Exploring the Potential of Polyploidization as a Breeding Tool for Medicinal Plants: A Case Study on Cumin (Cuminum cyminum L.)

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Abstract

Polyploidization is a major trend in plant evolution that has many advantages over diploidization. Ploidy level manipulation is a powerful breeding tool for many plant species, including medicinal plants. Cumin (*Cuminum cyminum*L.), as an annual diploid plant belonging to the Apiaceae family, is the second most popular spice worldwide. To identify effective concentrations and target tissues for inducing polyploidy in cumin, three methods of seed treatment, root treatment and apical meristem treatment were tested on a cumin ecotype from South Khorasan under greenhouse conditions. Colchicine was used in varying concentrations (0.5, 0.2, 0.1, 0.05, 0.025% and 0%) for all assays. Different treatment times (12, 24, 36 and 48 hours) were considered for seed and root treatments, while the droplet method was used to treat the apical meristem. The ploidy level of the plantlets was verified by chromosome counts, flow cytometry, and cytology traits. The results showed that the seed and root treatments were not suitable for inducing polyploidy. The most effective method for inducing polyploidy in cumin was the application of colchicine (0.05%) on the apical meristem. However, applying 0.5% and 0.2% concentrations of colchicine on the apical meristem resulted in the wilting of the majority of seedlings. The tetraploid plants showed a significant difference in stomata size and pollen grain size and shape compared to the diploid mother plants.

1. Introduction

Polyploidization is a slow and gradual process that has driven plant evolution and speciation over time. However, artificial polyploidization can induce polyploidy in a shorter period (Eng & Ho, 2019). There are several methods of inducing polyploidy in plants, such as inducing polyploidy in somatic cells by the disrupting chromosome disjunction at anaphase of mitosis (Trojak-Goluch et al., 2021).

Colchicine, an alkaloid extracted from the seeds and roots of autumn crocus (*Colchicum autumnale* L.), is the most effective and widely used antimitotic agent to induce polyploidy in medicinal plants (Adaniya and Shirai 2001; Rubuluza et al. 2007; Sadat et al. 2011; Widoretno 2016; Noori et al., 2017; Ahmadi et al., 2021; Purbiya et al., 2021; Tsai et al., 2021). Colchicine dissolves well in alcohol and chloroform, less well in water and glycerol, and has multiple properties and a complex mechanism of action (Karamanou et al., 2018). Its action disrupts the function of microtubules responsible for the propagation of chromosomes to daughter cells, leading to the formation of polyploid nuclei (Manzoor et al., 2019).

Inducing chromosome doubling involves a series of steps, including an induction phase, growth phase, and a confirmation technique to evaluate the rate of achievement. The induction phase depends on different factors, such as method type, type of plant tissue, antimitotic agents, their different concentrations, and exposure durations. In cases of in vivo treatment, the antimitotic agent is generally applied through syringe injection, foliar spray, or cotton plug method. Another method for inducing polyploidy is soaking seeds and plunging the roots in aqueous solutions of mitotic inhibitors (Salma et al., 2017). The colchicine concentration and treatment duration are interdependant and crucial in polyploidization induction. The colchicine concentration applied ranges from 0.05–1.0% and duration of

treatment ranges from hours to weeks, depending on the method of application. Higher concentrations and longer colchicine treatment often result in higher lethality of explants (Tavan et al., 2015; Zhou et al., 2017).

High concentrations are toxic to plant cells and usually cause abnormal plant growth or reduced viability, while low concentrations may be ineffective or induce many mixoploids or aneuploids (Manzoor et al., 2019). There are two ways to identify polyploids, namely indirect identification and direct identification. Most polyploidization studies conduct indirect identification first, followed by direct identification to accurately identify polyploids (Moghbel et al., 2015). Indirect methods are easy, less time-consuming, and use simple instruments for screening (Eng & Ho, 2019). Different morphological and physiological traits, particularly pollen diameter, number of chloroplasts, stomatal size, and stomatal density, can be studied through these indirect methods (Moghbel et al., 2015). The measurement of stomatal dimensions is effortless and cost-effective (Salma et al., 2017). In direct methods, techniques like chromosome counting have been examined as an effective and reliable method, but it is a laborious process, particularly for plant species with highly dense cytoplasm consisting of a large number of chromosomes (Sakhanokho et al., 2015). Moreover, a specific protocol is required for each plant species. Thus, flow cytometry (as an indirect method) is considered to be a more reliable, rapid and simple method to analyze a large number of samples in a very short time period (Sattler et al., 2016). Combination of chromosome counting and flow cytometry analysis provides an overall picture of chromosome composition of the cells (Ochatt et al., 2011, Sign., 2017). Based on Eng & Ho (2019) findings, the most commonly used method to determine chromosome number is flow cytometry analysis (70.0%), followed by chromosome counting procedure (56.7%). 46.7% of studies used combination of both methods. Previous studies have established polyploidization protocols in several medicinal and aromatic herbs, although the protocols are rather species-specific and may not be effective on other species. Thus, proper species-specific method development for polyploidization is a prerequisite to achieving polyploids effectively (Julião et al., 2020). Successful application of the in vivo system for induced polyploidy has been reported in several studies, including *Gossypium arboreum* (Yang et al., 2015), *Jatropha curcas* (Chiangmai et al., 2014), *Eribotrya japonica* (Blasco et al., 2015), *Stevia rebaudiana* (Zhang et al., 2018), *Carum copticum* (Akbari et al., 2021), *Trachyspermum ammi* (Dwivedi and Kumar, 2021) and etc.

