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Evaluation of Colchicine Dosage Effects on Autopolyploid Induction in Diploid *Medicago sativa ssp. Falcata*

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ABSTRACT

Background: *Medicago sativa subsp.falcata* (yellow-flowered alfalfa), a perennial leguminous forage crop, has inherent agronomic limitations, including slow growth, low biomass yield and difficulties in cultivation. The development of polyploid germplasm is a promising strategy for varietal improvement, requiring systematic optimization of colchicine induction protocols.

Methods: Using imbibed seeds of diploid *Medicago sativa subsp. falcata* (2n=16), we performed colchicine soak treatments and systematically characterized mutagenized plants *via* stomatal morphology analysis and chromosome counting. Our quantitative assessment revealed concentration and exposure time-dependent effects on seed germination, seedling development and polyploidization efficiency.

Result: The results showed that increasing colchicine concentrations led to a progressive inhibition of seed germination and seedling growth. This was evidenced by a delay in germination initiation, a reduction in germination rates, an increase in hypocotyl diameter and a shortening of radicle length. After treatment with 0.15% colchicine for 6 hours, chromosome doubling was observed at a frequency of 10%, with a seedling survival rate of 39%. Simultaneously, the stomatal density of the treated polyploid plants reached the lowest. These results indicate that this method represents a feasible approach for polyploid breeding in this germplasm.

Key words: Chromosome number, Colchicine, Imbibed seeds, Medicago *sativa subsp falcata*, Mutagenic dosage, Stomatal density.

INTRODUCTION

Chemical mutagens are specialized compounds that alter DNA structure or function in organisms. These agents induce genetic mutations through diverse mechanisms, including chromosome doubling and the insertion or deletion of DNA segments (Ren, 2023). Among diverse chemical mutagens, colchicine exhibits a unique mode of action: it disrupts spindle formation during mitosis, thereby inducing chromosome doubling (Zhang *et al*., 2016). Owing to its low cost, high mutagenic efficiency and broad applicability across genetic materials, colchicine has become one of the most widely used chemical mutagens in practice. Following genome doubling, plants typically undergo a series of phenotypic and physiological modifications. These alterations include enhanced growth rates, improved genetic traits and increased metabolite accumulation (Li *et al*., 2024). Consequently, induced autotetraploids exhibit multidimensional differences compared to their diploid progenitors, spanning morphological, anatomical, physiological and agronomic characteristics. Polyploid plants exhibit characteristic phenotypic enhancements, including thicker, larger leaves with intensified pigmentation, elevated chlorophyll content, enlarged floral organs and fruits, thickened vascular bundles, as well as significantly improved stress tolerance and increased biomass and bioactive compound production (Lei *et al*., 2023; Veronesi *et al*., 1986; Zhang *et al*., 2024; Qiu *et al*., 2020).

Researchers have successfully induced polyploidy in various forage species-including *Melilotoides ruthenica* (Wang *et al*., 2013), *Medicago sativa* (Li *et al*., 2016) and *Lolium perenne* (Ferreres *et al*., 2019) -using colchicine and established corresponding mutant libraries (Darmadi *et al*., 2025). These mutagenized collections have not only provided valuable materials for functional genomic studies but also served as novel germplasm resources for cultivar development (Francis *et al*., 1989).

Yellow-flowered alfalfa *(Medicago sativa subsp. falcata*), a perennial leguminous forage crop (Wang *et al*., 2008), has been widely cultivated for its exceptional cold and drought tolerance. In China, extensive research efforts have focused on this species, including analyses of genetic diversity in germplasm resources (Li *et al*., 2022; Paun *et al*., 2024), improvement of local cultivars (Zhou *et al*., 2022; Li *et al*., 2025) and investigations into stress resistance mechanisms (Cui *et al*., 2019; Jia *et al*., 2025; Han *et al*., 2025).

