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Evaluation of Morphological and Cytological Variations in Colchicine-Induced M_1 and M_2 Generations of Bambara Groundnut (*Vigna subterranea* L.)

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ABSTRACT/ABSTRAK

Evaluasi keragaman morfologi dan sitologi generasi M_1 dan M_2 kacang bambara (*Vigna subterranea* L.) yang diinduksi dengan kolkisin

Bambara groundnut (*Vigna subterranea* L.) has the potential to secure food supply in the face of climate change. However, limited genetic resources hinder the development of superior bambara groundnut varieties in Indonesia. Mutation breeding using colchicine is one method of increasing variability on bambara groundnut. This study aimed to evaluate the morphological and cytological variability in two generations of bambara groundnut induced by colchicine at different concentrations. The experiments were conducted in two different times and locations, i.e., March-July 2022 at experimental fields of the Agriculture Faculty Brawijaya University, and March-July 2023 in a greenhouse in Joyo Agung, Lowokwaru District, Malang City, East Java. Seedlings were treated with different concentrations of colchicine (0, 0.1, 0.2, 0.3, and 0.4%) using the drip method on the apical bud. The morphological and cytological characteristics were studied for M_1 and M_2 generations. The results showed that colchicine induction increased the variability of morphological and cytological characters in two generations. Colchicine treatment affected changes in the morphology and cytology of bambara groundnut plants in the M_1 and M_2 generations. In the M_2 generation, colchicine treatment with a concentration of 0.4% increased leaf width by 31%, leaf length by 32%, flowering time by 28%, seed width by 29%, seed length by 47%, weight of 100 seeds by 3%, stomata width by 20%, and stomata length by 25% compared to control plants. However, it also reduced the number of leaves by 30%, plant height by 1%, number of seeds per plant by 58%, seed weight per plant by 56%, and stomata density by 15% compared to control plants. The colchicine treatment increased chromosome number in the M_2 generation so that it can be used to increase variability and overcome the limitations of the genetic resources of bambara groundnut.

Kata Kunci:
Jumlah kromosom, Kerapatan stomata, Mutasi, Panjang daun, Sumberdaya genetik

Kacang bambara (*Vigna subterranea* L.) memiliki potensi dalam mengamankan pasokan pangan dalam menghadapi perubahan iklim karena memiliki manfaat dari aspek kandungan gizi, kesehatan, dan agronominya. Keterbatasan sumberdaya genetik menghambat pengembangan varietas unggul kacang bambara di Indonesia. Pemuliaan mutasi menggunakan kolkisin merupakan salah satu metode untuk meningkatkan keragaman kacang bambara. Penelitian ini bertujuan untuk mengevaluasi keragaman morfologi dan sitologi pada dua generasi kacang bambara yang diinduksi oleh kolkisin pada konsentrasi yang berbeda. Eksperimen dilakukan di dua waktu dan lokasi yang berbeda, yaitu

Maret-Juli 2022 di lapangan percobaan Fakultas Pertanian Universitas Brawijaya dan Maret-Juli 2023 di rumah kaca di wilayah Joyo Agung, Lowokwaru, Kota Malang, Jawa Timur. Perlakuan kolkisin dilakukan dengan metode tetes pada pucuk apikal bibit dengan konsentrasi yang berbeda (0, 0,1, 0,2, 0,3, dan 0,4%). Pengamatan karakteristik morfologi dan sitologi dilakukan pada generasi M_1 dan M_2. Perlakuan kolkisin mempengaruhi perubahan morfologi dan sitologi tanaman kacang bambara pada generasi M_1 dan M_2. Pada generasi M_2, perlakuan kolkisin konsentrasi 0,4% meningkatkan lebar daun sebesar 31%, panjang daun sebesar 32%, waktu berbunga sebesar 28%, lebar biji sebesar 29%, panjang biji sebesar 47%, bobot 100 biji sebesar 3%, lebar stomata sebesar 20%, dan panjang stomata sebesar 25% dibandingkan dengan kontrol. Namun, perlakuan kolkisin menurunkan jumlah daun sebesar 30%, tinggi tanaman sebesar 1%, jumlah biji pertanaman sebesar 58%, bobot biji pertanaman sebesar 56%, dan kerapatan stomata sebesar 15% dibandingkan dengan kontrol. Perlakuan induksi kolkisin menyebabkan peningkatan jumlah kromosom pada generasi M_2 sehingga dapat digunakan untuk meningkatkan variasi dan mengatasi keterbatasan sumber daya genetik kacang Bambara.

