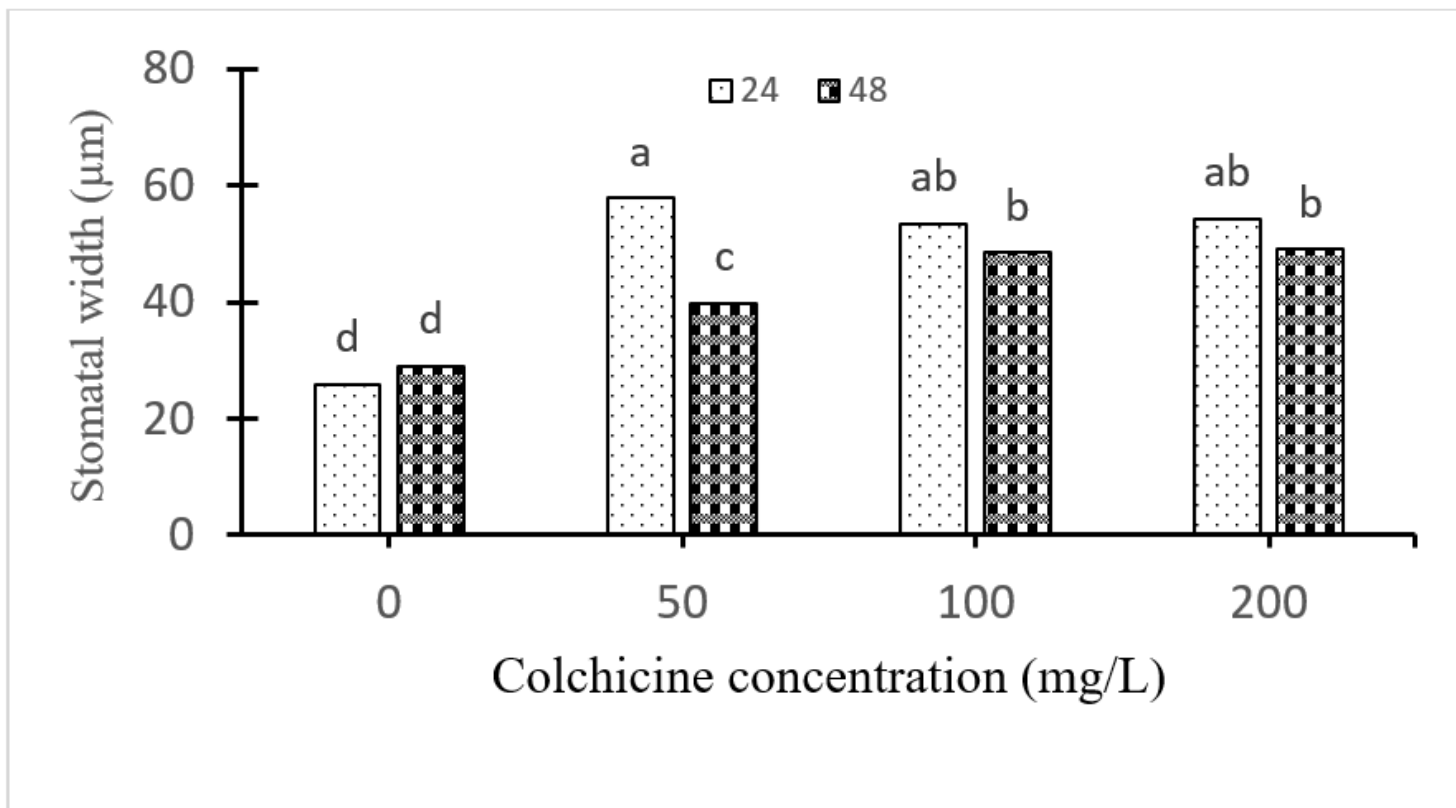


Figure 5

Stomatal width variations under different colchicine concentrations and durations.