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In this study, diploid *Medicago sativa* subsp. *Falcata* (2n = 16) germplasm from Xinjiang was used to investigate the effects of colchicine on polyploidization. Seeds were treated with various concentrations of colchicine for different durations to evaluate the following parameters: (i) seed germination rate, (ii) radicle growth dynamics, (iii) seedling survival rate, (iv) frequency of chromosome doubling and (v) stomatal morphological characteristics. This comprehensive profiling establishes an optimized colchicine induction protocol for efficient polyploid generation, providing a foundational mutagenized population for genetic improvement of this stress-tolerant forage crop.

MATERIALS AND METHODS

Plant material

The plant material used in this study consisted of wild *Medicago sativa subsp. falcata*. seeds (2n=16) collected from Xinjiang, China in 2021. Seeds were stored at 4°C with a thousand-seed weight of 0.9096 g. All experiments were conducted in 2024 at the Life Sciences Building ,Inner Mongolia Agricultural University (geographic coordinates: 40°83′N, 111°73′E).

Seed pre treatment

Plump, uniformly-sized diploid *Medicago sativa subsp. falcata*. seeds were selected and scarified with 30-grit sandpaper to disrupt the cuticular layer and remove surface contaminants. Seeds were subsequently surface-sterilized in 70% (v/v) ethanol for 30 s to mitigate fungal contamination during germination, followed by three thorough rinses with sterile distilled water and air-drying in sterile Petri dishes until no surface moisture remained. Pretreated seeds were transferred to sterile 90-mm dishes (100 seeds per dish; three biological replicates per treatment) and stratified at 4°C in a controlled climate chamber for 24 h to synchronize imbibition. Seeds exhibiting >90% volumetric expansion were identified as fully imbibed, blot-dried on Whatman ® No.1 sterile filter paper and immediately exposed to colchicine solutions to initiate polyploid mutagenesis.

Colchicine treatment

Imbibed *Medicago falcata* seeds were treated with colchicine solutions prepared in distilled water at concentrations of 0.05%, 0.1%, 0.15% and 0.2% (v/v), each supplemented with 1% DMSO as a penetration enhancer (Table 1 for experimental design). Seeds were immersed in sterile conical flasks containing 10 ml of the assigned colchicine solution per flask, with distilled water-treated samples serving as controls (CK). All flasks were sealed with Parafilm®, wrapped in aluminum foil for light exclusion and agitated at 100 rpm and 20°C for specified durations (3, 6, 9, 12, or 24 h). Post-treatment, seeds were rinsed under running water for 40 min followed by three sterile-water washes. Finally, seeds were plated on Petri dishes and incubated at 25°C under a 16-h light/8-h dark photoperiod.

Seed germination characteristics and radicle growth analysis

Germination was monitored daily over 7 days, with recordings of: (i) seed germination progress, (ii) cumulative number of germinated seeds and (iii) radicle development. Germination energy and seedling fresh weight were quantified on day 7. The experiment was terminated on day 15, at which point final germination percentages were calculated.

Germination energy (%) =

$$\frac{\text{Number of normally germinated seeds within 4 days}}{\text{Total number of tested seeds}} \times 100\%$$

Relative germination energy (%) =

$$\frac{\text{Germination energy of treated seeds}}{\text{Germination energy of control seeds}} \times 100\%$$

Germination rate (%) =

$$\frac{\text{Number of normally germinated seeds}}{\text{Total number of tested seeds}} \times 100\%$$

Relative germination rate (%) =

$$\frac{\text{Germination rate of treated seeds}}{\text{Germination rate of control seeds}} \times 100\%$$

$$\text{Germination index (GI)} = \Sigma \left(G_t / D_t\right)$$

Relative germination index (%) =

$$\frac{\text{Germination index of treated seeds}}{\text{Germination index of control seeds}} \times 100\%$$

(G_t = Number of seeds germinated on day t (dimensionless) D_t = Corresponding germination day (day)

Vigor index (%) = Germination index × Seedling fresh weight × 100%

Relative vigor index (%) =

$$\frac{\text{Vigor index of treatment}}{\text{Vigor index of control}} \times 100\%$$

Chromosomal analysis of root tip cells following colchicine treatment

(1) Root/shoot tip collection: Fresh root and shoot tips (~25 mm in length) were excised at 09:00 local time and immediately transferred to ice-chilled centrifuge tubes filled with distilled water. Samples were held at 4°C for 24 h to synchronize cell cycles before fixation.