INTRODUCTION

Bambara groundnut (*Vigna subterranea* L.) is a legume from Africa, now widely grown in America, Asia (mainly in India, Indonesia, Malaysia, Philippines, and Thailand), and Australia (Khan *et al*., 2021). Many studies reveal that this plant is a promising food crop due to its climate-smart features and essential nutrient contents. Often referred to as a 'complete food,' bambara groundnut contains macronutrients such as carbohydrates (64.4%), protein (23.6%), fat (6.6%) and fiber (5.5%) as well as micronutrients such as K (11.44–19.35 mg/100 g), Fe (4.9–48 mg/100 g), Na (2.9–12.0 mg/100 g), and Ca (95.8–99 mg/ 100 g) (Paliwal *et al.,* 2021). Additionally, it includes anthocyanins and flavonoids, which are cancer-curing antioxidants (Jideani & Diedericks, 2014). This plant is tolerant to drought and poor soil conditions such as salt stress and low nutrients (Mayes *et al.,* 2019; Paliwal *et al.,* 2020), and it can be cultivated under conditions where peanuts fail (Temegne, 2018), making it a climate-smart crop. Therefore, bambara groundnut is essential in food diversification programs to improve global food and nutrition security in the face of climate change.

Bambara groundnut is an underutilized crop. To date, superior varieties have yet to be developed in Indonesia, causing many farmers to rely on local genotypes for their cultivation. Consequently, productivity in Indonesia is low, with an average yield of only one ton per hectare of dry pods (Sari *et al*., 2022). The Underutilised Crop Research Center at the Faculty of Agriculture, Brawijaya University, has been conducting plant breeding efforts to produce superior varieties through exploration, collection, and selection activities for strains cultivated in Indonesia (Kuswanto *et al*., 2012; Nuryati *et al*., 2014; Arif *et al*., 2016; Nugraha *et al*., 2017; Saptadi *et al*., 2021). However, these methods have yet to achieve satisfactory production potential due to the low level of genetic variability and the limited number of high-yield genetic sources (Saptadi e*t al.,* 2021; Sari *et al*., 2022). Therefore, increasing genetic variability is essential to obtain high-yielding genotypes. One feasible technique for enhancing plant variability is polyploidization.

Polyploidization refers to the process of producing plants with two or more complete sets of chromosomes, known as polyploid (Soltis *et al*., 2015). Genomic changes due to polyploidization lead to polyploids exhibiting greater genetic variability than diploid organisms (Trojak-Goluch *et al*., 2021). This new genetic variability resulting from the induction of polyploidy in bambara groundnut is crucial for selection efforts to improve yield characteristics. Wide genetic variation provides the basis for breeders to select plants with enhanced traits, thereby increasing the potential for developing high-yielding varieties (He & Li, 2020). Polyploid plants often exhibit larger morphological characteristics, such as increased leaf, stem, and root size, which can benefit yield and crop production (Dhooghe *et al*., 2011).

Mitotic polyploidization is the most common method for doubling the number of chromosomes in

crop plants' somatic cells. This technique involves using chemicals that disrupt the process of mitosis in plant somatic cells (Trojak-Goluch *et al.*, 2021). One of the most widely used antimitotic for inducing polyploidy is colchicine, which has proven effective in many plant species (Urwin, 2014; Mo *et al.*, 2020). Colchicine prevents the formation of tubulin dimers by binding to β-tubulin, thereby inhibiting microtubule formation. The absence of microtubules during mitosis results in incomplete chromosome separation, leading to cells with doubled sets of chromosomes and the formation of a polyploid organism (Chaikam *et al.*, 2019). However, plant polyploidization using this mutagen is one of the unexplored approaches for varietal improvements and the generation of basic genetic information of grain legumes (Mangena & Mushadu, 2023), such as bambara groundnut.

The success rate of polyploidy induction can vary even within the same species. Key factors influencing the effectiveness of polyploidy induction include colchicine concentration, application duration, application method, and the materials used (Eng & Ho, 2019). Among these, colchicine concentration is particularly crucial. High concentrations can increase plant mortality, while low concentrations may result in insufficient induction or failure to induce mutations (Roslim *et al.*, 2015). Furthermore, mutations often appear in subsequent generations, such as M_2, V_2, or its continuation (Soedjono, 2003; Damanhuri & Adiredjo, 2018). The first mutation population (M_1) often experiences physiological disorders due to mutagen treatment, preventing phenotypic selection for mutations in the M_1 generation (FAO & IAEA, 2018). This study aims to evaluate the morphological and cytological variability in two generations of Bambara groundnut induced by colchicine at different concentrations.

MATERIALS AND METHODS

Location and Time

Bambara groundnut seeds (SS 3.4.2) were treated with various colchicine treatments and planted in experimental fields at the Faculty of Agriculture, Brawijaya University, Jatimulyo Village, Lowokwaru District, Malang City, East Java, with an altitude of 460 meters above sea level (MASL) and temperature of 20–28 °C. The experiment was conducted in March-July 2022 for the M_1 generation and March-July 2023 for the M_2 generation. In the M_2 generation, planting was carried out in a greenhouse located in Joyo Agung, Malang City, East Java.