Some researchers have reported very low numbers of polyploids in certain plant species, such as 3.7% in *Centella asiatica* (Kaensaksiri et al. 2011), 4% in *Salvia hains* (Sadat et al. 2011), and 5% in *Echinacea purpurea* (Abdoli et al. 2013). However, other studies have reported much higher percentage of tetraploids, such as 40% in *Scutellaria baicalensis* (Gao et al. 2002), 66.2% in *Pfaffia glomerata* (Gomes et al. 2014), and 100% in *Pogostemon cablin* (Widoretno 2016).

Cumin (*Cuminum cyminum* Linn.), the king of seed spices (Lal et al.,2014), which belongs to the Apiaceae family, is highly valued for its aroma, medicinal properties, and therapeutic benefits, even in long-time stored seeds (Sowbhagya,2013). It is the second most popular spice in the world, after black pepper (Lodha and Mawar,2014), and is widely used in the food, cosmetics, and perfume industries

(Dubey et al., 2017). Despite its economic and medicinal importance, no established protocol for polyploidization in *C. cyminum* has been reported to date.

Here, we describe a successful method for producing synthetic polyploid plants of *C.cyminum* using colchicine. The results of our study provide a foundation for future breeding efforts aimed at enhancing essential oil yields in medicinal and aromatic herbs through polyploidization. Additionally, our protocol may serve as an optimized approach for artificial polyploidization in *C.cyminum* and related species, leading to the development of improved genotypes.

2. Materials and methods

2.1. Plant material

The cumin seeds used in this study were obtained from the South Khorasan ecotype and collected from the gene bank at the Aburaihan Faculty of Agriculture, University of Tehran. Prior to use, the seeds were disinfected by soaking them in a 0.2% solution of sodium hypochlorite (v/v) (NaClO, commercial bleach-SAVO) for 5 minutes. After disinfection, the seeds were rinsed three times with distilled water and then used in the subsequent steps of the study.

2.2. Polyploid induction

To induce polyploidy, five concentrations of colchicine (0.5, 0.2, 0.1. 0.05 and 0.025% v/w) were prepared by dissolving in distilled water. Three separate experiments were performed to determine the most effective treatment for polyploidy induction.

2.2.1. Root induction. Cumin seeds were planted in a seedling tray containing cocopeat perlite (3:1) (Fig. 1a) and were selected at the stage of 2–3 true leaves. Seedlings were washed under running water for 2 minutes and then the seedling roots exposed to 24 different treatments using varying concentrations of colchicine for 12, 24, 36 and 48 hours in the dark. Each treatment consisted of 9 seedlings (Fig. 1b). After exposure, the roots of the seedlings were carefully washed under running water and if healthy, they were transferred to pots filled with the same soil composition.

2.2.2. Seed induction. Cumin seeds were exposed to different concentrations of colchicine (0.5, 0.2, 0.1, 0.05, 0.025% and 0) for 12, 24, 36, and 48 hours in the dark. Each treatment consisted of 20 seeds, and distilled water was used as a control. All treated seeds were then rinsed under running water for 20 minutes before being planted in a seedling tray filled with cocopeat and perlite (3:1(and grown for 3 weeks before being transferred to pots containing soil, vegetable manure and fine sand (2:1:1) (Fig. 1d).

2.2.3. Apical meristem induction. Cumin seeds were planted in pots filled with soil, fine sand and vegetable manure (in a 2:1:1 ratio) and kept under greenhouse conditions with an average temperature of 25˚C and 20–30% relative humidity. Each pot contained 10 seeds, and when the seedlings reached the 2–3 true leaf stage, 3 seedlings were kept in each pot and the rest were removed. Colchicine treatments with

concentrations of 0, 0.025, 0.05, 0.1, 0.2 and 0.5% were applied by placing a drop of colchicine solution on apical meristem and growth points. The pots were covered with opaque disposable container to prevent evaporation. To ensure absorption of colchicine, this procedure was repeated for three consecutive days. For each concentration, 3 pots containing 3 seedlings were considered.