(2) Fixation: Samples were immersed in freshly prepared Carnoy's fixative (95% ethanol: glacial acetic acid, 3:1 v/v) for 24 h at room temperature. After fixation, residual fixative was thoroughly removed by three 5-minute washes with distilled water under gentle agitation.

(3) Acid hydrolysis: Fixed root and shoot tips were transferred to 1.5-mL microcentrifuge tubes containing 1 M hydrochloric acid (HCl) and incubated at 60°C for 8 min in a precision

water bath (Julabo SW23). Following hydrolysis, residual acid was removed by three 2-minute washes with distilled water under gentle agitation (100 rpm).

(4) Slide preparation: The excised root/shoot tips were placed on glass slides and treated with 45% acetic acid solution. Cover slips were then applied and the samples were gently tapped with forceps until the tips dispersed into a homogeneous cloud-like suspension.

(5) Cytogenetic analysis: Ten cells per root tip were analyzed systematically at 1000× magnification. Chromosome counts were recorded for each cell and the proportions of diploid (2 n), mixoploid and tetraploid (4 n) cells were calculated relative to the total cell population.

Treatment intensity (TI) = Exposure duration (h) × Colchicine concentration (%)

Seedling emergence rate

Germinated seeds were transplanted into 10-cm-deep nursery trays filled with growth substrate (fertilizer: vermiculite: volcanic rock, 1:1:1 w/v). The relative survival rate of seedlings was quantified 30 days after transplantation.

$$\text{Survival rate (\%)} = \frac{\text{Number of surviving seedlings}}{\text{Total number of sown seeds}} \times 100\%$$

$$\text{Relative survival rate (\%)} = \frac{\text{Survival rate of treated seedlings}}{\text{Survival rate of control seedlings}} \times 100\%$$

Stomatal density quantification

For stomatal observations, fully expanded leaves of comparable size were sampled simultaneously from identical nodal positions. The abaxial epidermis was prepared using transparent adhesive tape (Xu, 2020) and imaged under an Olympus BX53 microscope with a 40× objective. Wild-type diploid Medicago sativa subsp. falcata from Xinjiang was used as the control.

Statistical and computational analyses

Statistical analyses were performed using Microsoft Excel 2021 and SPSS 26.0, with data visualization conducted in Origin 2022. Chromosomal images of diploid Medicago sativa subsp.falcata were processed using Photoshop for enhanced clarity.

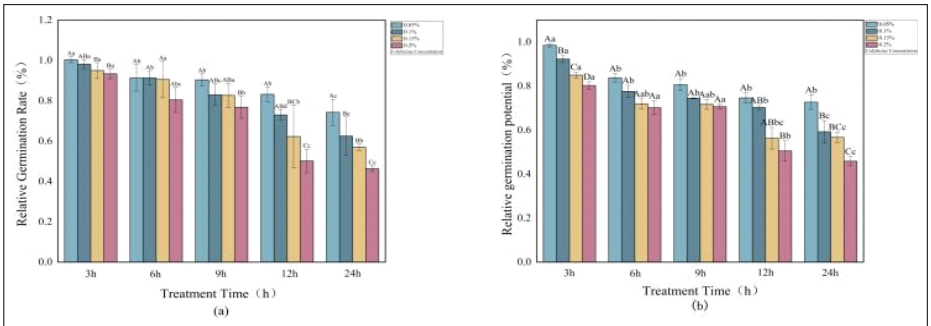
RESULTS AND DISCUSSION

Dose-and time-dependent effects of colchicine on germination in diploid *M. sativa subsp.falcata*