Research Design and Colchicine Treatment

The experiment was arranged in a Single Plot Design, and data was collected using the single-plant method. Colchicine concentration, i.e., 0 (control), 0.1, 0.2, 0.3, and 0.4%, was used as a treatment. Colchicine was applied to the apical buds using a pipette, with two drops administered each day for three consecutive days. Applications were performed at 07.00 AM and 4.00 PM. After colchicine treatment, the M_1 generation seeds were sown in seedling trays in March 2022 for seven days. The number of seeds used was 125 (5 plants x 25 experimental sets). Seedlings were transplanted into 30 cm diameter plastic pots (one seedling/pot) containing soil and manure in a ratio of 2 : 1. From each M_1 generation treatment, 39 seeds with the largest seed size were selected and planted in the M_2 generation in March 2023.

Morphological and Cytological Trait Observation

The observed characteristics included morphological and cytological traits in the M_1 and M_2 generations. Morphological characters were the seed germination (%), plant height (cm), number of leaves, leaf width (cm), leaf length (cm), flowering days (DAP), number of seeds per plant (seeds), the weight of 100 seeds (g), seeds weight per plant (g), seed width (nm), and length seeds (nm). Cytological characters were stomata length (µm) and stomata width (µm). In the M_1 generation, additional observations included stomata density (mm²) and number of chromosomes.

The number of chromosomes in the M_2 generation was assessed using the squashing method, adapted from Jahier *et al.* (1996). Five plant samples from each treatment were selected, with two root tips collected from each sample. Root tip cutting was performed at between 10.30 and 11.00 AM. Five plant samples from each treatment were selected, with two root tips collected from each sample. Root tip cutting was performed between 10.30 and 11.00 AM, as this period is optimal for observing the number of chromosomes in bambara groundnut during the mitosis phase. Chromosome observation using the squashing method consists of pretreatment, fixation, hydrolysis, and staining. Pretreatment: the root tip was cut 0.5-1 cm, soaked in distilled water, and stored in the refrigerator (±4 ˚C) for 24 h. Fixation: the root tips were inserted into a microtube containing Carnoy's solution (acetic acid : ethanol = 1 : 3) and

stored in the refrigerator (±4 ˚C) for 1 h. Hydrolysis: the root tips were hydrolysed by HCl 1N in a water bath (±60 ˚C) for 30 min. Every time the solution changed, the root tips were cleaned with distilled water and repeated three times. Staining: cleaned root tips were stained using aceto-orcein 2% and stored at room temperature for 15 min. The root tip was cut ±0.5 mm and placed on a slide glass. Then, the root tip was dripped with glycerol and covered with a cover slip. The coverslip was tapped with a pencil tip and then the slide was heated over an alcohol flame for several seconds. Labelling: To seal the coverslip, clear nail polish was used on the edge of the coverslip. The finished slides were then observed using an Olympus BX51 microscope with 1000x magnification under an oil immersion objective lens. Chromosome calculations were carried out manually with the help of the Image J application.

Data Analysis

Data obtained from the M_1 generation were analyzed using descriptive analysis, while data from the M_2 generation was analyzed using the t-test at the 5% level provided by Acquaah (2007a):

$$t = \frac{\bar{x}_1 - \bar{x}_2}{Sp\sqrt{\left(\frac{1}{n_1}\right) + \left(\frac{1}{n_1}\right)}}$$

$$Sp = \frac{(n_1 - 1)S_1^2 - (n_2 - 1)S_2^2}{n_1 + n_2 - 2}$$

Explanation:
$\bar{x}_1; \bar{x}_2$ = the means of samples 1 and 2
Sp = pooled variance
$s_1^2; s_1^2$ = variance of samples 1 and 2
$n_1; n_1$ = the number of samples 1 and 2

Variability analysis was carried out by calculating the coefficient of variation (CV) using the formulation provided by Acquaah (2007b):

$$CV = \frac{\sigma}{\bar{x}} x\ 100\%$$

Explanation:
σ = standard deviation
$\bar{x}$ = mean

RESULTS AND DISCUSSION

Percentage of Seed Germination

The response of bambara groundnut plants to colchicine treatment can be observed through the seed germination percentage. Colchicine inhibits spindle fiber development and alters the differentiation process. It is highly toxic to plants and, at high concentrations, can lead to the death of plant cells (Trojak-Goluch & Skomra, 2013). The colchicine-induced M_1 and M_2 generations exhibited lower seed germination rates than the control (Table 1). Similar findings were reported by Dheer *et al.* (2014) in the C_1 and C_2 generations of *Lablab purpureus*, where colchicine-induced plants showed a lower seedling survival rate than control plants. The control treatment had the highest seed germination percentage, whereas the treatment with the highest colchicine concentration (0.4%) had the lowest germination rate. An increase in colchicine concentration correlated with a decrease in seed germination percentage. This diminished growth capacity may be due to chromosomal imbalances caused by colchicine, leading to irregular cell division and impaired cell differentiation, ultimately inhibiting plant germination. Choopeng *et al.* (2019) stated that elevated colchicine concentrations likely induce toxicity in plant cells, causing cell imbalance and affecting internal cell processes, such as cell division, resulting in plant mortality.