2.3. Measurement of Stomata

To compare the size and density of stomata between diploid control and tetraploid plants, the nail polish impression method was used (Grant and Vatnick, 2004; Parsons et al., 2019). Transparent nail polish was applied to the back of the leaves and allowed to dry. Impressions were taken using transparent tape, and the size of stomata was recorded under a light microscope (Nikon eslipse E200) at 40x magnification. To analyze the length and width of stomata, approximately 50 stomata were randomly selected and analyzed using the software (Digimizer). Additionally, images of the number of chloroplasts in the guard cells of control and treated plants were recorded.

2.4. Pollen grain characteristics

During flowering another selection was applied on the basis of the pollen characteristics. Pollen was collected from the main umbels and stained with orcein. Approximately 10 flower buds were collected from at least five different plant per treatment, then observed and photographed at 40x and 100x magnification using a light microscope.

2.5. Ploidy level determination

The ploidy level of the treated plants was assessed using both flow cytometry and chromosome counting.

2.5.1. Flowcytometry analysis

Flow cytometry analysis was performed on treated plants at two different time points. The first analysis was conducted 25 days after colchicine application, and the second analysis was performed after the seeds were replanted. For the analysis, approximately 50 mg of leaves were taken and chopped using a sharp razor blade in 1000 µL of Galbrith buffer. The crude suspension was then filtered through a 30 µm nylon mesh, and 1 mL of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$) containing 2 µg/mL of fluorescent dye (PI) was added to the filtered crude suspension. The mixture was incubated at room temperature for 10 minutes. A minimum of 10000 nuclei per sample were measured using a FACSCalliber flow cytometer, and the ploidal level was determined by referencing the position of the G1 peak of the diploid plant.

2.5.2. Chromosomes counting

Fresh root tips (approximately 12-15mm) were collected between 7 and 8 o' clock in the morning and placed in a 0.002 M solution of 8-hydroxyquinoline for 2 hours at either 4˚C or in ice water (0˚C) for 48 hours. The root tips were then rinsed tree times with distilled water and placed in a freshly prepared solution of ethyl alcohol and acetic acid (3:1) for 24 hours at 4˚C. After removal from the solution, the

roots were washed three times with distilled water before undergoing hydrolysis and staining. The root tips were incubated in a 1 N HCl solution at 55˚C for 6 minutes, followed by staining with 1% orcein for 15 minutes. The root tips were then excised to approximately 0.1 cm and placed on a glass slide with a drop of acetic acid, before being visualized under a light microscope (Nikon eclipse E200, Japan) at 100x magnification.

2.6. Meiosis

To investigate the effect of colchicine on chromosomes and confirm tetraploid induction, chromosome counting and observation of pollen mother cells (PMCs) during meiosis were performed under a light microscope. We also stained PMCs with orcein to examine meiosis in some plants. Our goal was to observe any changes in chromosomal behavior to confirm the successful induction of tetraploidy.

2.7. Statistical analysis

Paired Student's t-test was conducted to compare means using Excel software. Stomata properties were analyzed using Digimizer software, which enabled the measurement of properties from images.

3. Results and Discussion

In recent decades, chemical agents have been used to artificially induce autopolyploidy and develop improved cultivars of economically important species (Miri, 2020). To determine the effect of colchicine on seedling viability, we assessed plant survival and seed germination approximately one month after treatment. Our results showed that both colchicine concentration and treatment duration had a significant effect on plant survival and seed germination. Specifically, we observed differences in survival rates among plants treated with different concentrations of colchicine and exposed to different treatment durations (Table 1).

Table 1
Survival rates and tetraploid induction efficiencies in cumin using different concentrations and methods

Treatment type	Colchicine concentration (%)	Duration of treatment (h)	Survival rate (%)[a]	No. of tetraploid
Root induction	0.025	12	11.11	0
control	0	12	55.56	
Seed induction	0.05	24	10	0
control	0	24	95	
Seed induction	0.05	12	25	0
control	0	12	95	
Seed induction	0.025	24	20	0
control	0	24	100	
Seed induction	0.025	12	35	0
control	0	12	90	
Apical meristem	0.5	Rep 3 days	11.11	0
Apical meristem	0.2	Rep 3 days	33.33	1
Apical meristem	0.1	Rep 3 days	66.67	3
Apical meristem	0.05	Rep 3 days	88.89	6
Apical meristem	0.025	Rep 3 days	100	5
control	0	Rep 3 days	100	

a Number of survived plants out of 9 treated seedlings. The table only includes data from treatments that survived after one month.