As shown in Fig 1, seeds treated with colchicine for 3 hours exhibited no significant difference in relative germination rate (RGR) compared to the untreated control group (P >0.05) at any of the tested concentrations, which may be attributed to the limited physiological impact of short-duration, low-concentration treatments (Xu, 2020). In contrast, extended exposure durations (6, 9, 12 and 24 hours) resulted in concentration-dependent decreases in RGR, with the most substantial inhibition observed at the highest concentration-time combination. The lowest RGR

Table 1: Colchicine treatment conditions.

Treatment concentration (w/v)	Contact time				
	3 h (B1)	6 h (B2)	9 h (B3)	12 h (B4)	24 h (B5)
0.05% (A1)	A1B1	A1B2	A1B3	A1B4	A1B5
0.1% (A2)	A2B1	A2B2	A2B3	A2B4	A2B5
0.15% (A3)	A3B1	A3B2	A3B3	A3B4	A3B5
0.2% (A4)	A4B1	A4B2	A4B3	A4B4	A4B5



Note: (a) Relative germination rate (RGR); (b) Relative germination potential (RGP). Capital letters indicate significant differences among concentrations at the same exposure time (P<0.05), whereas lowercase letters denote significant differences among exposure times at the same concentration (P<0.05). This notation applies to all subsequent figures.

Fig 1: Germination responses to colchicine concentration and duration.

(46.40%) was recorded under the most severe treatment conditions (0.2% colchicine for 24 hours). Furthermore, within each concentration group, prolonged treatment also led to time-dependent reductions in germination performance. Similarly, relative germination energy (RGE) showed significant dose- and time-dependent declines (P<0.05), demonstrating a progressive decrease with increasing colchicine concentration and exposure duration. The inhibitory effects of prolonged high-concentration treatments on RGR, RGE and relative vigor index were consistent with previous findings in *Medicago erecta* (Wang, 2013), *Medicago lupulina* and *Medicago sativa* (Liu, 2014). The phytotoxicity of colchicine appears to be positively correlated with both concentration and treatment duration, wherein excessively high concentrations or overly extended exposure times cause substantial damage to seeds (Harbard, 2012).

Dose- and time-dependent effects of colchicine on seed vigor in diploid *M. sativa subsp. falcata*

Fig 2 demonstrates that the relative seed vigor index (RSVI) was significantly lower in all treatment groups compared to the control (P<0.05). The RSVI progressively decreased with increasing colchicine concentrations and prolonged exposure durations. The most severe suppression of RSVI (23.10%) was observed at the highest concentration and longest duration (0.2% for 24 h).

Dose- and time-dependent effects of colchicine on radicle growth in diploid *M. sativa subsp. falcata*

During polyploid induction in plants using the seed-soaking method, colchicine often causes significant toxic effects on the root system. In this study, treatment of Medicago falcata seeds with colchicine resulted in a significant reduction in the average radicle length-by more than 65% compared with the untreated control group (P<0.05; Fig 3). Concurrently, the hypocotyl diameter showed a gradual increase with higher colchicine concentrations and longer exposure durations. Under the treatment with 0.2% colchicine for 9 h, the average hypocotyl diameter reached a maximum value of 1.33 mm, representing an increase of 0.83 mm compared to the control group (Fig 4). These morphological responses are consistent with reports by (Zhang, 2006) in Psathyrostachys juncea, as well as in Agropyron cristatum (Qalby *et al*., 2020) and *Medicago sativa subsp. falcata* (Liu, 2014), which described typical cytological abnormalities such as swollen coleoptiles and bulbous root tips. Further analysis indicated that higher concentrations and extended treatment durations exacerbated colchicine-induced damage to hypocotyl tissues. Treated radicles commonly exhibited yellowing and necrosis, loss of regenerative capacity and failure of hypocotyls to initiate new roots, ultimately leading to severely impeded root development and significantly reduced seedling survival. Similar phenomena have been reported in colchicine mutagenesis experiments in Taraxacum (Zhu, 2022). The underlying mechanism involves colchicine's disruption of spindle formation, which inhibits normal cell division. Meanwhile, ongoing cellular metabolic activities promote abnormal cell