Table 1. Percentage of seed germination in 14 days after seedling

Treatment (%)	Percentage of seed germination (%)	
	M_1	M_2
Control	100	100
0.1	96	77
0.2	96	72
0.3	92	72
0.4	84	64

In the second generation, seed germination percentages still decreased due to the impact of colchicine use. According to Münzbergová (2017), colchicine's effects can persist into the second generation and its direct effects can last for several generations. Additionally, the low germination percentage may also be attributed to the SS 3.4.2 line's low resistance to colchicine toxicity. The SS 3.4.2 line is characterized by its low stability and sensitivity to environmental changes (Sari *et al.*, 2022), further contributing to its reduced germination rates under colchicine treatment. According to Zlesak *et al.*, (2005) in some cases, plant death is caused by low seed resistance, resulting in a diminished ability to overcome the toxic effects of colchicine.

Morphological Characteristics

An increase in the morphological characteristics values of the M_1 and M_2 due to colchicine induction was observable in leaf length, leaf width, flowering time, seed length, seed width, and weight of 100 seeds (Table 2 and 3). The variation of leaf size due to colchicine induction can be seen in Figure 1. This increase can be attributed to larger cell size resulting from the augmented number of chromosomes due to colchicine treatment. When the number of chromosomes increases, cells respond by enlarging their size. This confirms the finding of Robinson *et al.* (2018) that ploidy plays an important role in determining cell size. The mechanism that links ploidy to cell size involves increased DNA bulk and related proteins, as well as an increased copy number at every locus. Genome size is positively associated with minimal cell size (Jovtchev *et al.*, 2006; Beaulieu *et al.*, 2008; Wang *et al.*, 2021), as the amount of nuclear DNA influences the lower limit of nuclear volume, which in turn sets a lower limit for the size of the cell that contains and supports it (Doyle & Coate, 2019; Wang *et al.*, 2021). Handayani *et al.* (2018) added that colchicine induction results in chromosomes doubling, leading to a higher number of chromosomes, causing larger cell and nuclei and, consequently, larger plant parts such as leaves.

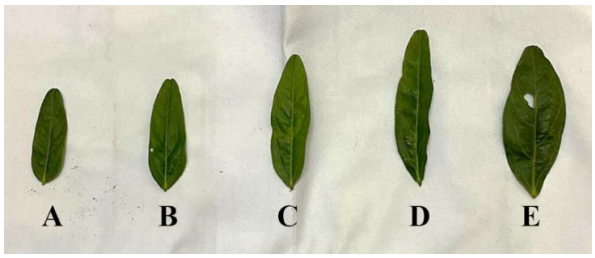

Figure 1. Variation of leaf size in the M_1 generation of bambara groundnut treated with different colchicine concentrations (A) Control, (B) 0.1%, (C) 0.2%, (D) 0.3%, (E) 0.4%

The impact of using chemical antimitotic agents was also observed in the M_1 and M_2 generations of bambara groundnut. The specific effects were seen in the colchicine-induced plants, which exhibited lower values in the number of leaves, plant height, number of seeds, and seed weight compared to the control (Table 2 and 3). A similar decreases in quantitative characteristics values were found in the M_1 generation of cowpea (Ajayi *et al.*, 2014). Syukur *et al.* (2013) stated that chromosome duplication or polyploidy hinders chromosome pairing. In diploid cells, mitosis involve the pairing of homologous chromosomes before cytokinesis. Conversely, in polyploid cells, the extended pairing process is the results of the increased number of chromosome sets (Syukur *et al.*, 2019), leading to reduced plant tissue growth. Moreover, chromosome doubling can disrupt endogenous hormonal balance and normal physiological and biochemical processes (Wu *et al.*, 2023). The induction of colchicine also affects the flowering time of Bambara groundnut in both the M_1 and M_2 generations. Colchicine-induced plants showed longer flowering times than control plants, with a longer flowering time associated with higher colchicine concentrations. Delayed flowering due to colchicine induction has also been observed in cowpea, mungbean and peanut (Khan & Wani, 2005; Obute *et al.*, 2007; Ajayi *et al.*, 2014). Chromosome doubling induced by colchicine inhibits mitotic division and slows the vegetative growth rate (Vichiato *et al.*, 2016), leading to delayed flower emergence. Moreover, chromosome doubling can disrupt endogenous hormonal balance and normal physiological and biochemical processes (Wu *et al.*, 2023).