3.1. Root induction. We obtained the higher survival rates when lower colchicine concentrations and shorter exposure times were applied. Concentrations higher than 0.05% and exposure times longer than 12 hours resulted in the destruction of all seedlings in the colchicine solution, making them unsuitable for transfer to pots. Of the remaining seedlings that were transferred to pots, only half of the seedlings from control treatment continued to grow until seeding (Fig. 1c). The root treatment method for cumin, which

involves repeated washing and transplanting of the roots, strongly affects the survival of the seedling and is not recommended. Similar effects have been reported for other species and were mainly attributed to the toxic effect of colchicine (Niel and Scherrmann, 2006; Lehrer et al., 2008; Sajjad et al., 2013, Dijkstra and Speckmann., 1980, Hanzelka and Kobza., 2004).

3.2. Seed induction. The seed induction experiment showed that as the colchicine concentration increased, seed germination decreased. No seeds germinated for colchicine concentrations higher than 0.1% and exposure times longer than 24 hours. For lower concentrations and times, some seeds germinated, but the resulting seedlings were weak and did not reach maturity, eventually being destroyed. Only the control treatment produced plants that continued to grow until the physiological maturity stage (Fig. 1e). These findings suggest that the treatment of cumin seeds with the tested concentrations and times exceeded the plant's tolerance, and further investigation with lower concentrations and shorter times is necessary. The observed results indicate that the use of colchicine on both seeds and roots of cumin is not suitable, as the plant is highly sensitive to chemicals and the treatments resulted in the death of the seedlings. As no survival was observed in experiments 1 and 2, statistical analysis was not performed for this section.

3.3. Apical meristem. All seedlings treated with a 0.5% concentration of colchicine, as well as some seedlings treated with 0.2% concentration, were wilted. Seedling growth retardation was the first visible effect of colchicine treatment, with treated seedlings showing a growth delay of approximately 10 days compared to control plants (Fig. 1g). Morphological changes were observed in all concentrations used in the apical meristem treatment, including an increase in plant height (Fig. 1h, i) and seed size (Fig. 1j) in polyploid plants that was visually observable. The highest number of tetraploid plants was obtained using a concentration of 0.05%. Given the success of polyploidy induction through terminal meristem treatment, all measurements, including flow cytometry, stomatal and cytological characteristics, were conducted only on terminal meristem treated plants. Our results suggest that terminal meristem treatment is the only appropriate method for in vivo tetraploidy induction in cumin, which is consistent with the findings of Dijkstra et al. (1980) on Caraway.

3.4 Difference in Stomatal cells

The morphological differences between stomatal cells of diploid and tetraploid *Cuminum cyminum* plants were investigated (Table 2). The stomatal cells of tetraploid plants exhibited a significantly larger size than those of diploid plants, also, more chloroplasts were observed in the guard cells of tetraploid plants, as demonstrated in Fig. 2. This morphological characteristic can be regarded as a reliable criterion for the diagnosis and identification of cumin with different ploidy levels. The average length and width of stomata in tetraploid plants were 69.4 and 44.45 µm, respectively, whereas these parameters were 53.3 and 28.35 µm, respectively, in diploid plants. The observed increase in stomatal size and decrease in stomatal density in tetraploid plants is consistent with previous studies on ploidy induction (Salma et al., 2017; Aina et al., 2012; Majdi et al., 2010; Xu et al., 2016; Noori et al., 2017; Zhou et al., 2017; Zhang et al., 2018; Eng and Ho, 2019; Pei et al., 2019; Purbiya et al., 2021; Akbari et al., 2021). According to Salma et

al. (2017), polyploid plants typically exhibit larger stomata than diploid plants, although this trait occurs at a lower frequency in polyploids. This may be attributed to the fact that polyploid stomata are often larger in size and have a lower density per unit area than diploid stomata. This is likely due to the larger cell size of polyploids, which may require lower stomatal density to maintain adequate gas exchange. Additionally, larger stomata in polyploids may facilitate more efficient water uptake, as polyploids often have a higher demand for water than diploids. The lower stomatal density observed in tetraploid *C. cyminum* plants may be due to an increase in the size of stomatal and epidermal cells, as well as less stomatal differentiation, as reported by Gantait et al. (2011). Overall, the distinct morphological features of stomatal cells in diploid and tetraploid *C. cyminum* plants can be utilized as a reliable tool for identifying and distinguishing plants with different ploidy levels.