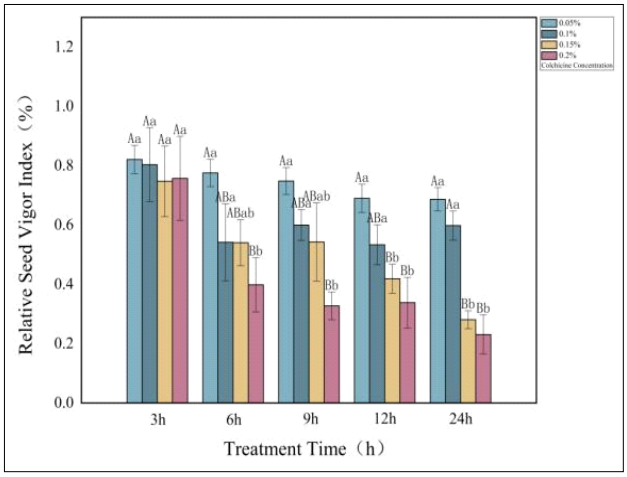


Fig 2: Seed vigor responses to colchicine gradients and exposure durations.

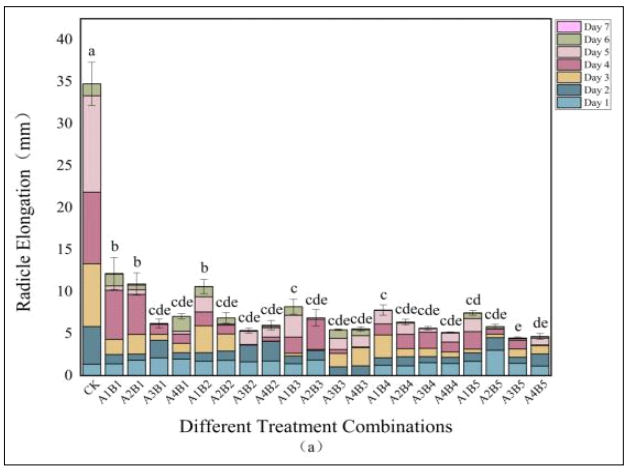


Fig 3: Radicle elongation under colchicine treatment.

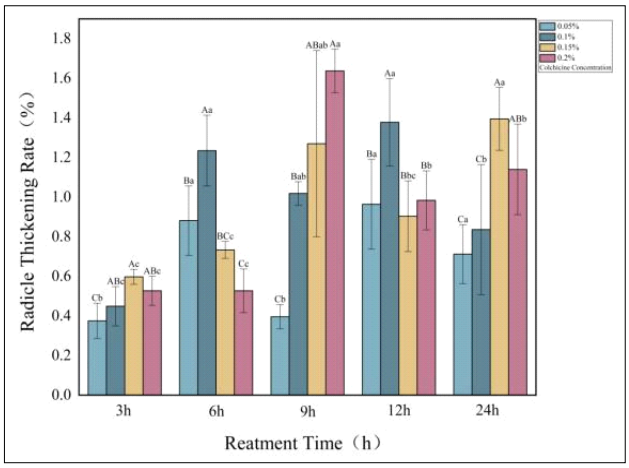
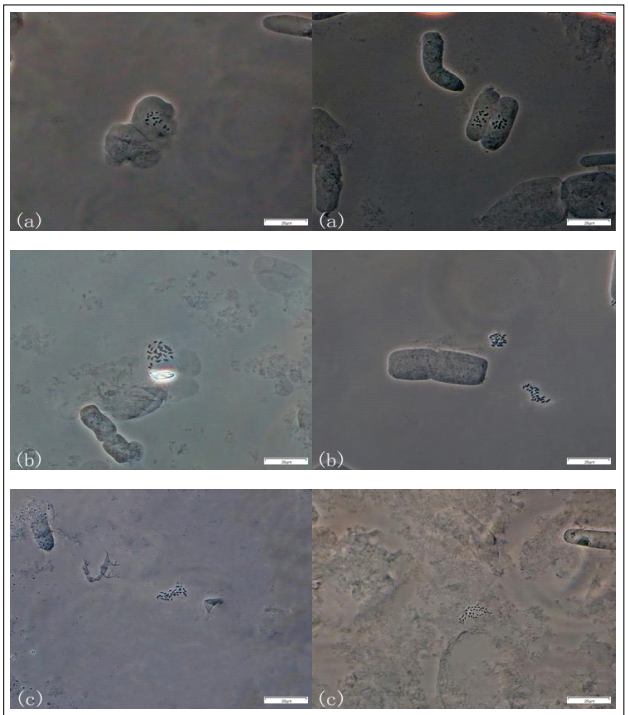