The concentration of colchicine treatment was shown to influence the morphological variability of bambara groundnut in both the M_1 and M_2 generations. The morphological variability in bambara groundnut increased as a result of colchicine induction, and this indicates the occurrence of polyploidy due to colchicine induction. According to Riddle *et al.* (2006), phenotypic changes due to alterations in ploidy levels are accompanied by genotype-specific responses to changes in genome dosage, indicating genetic variation in response to ploidy changes.

Based on data from observations of the yield characteristics of bambara groundnut plants in the M_1 and M_2 generation, it is evident that colchicine induction reduces yield (Table 2 and 3). The colchicine treatment with concentrations of 0.1%, 0.2%, 0.3%, and 0.4% resulted in fewer seeds per plant and lower seed weight per plant compared to those of the control treatment. A decrease in yield due to colchicine induction was also reported in the M_2 generation of *L. purpureus* and *Vicia sativa* (Dheer *et al.* 2014; Münzbergová, 2017). The reduced yield of colchicine-induced polyploid plants may be attributed to disrupted meiotic division, which hinders the fertilization process. Colchicine-

induced genome doubling leads to abnormal meiosis, resulting in multivalent formation. Multivalents often cause abnormal disjunction during anaphase 1, bridge formation, lagging chromosomes, and other irregularities that impact plant fertility (Pavlíková *et al.*, 2017).

Table 2. Morphological character in the M_1 generation of bambara groundnut

Character	Treatment (%)	$\bar{x}$	σ	σ^2
Number of leaves	Control	77.68	13.88	192.62
	0.1	65.25	12.54	157.19
	0.2	54.79	15.04	226.25
	0.3	55.00	9.76	95.30
	0.4	54.71	14.92	222.49
Plant height (cm)	Control	17.40	0.93	0.87
	0.1	19.08	1.83	3.35
	0.2	19.46	4.37	19.10
	0.3	17.90	1.90	3.62
	0.4	17.23	2.32	5.36
Leaf width (cm)	Control	2.26	0.38	0.15
	0.1	2.53	0.34	0.12
	0.2	2.75	0.78	0.61
	0.3	2.77	0.47	0.22
	0.4	2.96	0.54	0.30
Leaf length (cm)	Control	6.79	1.10	1.20
	0.1	7.08	1.16	1.35
	0.2	8.76	1.32	1.73
	0.3	8.73	1.18	1.38
	0.4	8.94	1.32	1.74
Flowering time (DAP)	Control	35.20	1.48	2.20
	0.1	37.00	2.45	6.00
	0.2	42.20	3.70	13.70
	0.3	43.40	2.70	7.30
	0.4	45.20	2.86	8.20
Number of seeds per plant	Control	10.64	3.72	13.83
	0.1	6.92	2.41	5.83
	0.2	3.29	1.14	1.29
	0.3	4.71	1.13	1.27
	0.4	4.46	1.39	1.94
Weight of 100 seeds (g)	Control	46.02	26.31	692.40
	0.1	51.55	17.88	319.68
	0.2	80.71	21.88	478.63
	0.3	54.01	19.59	428.76
	0.4	47.22	21.80	475.22
Seeds weight per plant (g)	Control	4.28	1.53	2.35
	0.1	3.27	1.11	1.24
	0.2	2.48	0.72	0.52
	0.3	2.47	0.89	0.80
	0.4	1.89	0.59	0.35
Seed width (nm)	Control	7.08	0.71	0.50
	0.1	9.30	0.99	0.97
	0.2	11.05	1.19	1.43
	0.3	9.08	0.81	0.65
	0.4	9.10	0.67	0.44
Seed length (nm)	Control	8.75	0.83	0.70
	0.1	12.83	1.61	2.58
	0.2	14.71	1.33	1.77
	0.3	13.16	1.39	1.93
	0.4	12.85	1.27	1.62

Note: ($\bar{x}$) mean, (σ) standard deviation, (σ^2) variance.