Table 2
Comparison of stomatal characteristics between diploid and tetraploid cumin plants

Stomatal parameters	Stomatal length (µm)		Stomatal width (µm)		Number of chloroplasts	
Ploidy level	2x	4x	2x	4x	2x	4x
Measure	53.3	69.4	28.3	44.4	11.2	17.6
T-test	0.0062**		0.008**		0.0171**	
** Significant at 1% probability level						

3.5. Ploidy level

The ploidy level of the treated plants was evaluated using flow cytometry and chromosome counting techniques. The analysis confirmed that the chromosome number in treated plants was twice that of the diploid control plants, with a tetraploid level of 2n = 4x = 28 chromosomes compared to the diploid level of 2n = 2x = 14 chromosomes in the control plants (Fig. 3). Additionally, examination of the meiotic chromosomes in various phases provided further evidence of the chromosomal doubling in the treated plants (Fig. 4).

During meiosis, the control plant showed seven bivalents (2n = 2x = 14), while the polyploid plant displayed fourteen bivalents (2n = 4x = 28) in most of the PMSs at diakinesis/metaphase-I. The distribution of chromosomes during telophase-I was normal in the control group, with a distribution of 7:7:7:7, whereas in the tetraploid group, the distribution was 14:14:14:14 at each pole (Fig. 4).

3.6. Pollen grain size

Pollen grain size is a commonly used indicator of ploidy level in plants, as there is a positive correlation between pollen grain size and ploidy level (Sanders, 2021). In this study, we observed that ploidy level affected the size and shape of pollen grains. As expected, there was significant variation in pollen size within the plants, with tetraploid plants producing larger pollen grains compared to diploids. Of note, the larger pollen grains in tetraploid plants exhibited a striking triangular and penta angular shape (Fig. 5),

which may be a result of the mutagenic effect of the polyploidizing agent (Joshi & Raghuvanshi, 1967). This trend has also been reported in other studies (Tate et al., 2009; Omidbaigi et al., 2010; Abdoli et al., 2013; Godfree et al., 2017; Siqueiros-Delgado et al., 2017; Singhal et al., 2017; Wendel et al., 2017; Ahmad et al., 2018; Martin et al., 2019; Zhou et al., 2019).

4. Conclusion

Polyploidy induction through the use of colchicine is a well-established technique that has been widely reported in the literature (Sanders, 2021). Medicinal plants are in high demand globally, and there has been a growing interest in polyploidization using colchicine in this field of research (Salma et al., 2017; Miri, 2020). In this study (root and seed induction), we observed that the application of colchicine caused almost all the treated material to wilt in the seedling stage, even at a low concentration of 0.025%. This suggests that *Cuminum cyminum* is extremely sensitive to the chemical. However, the highest efficiency of tetraploidy induction was achieved using the apical meristem method with 0.05% colchicine. Chromosomal observations and flow cytometric analysis confirmed that colchicine induced DNA content duplication in tetraploid plants compared to diploid intact plants. Interestingly, the tetraploid plants showed significantly delayed flowering, with the flower buds appearing 10 days later than in diploid plants. Stomatal length and width were increased in tetraploid plants, while the stomatal density of diploid plants was greater than that of induced tetraploid plants. Additionally, there was clear variation in pollen size within the treated plants, with some pollen grains exhibiting a triangular shape. In summary, we have established an in vivo procedure for tetraploidy induction in *C. cyminum*. This procedure can provide valuable insights for the future cultivation and industrial-pharmaceutical processing of this plant, as it can create more genetic diversity and increase essential oil production and trade.

Declarations

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Ethical Approval

Not required as no human data or animal samples were used.

Consent to Participate

All authors agreed to participate in the present work.

Consent to Publish

All the authors agreed with the present publication.

Authors Contributions

Seyed Mohammad Mahdi Mortazavian (SMMM) is the supervisor and suggested the research idea; Zahra Sanaei Hoveida (ZSH) and SMMM designed the experiments, collected data, and contributed to interpreting the results. Maryam Norouzi (MN) and Seyed Ahmad Sadat-Noori (SASN) contributed in collecting data and statistical analysis. ZSH wrote the paper and SMMM edited the original draft. All authors approved the final manuscript for publication.

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Competing Interests

The authors declare that they have no conflict of interests.

Availability of data and materials

All data generated or analyzed during this study are included in the article and its supplementary information.

