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Comparison of comet assay parameter patterns between self-pollinated and cross-pollinated diploid *Medicago* species, their resulting tetraploids and cultivated cultivars

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Original paper

Abstract: Three parameters of comet assay (tail length, tail intensity, and tail moment) were used to compare the autotetraploids produced from three populations of cross pollinated *Medicago sativa* spp. *caerulea* and five diploid self-pollinated species from *Medicago* genus. These specimens were subjected to three concentrations of colchicine (0.1, 0.5 and 1%) alongside five alfalfa cultivated cultivars. In the concentrations of 0.1% and 0.5%, a similar level of increase and pattern was observed in the two autotetraploids medic categories. Increasing of concentration from 0.1% to 0.5% resulted in a more pronounced augmentation of comet parameters. Autotetraploids induced by the two mentioned colchicine concentrations exhibited increases in the value and pattern of the three comet parameters compared to the cultivated cultivars and the two categories medics in diploid level. At the concentration of 1% colchicine, only two annual medic species produced tetraploids showing very pronounced augmentation of comet parameters in comparison with 0.1% and 0.5% of colchicine. Changes in patterns and values of the three parameters in induced tetraploids compared to cultivated alfalfa and the two categories medics in diploid level, demonstrate differential effects of damages of colchicines from one concentration to another. A new variability in each concentration change will be expected.

Keywords: comet analysis, autopolyploidy, alfalfa, colchicine, medic species.

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Introduction

The pattern of DNA migration from a single cell on the electrophoresis gel, resembling a comet, has been designated as the "comet assay" by Ostling O. and Johansson K. J. (Ostling and Johanson, 1984). Currently, the comet assay (i.e., the single-cell gel electrophoresis assay) is being used as a quick, popular, sensitive, and relatively cost-effective technique to detect transient genetic damages and their repair at the DNA level in eukaryotic cells. This technique has been used to detect the genotoxic effects of toxic agents (Forchhammer et al., 2012; Amaeze et al., 2015). The procedure was initially developed by Ostling and Johansson (Ostling and Johanson, 1984) at the level of the individual mammalian cells after irradiation, and it was later modified by Singh and coworkers (Singh et al., 1988). The use of higher plants for the detection of cytotoxicity and genotoxicity and monitoring its mutagenesis was recommended by the Royal Swedish Academy of Sciences (1973), the Council of the Environmental Mutagen Society (1975), and the World Health Organization (1985). In 1989, the assay was approved by the Swedish Board of the Protection of the Environment (Fiskesjó, 1993; Lanier et al., 2015). Before using plants in comet assay, some cytotoxic and genotoxic agents were used in plant breeding programs as physical or chemical mutagens. For many years, cytogenetic techniques have been used as a practical tool for detecting genetic and chromosomal damages and changes caused by genotoxic agents in crops, independent of the change being useful or useless. Such studies have focused on different species, including *Allium cepa* (onion) (Levan, 1938; Firbas and Amon, 2014) tobacco and eggplants (Kostoff and Kendall, 1931), or the gene mutation test in *Hordeum vulgare* (barley) (Gustafsson, 1940; 1947). Nowadays, the comet assay plays an important role in detecting the cytotoxic and genotoxic effects of mutagen agents. Duplication of the plant genomes, or polyploidy induction, which leads to changes in some horticultural, pharmaceutical, and agronomic traits in the plant species, is based on the application of different drugs, such as colchicine, oryzalin, trifluralin, and so on. Colchicine is the main chromosome doubling agent, which is extracted from the bulbs and seeds of the autumn crocus (*Colchicum autumnal*). It prevents the polymerization of tubulin, preventing spindle formation; hence, chromosomal segregation cannot occur in the dividing cells (Ade and RAI, 2010; Kumar and Rani, 2013; Manzoor et al., 2019). Taking into account the importance of polyploidy in plant improvement and the existence of two main categories of crops (i.e., self-pollinated and cross-pollinated crops), and endeavor to obtain more and healthier food and the importance of *Medicago* species in livestock feeding, it was important to study the behavior of two different categories of medic species versus using colchicine as a chromosome doubling agent.

Therefore, on the one hand, considering the importance of polyploidy in improving plants and on the other hand, as a factor in increasing the number of chromosomes, it is important to investigate the effects of colchicine as a genotoxic agent in these two categories of products. Due to the simplicity and sensitivity of the comet assay, it will be possible to demonstrate the genotoxic effects of colchicine in self-pollinating and cross-pollinating species of *Medicago*, where these two categories of products have differences in plant breeding methods.

Materials and Methods

The design of the study

The current study was carried out in the Genetic Laboratory of the Ahvaz Branch of the Islamic Azad University, Ahvaz, Iran. Three populations of *Medicago sativa* ssp. *caerulea* (i.e., Karaj1, Karaj2, and Tehran /IRAN as perennial diploid *Medicago*); five species of annual diploid *Medicago* (i.e., *M. radiata, M. lupulina, M. rigidula, M. truncatula, and M. turbinate*); four native alfalfa cultivars (i.e., Bamy, Hamadany, Bagdady, and ghareyonjeh); and one exotic cultivar (i.e., Ranger) were used in this study (Table 1). The polyploidy induction was already performed on the aforementioned diploid *Medicago* species by application of three concentrations of colchicine (0.1, 0.5, and 1%) in the auxiliary buds of the one node cuttings in our previous experiments (Ansari et al., 2021; Ansari et al., 2022).