Table 3. Morphological character in the M_2 generation of bambara groundnut

Character	Treatment (%)	Mean	T-Test	CV (%)
Number of leaves	Control	28.18		20.33
	0.1	27.47	0.50 tn	22.44
	0.2	26.11	1.42 tn	23.5
	0.3	24.14	2.94 **	21.85
	0.4	18.72	6.99 **	23.92
Plant height (cm)	Control	17.93		8.62
	0.1	17.04	2.25 *	10.18
	0.2	16.98	2.23 *	11.47
	0.3	16.73	3.02 **	10.01
	0.4	16.26	3.76 **	12.21
Leaf width (cm)	Control	1.60		11.2
	0.1	1.72	2.21 *	16.39
	0.2	1.83	3.68 **	17.85
	0.3	1.98	4.75 **	22.59
	0.4	2.08	8.15 **	14.24
Leaf length (cm)	Control	3.50		8.08
	0.1	4.70	8.93 **	16.55
	0.2	4.94	8.83 **	19.57
	0.3	5.13	12.32 **	14.76
	0.4	5.19	11.98 **	15.59
Flowering time (DAP)	Control	53		1.06
	0.1	58	35.92 **	1.15
	0.2	60	44.50 **	1.16
	0.3	63	65.50 **	1.08
	0.4	63	58.20 **	1.44
Number of seeds per plant	Control	9.57		17.63
	0.1	4.85	11.81 **	25.37
	0.2	3.67	14.99 **	19.92
	0.3	4.14	13.34 **	21.97
	0.4	3.32	16.53 **	27.11
Weight of 100 seeds (g)	Control	27.99		19.25
	0.1	34.72	2.67 *	35.12
	0.2	37.57	4.73 **	23.29
	0.3	37.40	3.21 **	38.06
	0.4	57.67	6.15 **	43.19
Seeds weight per plant (g)	Control	2.64		20.81
	0.1	1.64	6.51 **	36.5
	0.2	1.39	8.33 **	34.71
	0.3	1.47	7.81 **	32.18
	0.4	1.79	4.92 **	39.30
Seed width (nm)	Control	7.15		8.03
	0.1	7.65	2.46 *	11.97
	0.2	7.9	3.82 **	10
	0.3	7.61	2.38 *	10.4
	0.4	8.89	6.61 **	14.15
Seed length (nm)	Control	9.05		9.44
	0.1	10.43	4.5 **	13.08
	0.2	10.7	5.35 **	12.1
	0.3	10.28	4.05 **	12.69
	0.4	12.1	7.81 **	15.33

Note: CV (coefficient of variation); Values with symbols (**) very significant difference, (*) significant difference, (tn) not significant difference on student t-test at 5% level with control as a comparison.

The quality of bambara groundnut seeds, such as seed size, is essential in determining economic value, as the bambara groundnut plant is mainly consumed for its seeds. Although colchicine induction reduced seed number and seed weight per plant, it increased the size of seeds. The size of the seeds affects the weight of 100 seeds. Colchicine-induced plants produced higher 100-seed weights than control. This aligns with Biçer (2009), that the weight of 100 seeds is influenced by seed size, with larger seeds producing a greater weight of 100 seeds. Mallikarjuna *et al.* (2011) found that colchicine-induced tetraploid peanut plants had larger pods, but the number of pods was less than that of diploid plants. The most common effect of polyploidy in plants is an increase in cell size due to an increase in the number of gene copies, known as the gigas effect (Sattler *et al.*, 2016), which leads to an increase in the size of Bambara groundnut seeds.

Cytological Characteristics

Stomatal characteristics were observed as an indicator of polyploidy. Based on the observations of stomatal characteristics, colchicine induction treatment produced higher stomata size than the control (Table 4 and Table 5). Colchicine-induced plants had greater stomata length and width than controls in bambara groundnut's M_1 and M_2 generation. Dheer *et al.* (2014) and Nagat *et al.* (2020) reported similar results in *L. purpureus* and *V. vaba*. In the M_2 generation, the highest stomata length and width were found at the highest concentration (Figure 2). There was an increase in stomata length and width in M_2 generation bambara peanuts, along with an increase in the concentration of induced colchicine. Tiwari and Mishra (2012) demonstrated a similar pattern in M_1, M_2, and M_3 generations of *Phlox drummondi*, where stomatal size increased with higher concentrations of induced colchicine. The relationship between genome size and stomatal size is positive (Lattier *et al.*, 2019), so increased stomata size is an indication of genome doubling. This aligns with previous observations in synthetic polyploids (Münzbergová, 2017; Wei *et al.*, 2020) where genome doubling resulted in enlarged stomata along with a decrease in stomatal density. Precisely, the observed average stomatal expansion aligns with the scaling prediction of the relationship between genome size and cell length (Wei *et al.*, 2020). Stomatal size is inversely proportional to stomatal density because larger stomata lead to fewer stomata, resulting in low density. Fetouh *et al.* (2020) reported that tetraploid *Ligustrum sinense* had a 45.7% lower density than control plants and a 32.2% larger size than control plants.

Table 4. The stomata character in the M_1 generation of bambara groundnut

Character	Treatment (%)	$\bar{x}$	σ	σ^2
Stomata width (µm)	Control	42.64	5.58	31.10
	0.1	49.75	9.89	97.83
	0.2	54.05	11.29	127.42
	0.3	56.36	5.78	33.46
	0.4	54.94	5.74	32.95
Stomata length (µm)	Control	46.18	5.56	30.97
	0.1	53.29	10.08	101.64
	0.2	60.05	11.90	141.69
	0.3	60.42	8.65	74.76
	0.4	61.57	9.69	93.84

Note: ($\bar{x}$) mean, (σ) standard deviation, (σ^2) variance.