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Figures

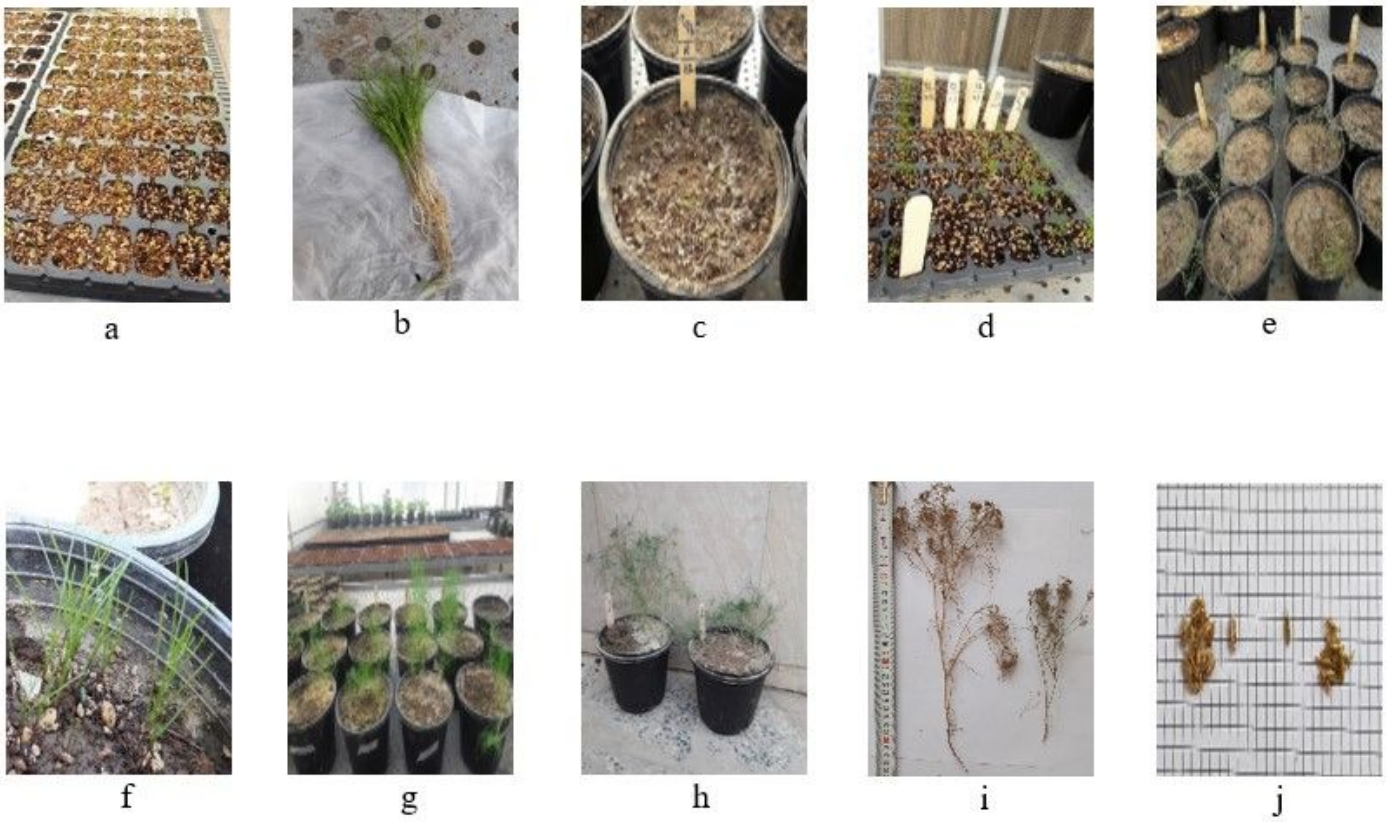

Figure 1

Effects of tetraploidy induction using different methods on cumin. (a-c) Root induction, (d-e) seed induction, with fewer seeds germinating as colchicine concentration and treatment time increased. (f-g) Apical meristem induction. (h-i) Comparison of plant height between diploid (right) and tetraploid (left) plants. (j) Comparison of seed size between tetraploid (left) and diploid (right) plants.