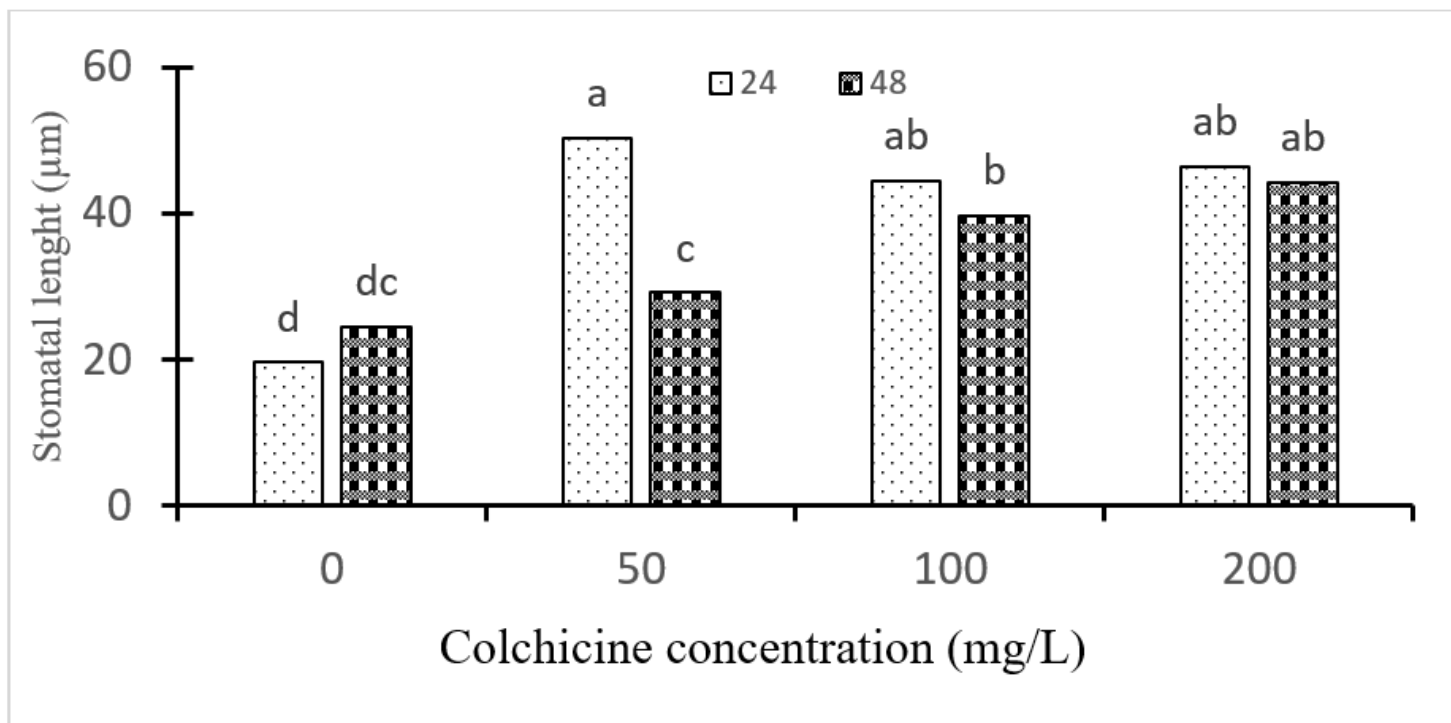


Figure 6

Stomatal length variations under different colchicine concentrations and durations.