Fig 4: Hypocotyl thickening rate under colchicine treatment.

expansion, resulting in structural thickening of the hypocotyl and radicle. Notably, the increased radicle diameter may serve as an indirect morphological indicator of chromosomal variation, providing preliminary phenotypic evidence for successful polyploid induction.



Note: (a) Diploid (2 n = 16); (b) Mixoploid; (c) Tetraploid (2n = 4x = 32±3).

Fig 5: Ploidy identification by root tip squashing method.

Colchicine effects on chromosome numbers in diploid *M.sativa subsp. falcata* root tips

Polyploidy is inherently characterized by chromosome number doubling and chromosome microscopic observation and counting thus represent one of the most accurate identification methods (Li *et al*., 2016). The success of this technique hinges on the selection of an optimal fixation time to ensure that the observed materials are in an active stage of cell division. In this study, radicles were excised daily from 9:00 to 10:00, a time window corresponding to the peak of cell division during *Medicago falcata* seed germination (Liu *et al*., 2015). By comparing the chromosome numbers in the root tips of *M. falcata* treated with colchicine at different concentrations and for various durations, the tetraploid induction rate was found to increase progressively with rising colchicine concentrations and extended treatment times. Specifically, the results (Table 2) showed that treatment with 0.15% colchicine for 6 h resulted in a tetraploid cell proportion of 10.00%, while the maximum tetraploid ratio (30.00%) was achieved at an elevated concentration of 0.2% with a prolonged treatment duration of 12 h. These findings are consistent with those reported in colchicine-induced polyploidy studies on *Medicago sativa* (Basu *et al*., 2018), *Medicago sativa* cv. Jinda (Ji *et al*., 2012) and *Melilotoides ruthenica* (Kong *et al*., 2010). Furthermore, mixoploids first appeared when the treatment intensity (concentration × time) reached or exceeded 0.0045, whereas tetraploids could be induced when the treatment intensity surpassed 0.009 (Fig 5).

Table 2: Chromosomal analysis of diploid *M. falcata* under colchicine treatment.

Treatment concentration (%)	Treatment duration (h)	Treatment intensity	Diploid frequency (%)	Mixoploid frequency (%)	Tetraploid frequency (%)
CK	0	100.00	0.00	0.00	
0.05%	3 h	0.00015	100.00	0.00	0.00
0.1%		0.003	100.00	0.00	0.00
0.15%		0.0045	93.33	6.67	0.00
0.2%		0.006	80.00	20.00	0.00
0.05%	6 h	0.003	86.67	13.33	0.00
0.1%		0.006	83.33	16.67	0.00
0.15%		0.009	73.33	16.67	10.00
0.2%		0.012	70.00	16.67	13.33
0.05%	9 h	0.0045	83.33	16.67	0.00
0.1%		0.009	60.00	30.00	10.00
0.15%		0.0135	56.67	23.33	20.00
0.2%		0.018	26.67	53.33	20.00
0.05%	12 h	0.006	60.00	40.00	0.00
0.1%		0.012	53.33	26.67	20.00
0.15%		0.018	56.67	16.67	26.67
0.2%		0.024	56.67	13.33	30.00