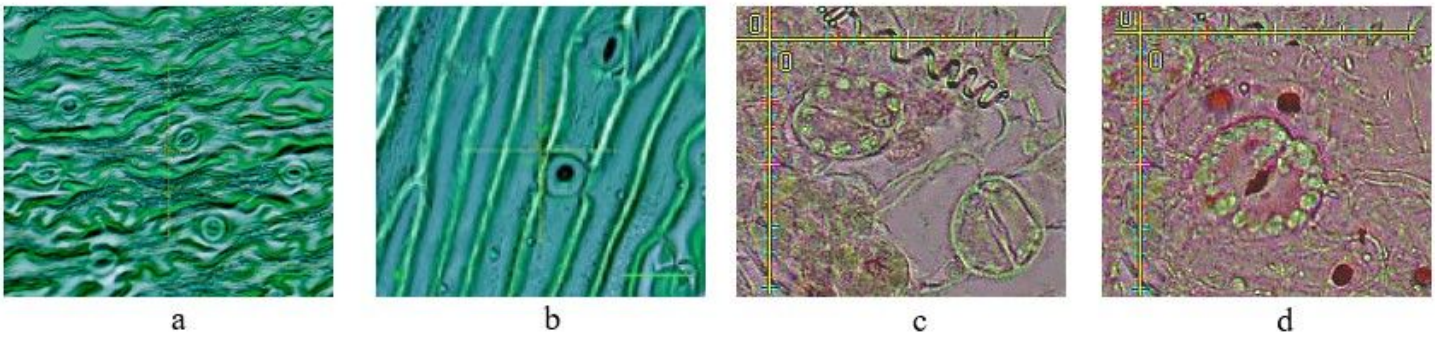

Figure 2

Stomata and chloroplast characteristics of cumin. (**a**) Stomata of tetraploid cumin plants (scale: 100 μm). (**b**) Stomata of diploid cumin plants (scale: 100 μm). (**c**) Number of chloroplasts in diploid plants (scale: 100 μm). (**d**) Number of chloroplasts in tetraploid plants (scale: 100 μm).

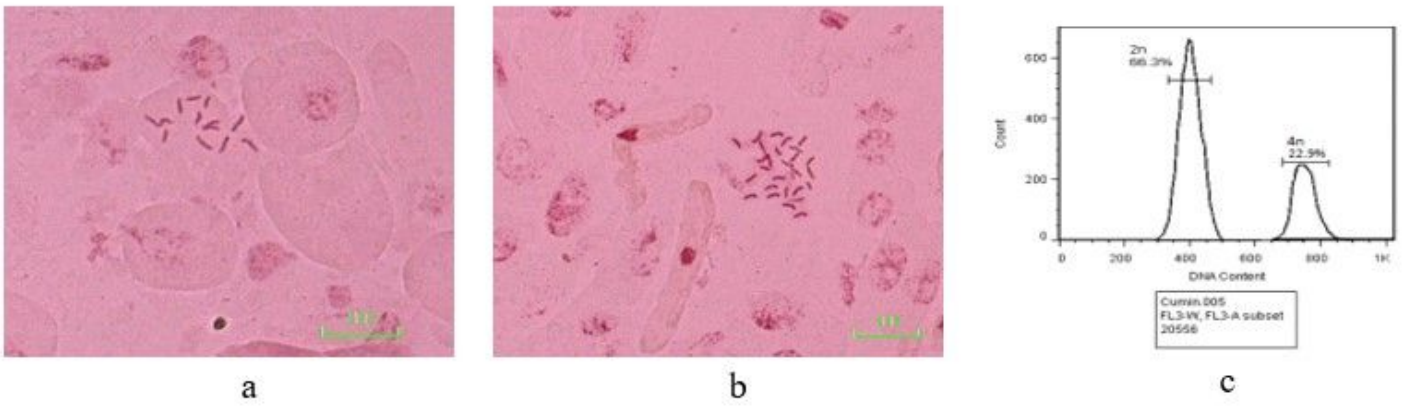

Figure 3

Chromosome counting and flow cytometry analysis of cumin. (**a**) Chromosome number of diploid plants (2n = 2x = 14) (scale: 50 μm). (**b**) Chromosome number of colchicine-induced tetraploid plants (2n = 4x = 28) (scale: 50 μm). (**c**) Flow cytometry results for diploid and tetraploid plants of cumin.