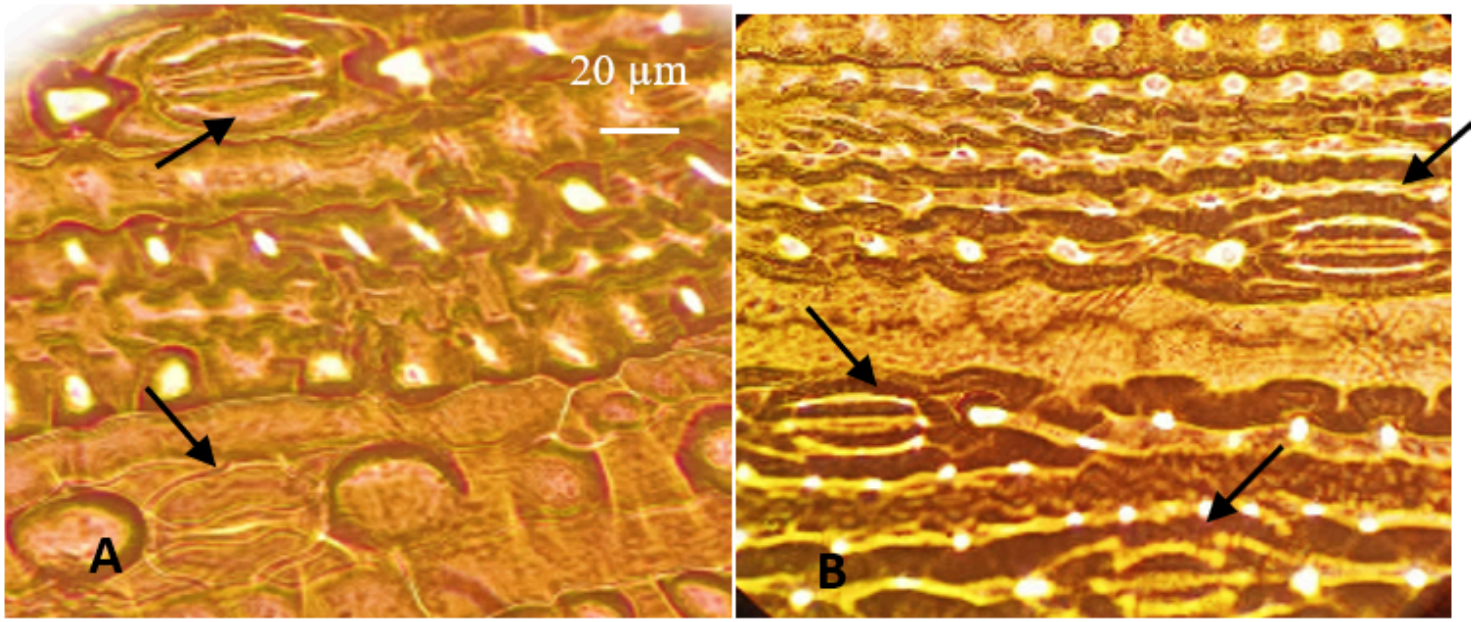


Figure 7

Stomatal morphology at ×100 magnification. (a) Stomatal structure in control (untreated) plant. (b) Stomatal structure in colchicine-treated plant (50 mg L⁻¹ colchicine for 24 h).