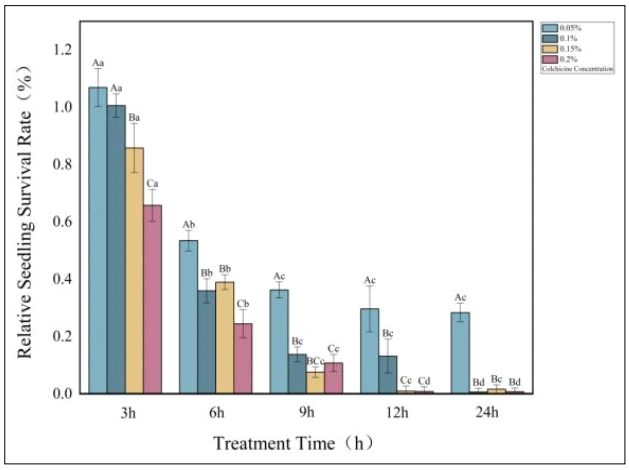


Fig 6: Seedling survival under colchicine treatment.

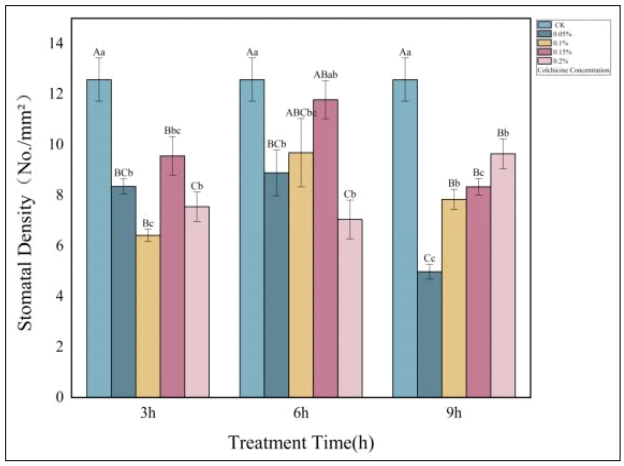


Fig 7: Stomatal density responses to colchicine treatment.

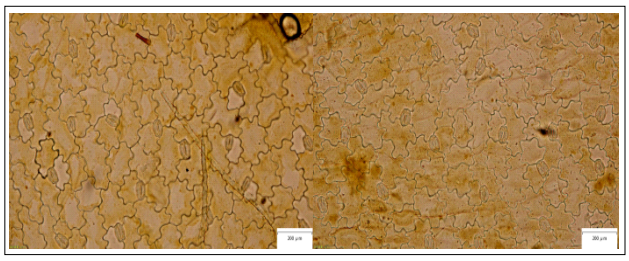


Fig 8.1: Stomatal morphology in untreated leaf epidermis of *M. sativa subsp. falcata.*

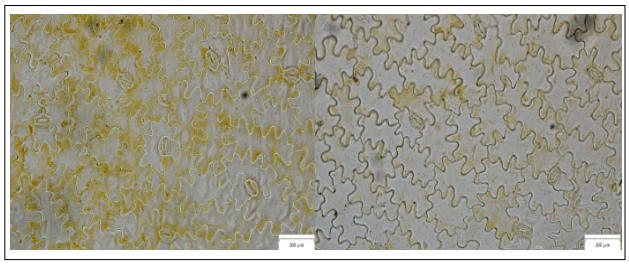


Fig 8.2: Stomatal morphology in colchicine-treated leaf epidermis of *M. sativa subsp. falcata.*