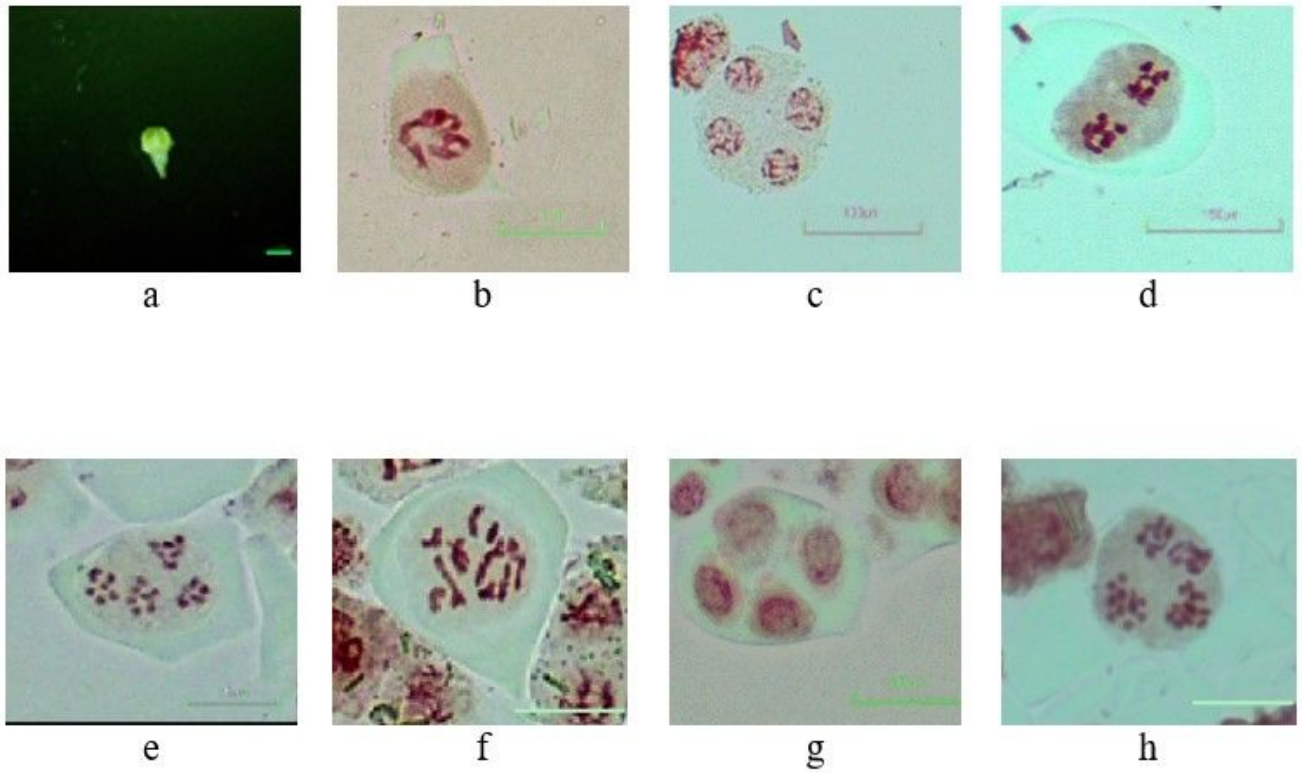

Figure 4

Meiosis in cumin plants. (**a**) Bud size used to check meiosis. (**b-e**) Meiosis in control (2x) plants (scale: 50 µm): (**b**) Diakinesis with 7II (2n=14), (**c**) Tetrad, (**d**) Normal anaphase with 7:7 segregation, and (**e**) Telophase-II with 7 chromosome distributions at each pole. (**f-l**) Meiosis in colchicine-induced tetraploid (4x) plants (scale: 50 µm): (**f**) Diakinesis with 14II (2n=28), (**g**) Tetrad, and (**h**) Telophase-II with 14 chromosome distributions at each pole.

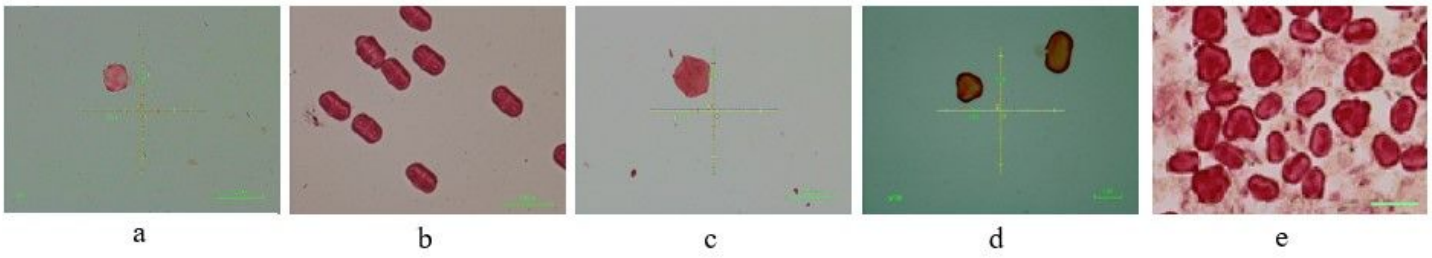

Figure 5

Pollen grain characteristics of cumin (scale: 10 µm). (**a-b**) Pollen grains from tetraploid cumin plants. (**c-e**) Pollen grains from diploid cumin plants.