Observations on the number of chromosome number in the M_2 generation were conducted on five plants selected from each treatment. which were selected based on the highest stomata length and width values from each treatment. The results of observations of the number of chromosomes in control plants found 11 pairs of chromosomes, and the chromosome number of Bambara groundnut plants was 2n = 2x = 22 (Table 6), consistent with the findings reported by Osundare *et al.* (2023). Colchicine treatment increased the number of Bambara groundnut chromosomes in the M_2 generation (Figure 3). Bambara groundnut plants induced by colchicine exhibited a higher number of chromosomes than control plants. Many researchers have shown that colchicine treatment is efficient for plant chromosomal doubling. However, their study found that the reaction to colchicine varies

depending on the plant species (Blasco *et al*., 2015; Jeloudar *et al*., 2019). Most plants suspected to be mixoploid were found in this study (Table 6). Udensi & Ontui (2013) reported that colchicine-induced Winged bean produced the two most common types of polyploids: mixoploid and tetraploid. The formation of mixoploid plants is one of the adverse effects of inducing polyploids with the mutagen colchicine, as these mixoploids can be inherited in subsequent generations. Sinta (2018) reported that there are still many aneuploid and mixoploid individuals in the fourth and fifth generations of *Stevia rebaudiana* Bert plant. A common problem from polyploid induction in meristematic tissue is polyploid changes that occur only in one or a few cells. As a result, the resulting plants often exhibit changes in cell tissue with varying numbers of chromosomes or a mixture of diploid and polyploid cells (mixoploid) (Azmi *et al*., 2016).

Table 5. The stomata character in the M_2 generation of bambara groundnut

Character	Treatment (%)	Mean	T-Test	CV (%)
Stomata width (µm)	Control	32.02		5.81
	0.1	36.55	8.05**	7.66
	0.2	36.65	7.39**	8.85
	0.3	38.03	9.48**	8.67
	0.4	38.58	10.73**	7.87
Stomata length (µm)	Control	47.72		4.30
	0.1	49.86	2.91**	7.91
	0.2	52.09	4.46**	10.77
	0.3	55.30	9.65**	7.73
	0.4	59.46	15.18**	6.90
Stomata density (mm²)	Control	72.29		5.33
	0.1	66.75	4.58**	9.20
	0.2	63.69	5.41**	13.87
	0.3	64.27	4.46**	16.01
	0.4	61.15	9.12**	9.70

Note: Values with symbols; (**) very significant difference, (*) significant difference on student t-test at 5% level with control as a comparison.

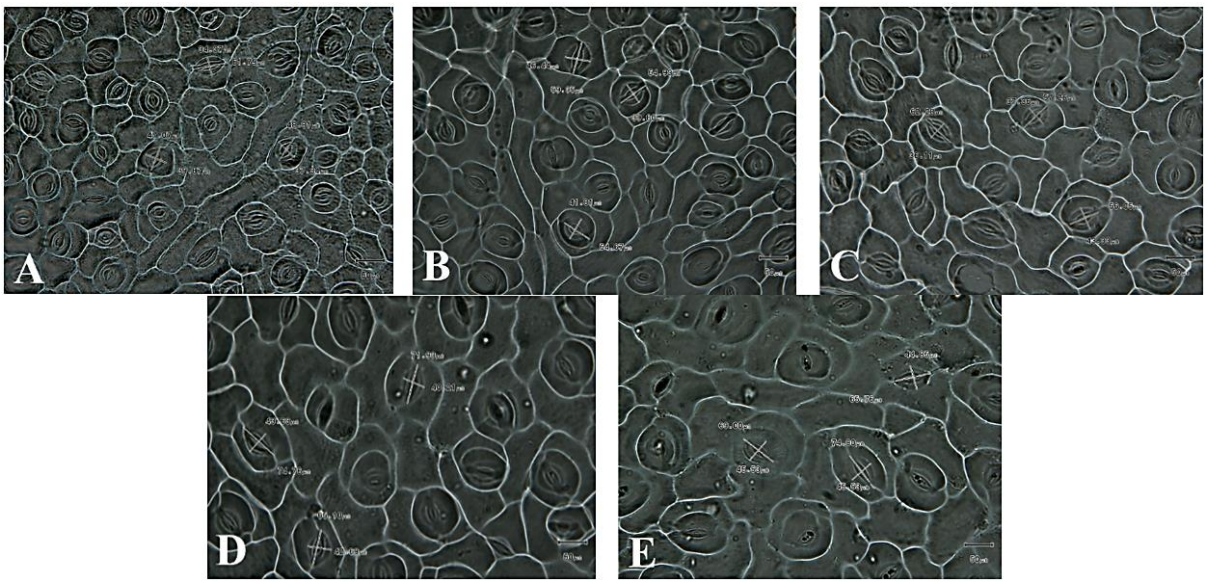

Figure 2. Variation of stomata in the M_2 generation of bambara groundnut treated with different colchicine concentrations (A) Control, (B) 0.1%, (C) 0.2%, (D) 0.3%, (E) 0.4%, Bar = 50 µm