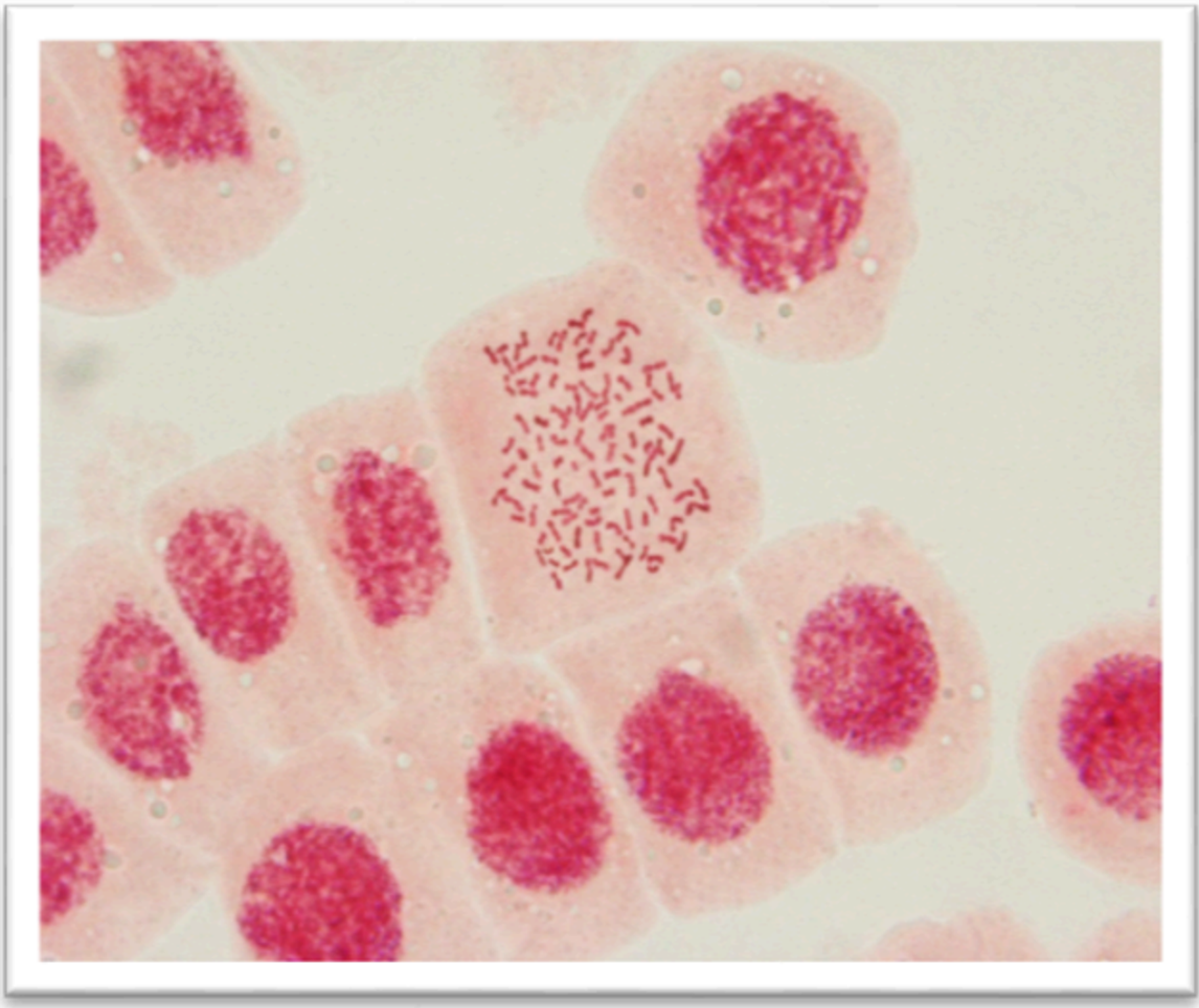


Figure 8

Representative diploid ($2n = 114$) metaphase spread (Control).