Dose-and time-dependent effects of colchicine on relative seedling survival in diploid *M. sativa subsp. falcata*

However, seedling survival rate showed a negative correlation with tetraploid induction rate, that is, a higher tetraploid induction rate was associated with a lower survival rate (Wang *et al*., 2024). This finding is consistent with the results of Li for colchicine-treated common alfalfa (*Medicago sativa*), where a negative correlation between tetraploidy rate and seedling survival was likewise observed (Li *et al*., 2016). Therefore, when determining the optimal mutagenic conditions for chromosome doubling in yellow-flowered alfalfa *(Medicago falcata*), it is essential to not only consider the tetraploid induction rate, but also to comprehensively evaluate key indicators such as seed relative vigor index and seedling survival rate. A comprehensive analysis in this study indicated that treatment with 0.15% colchicine for 6 h effectively induced tetraploidy, while resulting in a higher seedling survival rate compared to other treatment combinations that also induced tetraploids. As shown in Fig 6, the relative survival rate of yellow-flowered alfalfa seedlings exhibited a declining trend with increasing colchicine concentration and extended treatment duration. When the concentration reached 0.2% with treatment times of 12 h or 24 h, nearly complete mortality of the seedlings was observed.

Dose- and time-dependent effects of colchicine on stomatal morphology in diploid *M. sativa subsp. falcata*

Polyploid plants commonly exhibit increased cell volume. Normal cell growth and function rely on a dynamic balance between the nucleus and cytoplasm (Pacey *et al*., 2020). From Fig 8.1 and 8.2, as plant ploidy increases, the nuclear volume expands accordingly. To maintain a stable nucleo-cytoplasmic ratio and cellular functions, the cytoplasmic volume must also increase, leading to an overall enlargement of the cell. Stomata are formed by a pair of guard cells and the increase in their size directly results in larger stomatal dimensions (Zhang *et al*., 2024). Previous studies have confirmed that stomatal size, guard cell volume and chloroplast number are significantly positively correlated with ploidy level, whereas stomatal density is negatively correlated with ploidy (Padoan *et al*., 2013). For instance, (Francis *et al*., 1974) demonstrated that stomatal traits can be used effectively to differentiate ploidy levels in Cucurbita species. In the present study, stomatal density in leaves of mutagen-treated seedlings showed a significant difference compared to the control group (P<0.05). As shown in Fig 7, the lowest stomatal density was observed after treatment with 0.1% colchicine for 9 h, suggesting a higher chromosome doubling efficiency under this treatment condition.

FUTURE RESEARCH SCOPE AND DIRECTIONS

Based on the present findings, future research will focus on the following directions: First, the optimized treatment conditions will be adopted to induce polyploidy in diploid

Medicago sativa subsp. falcata seeds, with a systematic assessment of the intergenerational genetic stability of ploidy levels and key agronomic traits for screening stable, elite germplasm. Second, molecular markers coupled with whole-genome sequencing will be used to analyze genomic variation during colchicine-induced chromosome doubling and identify key genes associated with important agronomic traits.

CONCLUSION

(1) Colchicine treatment of imbibed diploid *Medicago sativa subsp. falcata* seeds (2 n=16) induced significant morphological alterations, including increased hypocotyl diameter (P<0.05) and reduced radicle length compared to untreated controls.

(2) Colchicine treatment induced chromosomal variations in the root tip cells of alfalfa, resulting in multiple cytotypes (2 n = 2x = 16±3, 2x < T < 4x, 2 n = 4x = 32±3). Among the tested regimens, treatment with 0.15% colchicine for 6 h was identified as the optimal dosage for tetraploid induction.

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Disclaimers

The views and conclusions presented in this article are those of all authors and do not necessarily represent the official positions of their affiliated institutions.The authors are responsible for the accuracy and completeness of the information provided, but shall not be held legally liable for any direct or indirect losses arising from the use of this content.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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