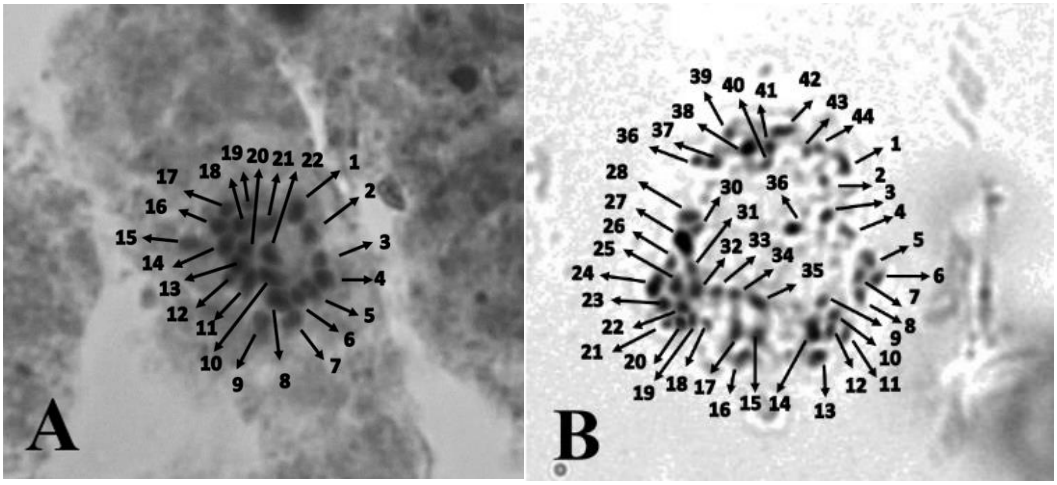

Figure 3. The increased number of chromosomes in the M_2 generation following the colchicine treatments (A) Diploid, B) Tetraploid

Table 6. The stomata character in the M_2 generation of bambara groundnut

Individual Plant Number	Number of chromosomes		Suspected Polyploid
	Sample 1	Sample 2	
Control	22	22	Diploid
0.1			
0.1-8	33	30	Mixoploid
0.1-19	27	28	Mixoploid
0.1-24	28	26	Mixoploid
0.1-30	32	36	Mixoploid
0.1-39	33	28	Mixoploid
0.2			
0.2-7	44	44	Tetraploid
0.2-11	33	32	Mixoploid
0.2-14	44	39	Mixoploid
0.2-25	33	33	Triploid
0.2-29	35	36	Mixoploid
0.3			
0.3-1	44	44	Tetraploid
0.3-21	33	33	Triploid
0.3-24	38	40	Mixoploid
0.3-26	44	42	Mixoploid
0.3-37	32	33	Mixoploid
0.4			
0.4-8	48	47	Mixoploid
0.4-24	44	44	Tetraploid
0.4-31	44	44	Tetraploid
0.4-35	47	48	Mixoploid
0.4-39	44	36	Mixoploid

The concentration of colchicine in the cell is an essential factor that influences how colchicine functions in the cell. Thus, the concentration of colchicine treatment highly influences variations in the cell division and division of chromosomes. Eigsti and Dustin (1955) reported that the effectiveness of colchicine in stopping, destroying, or inhibiting the formation of spindle threads is greatly influenced by the concentration level of colchicine. Tetraploid plants were often found in the 0.4% colchicine treatment, which constituted 40% of the sample plants, whereas at 0.3% and 0.2% colchicine

concentrations, both produced tetraploid plants, amounting to 20% of the sample plants. Therefore, it can be inferred that the higher the concentration of colchicine induced, the higher the percentage of tetraploids obtained. Wu *et al.* (2010) observed similar results in the M_4 generation of azuki beans treated with the highest colchicine concentration of 0.5%, which resulted in the highest percentage of mutants at 71.86%. Similarly, Nagat *et al.* (2020) found that 0.3% and 0.4% colchicine concentrations successfully induced triploid and tetraploid plants in *V. vaba*. Based on Table 6, six plants were selected in this study: 0.2-7, 0.2-25, 0.3-1, 0.3-21, 0.4-24, 0.4-31.

CONCLUSION

Based on this study, colchicine induction in bambara groundnut produced genotypes with higher variability in morphological and cytological characters than controls, consistent in two generations. Colchicine induction positively affected the increase in leaf size, stomata, seeds, and seed weight. However, it also had a negative impact on reducing germination rates, number of leaves, plant height, flowering age, number of seeds per plant, and seed weight per plant. Additionally, colchicine induction treatment increases the number of bambara groundnut chromosomes, so that it can be used to increase variation and address the issue of limited genetic resources. Colchicine treatment with a concentration of 0.4% produced more tetraploid plants than other colchicine treatments.

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