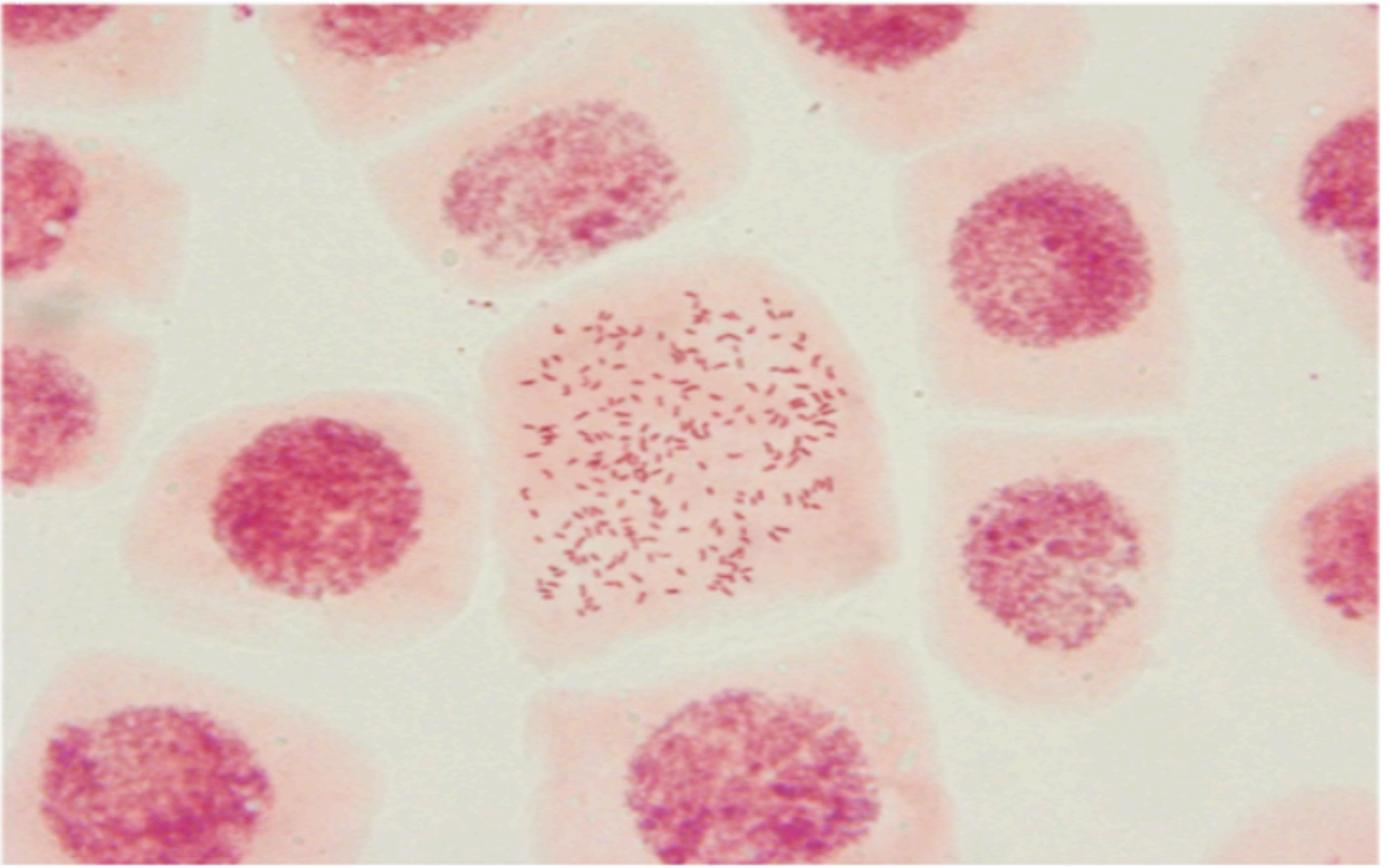


Figure 9

Representative tetraploid ($2n \approx 230$) metaphase spread (50 mg L^{-1} colchicine for 24 h).

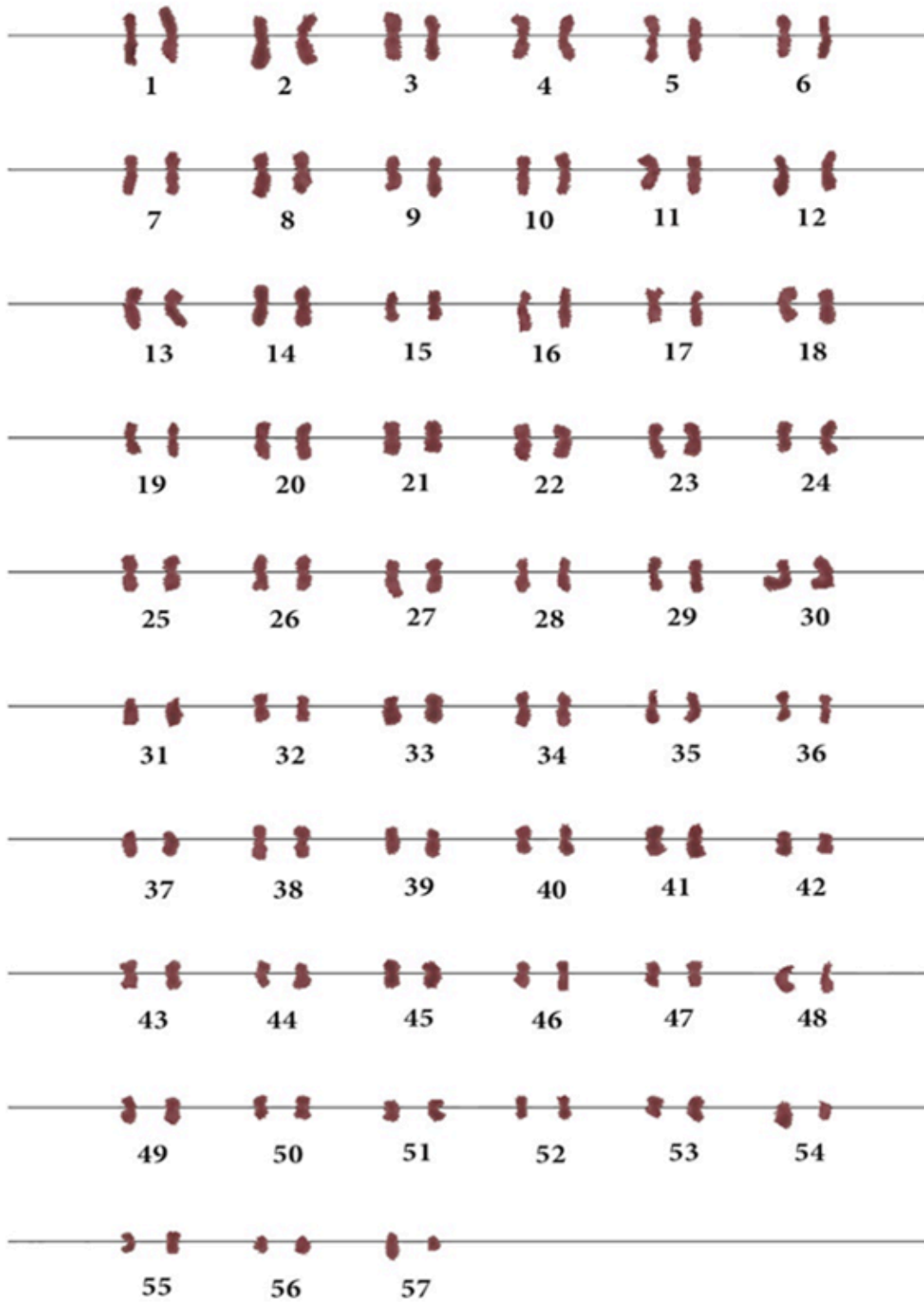


Figure 10

Karyotype of sugarcane cultivar CP-57 (control sample), $2n = 114$.

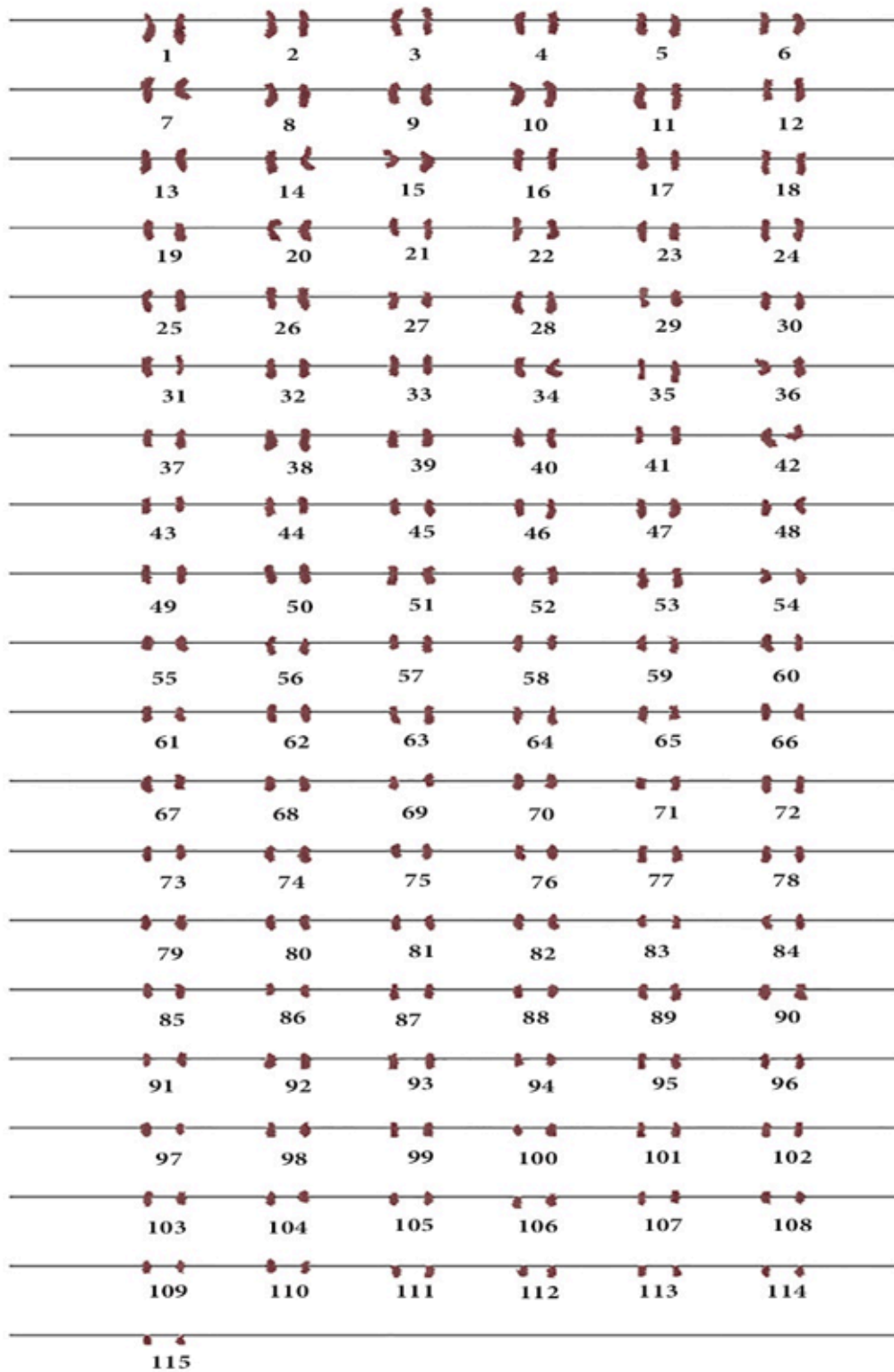


Figure 11

Karyotype of sugarcane cultivar CP-57 treated sample (50 mg L⁻¹ colchicine for 24 h), 2n = 230.



Figure 12

Karyogram of sugarcane cultivar CP-57 (control sample), $2n = 114$.

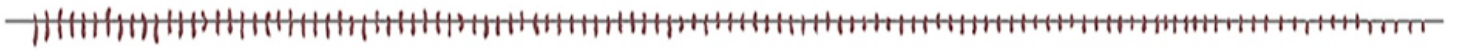


Figure 13

Karyogram of sugarcane cultivar CP-57 treated sample (50 mg L^{-1} colchicine for 24 h), $2n = 230$.

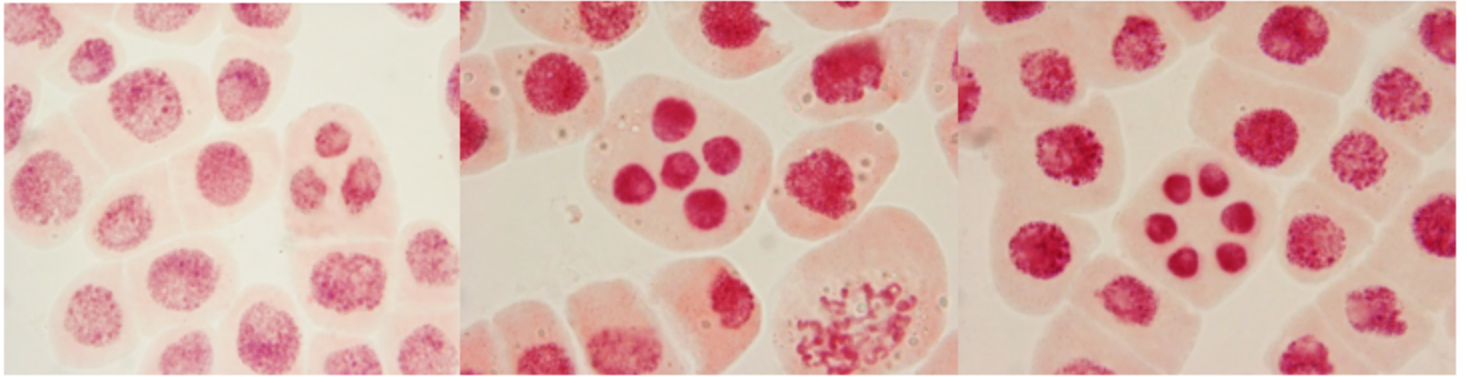


Figure 14

Interphase nuclear abnormalities observed in colchicine-treated CP57 cells.

Supplementary Files

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