The Comet assay

The seventh to twelfth leaves of the 12 to 18 cm cuttings of five annual diploid species, three populations of one perennial diploid species, eight tetraploid populations obtained from the induction of the diploid species, and five cutivated alfalfa cultivars were used for the comet

assay. The plant cell nuclei were isolated from the leaf tissues, and the alkaline single cell gel electrophoresis (SCGE) assay, developed for leaf tissues by Gichner and Plewa (1998) was used. The different steps of the comet assay for identifying the genotoxic and cytotoxic effects of three concentrations of colchicine in the diploid species, the tetraploids obtained from the diploids, and the cultivated tetraploid cultivars were followed. These steps are discussed in the following.

Step 1: Nuclei isolation from plants tissue

All operations were carried out under yellow or faint light. Leaves were removed from each species in three plant categories (annual diploid and perennial diploid species, induced tetraploids and cultivated tetraploids), and they were then placed in 60-mm petri dishes. Then, 500 ml of cold modified Sorensen buffer (50 mM of sodium phosphate at pH 6.8, 0.1 mM of ethylene diamine tetra acetic acid (EDTA), and 0.5% dimethyl sulfoxide (DMSO) was spread on the leaves that were kept on ice. Using a sharp razor blade, leaves were sliced to form fringes over most of the leaves' surface. The petri dishes were inclined in a way that the buffer was collected on the side, and the leaf fringes were immersed in the buffer and gently stirred five times. The fringes were spread over the bottom of the plate and rinsed with Sorensen buffer for several times using a cut plastic pipette tip. The plate was kept tilted in the ice so that the nuclei would be collected in the buffer.

Step 2: Slide preparation

The objective of the slide preparation step was to obtain a uniform gel to ensure easily viewable comets, a better attachment of the gel to the slides, and non-shedding. Regular microscopic slides were dipped into a solution of 1% normal melting point agarose (NMA) prepared with water at 50°C. The bottom of the slides was wiped to remove the agarose, then they were placed horizontally on a level surface and dried overnight at room temperature. Prepared slides were kept dry in slide boxes until use.

Step 3: Single Cell Gel Electrophoresis

Slides that were previously coated and dried with NMA were marked (labeling, numbering, and scoring).

The surface of each slide was supplemented with 30 µl of the nuclear suspension, then 60 µl of 0.75% low melting agarose (LMA), prepared with PBS, was added to each slide at 37 °C to bring the final concentration of LMA in the mixture to 0.5%. Using gentle and repeated pipetting with a disposable and cut micropipette tip, the cell nuclei and LMA were mixed, and then cover slips were placed on the mixtures.

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Table 1. Name and characteristics of plant material.

Medic characteristics	Species	Cultivars	Population
Annual, Diploid medics species/ Self-pollinated	*M. radiata*,	-	1
	M.rigidula,	-	1
	M. truncatula,	-	1
	M. turbinata,	-	1
	M. lupulina	-	1
Perennial, Diploid Medic species/ Cross-ollinated	*M. sativa* ssp. *caerulea*		3
Cultivated cultivars of alfalfa (Tetraploid)/ Cross pollinated	*M. sativa* L.	Bamy,	1
		Hamadany,	1
		Bagdady,	1
		Ghareyonjeh	1
		Ranger	1

The slides were placed on an ice surface for at least 5 minutes, after which the cover slips were removed, and a final layer of 90 µl of 0.5% LMA was placed on each slide. Cover slips were placed on the LMA, and the slides were maintained at 48°C for 5 min. Then, the cover slips were removed, and all the SCGE slides were immersed in a lysis solution composed of 2.5 M of NaCl, 1% sodium sarcosinate, 100 mM of Na_2 EDTA, and 10 mM of Tris (pH = 10) with 1% Triton X- 100 and 10% DMSO at 48°C. After a minimum of 1 h in the lysis solution, the slides were placed in a horizontal gel electrophoresis tank, containing a freshly prepared and cold electrophoresis buffer (1 mM of Na2 EDTA and 300 mM of NaOH, pH > 13). The nuclei were incubated for 20 min to allow the DNA to unwind, and then electrophoresis at 0.74 V/cm (25 V, 300 mA) at 48° C was carried out for 20 min. After electrophoresis, the slides were rinsed three times with 400 mM of tris at pH=7.5, and then they were stained with 60 µl of ethidium bromide (20 µ gram/ ml) for 5 min. The SCGE slides were immersed in ice water to remove excess ethidium bromide, and then they were covered with cover slips. For each slide, 15 cells were randomly selected to be analyzed under a fluorescence microscope with an excitation and a barrier filter. A computerized image analysis system (Open Comet version 1.3.1, 2016) was used to measure various parameters of the comets. SAS version 9.1 was used for statistical analysis and to draw the graphs. The tail length was measured from the leading edge of the head image. The most important parameters of the comet assay, i.e., the criteria for DNA damages, include the percentage of DNA in the tail (tail intensity), the length of the tail, and tail moment (tail intensity x the length of the tail), which were used in the statistical analysis.

Results

Comet assay parameters pattern and value in the diploid level of annual and perennial medic species

After performing the comet assay at the diploid level of annual and perennial *Medicago* species, three parameters of the comet tail (i.e., length, intensity, and moment) were measured in the annual and perennial diploid species of *Medicago*. The results showed no significant differences in comet parameters patterns and values between the genetic structures of the annual self-pollinated and perennial cross-pollinated species at the diploid level in terms of genome structural stability. For instance, the average of tail lengths for both cases were 10.81±0.26 and 10.78±0.26 microns, respectively (Table 2 and Fig. 1a).

Slides that were previously coated and dried with Eight distinct *CsCBL* were found in *C. sinensis* (Table 1). All *CsCBL* have 7 introns and 8 exons, except *SsCBL7*, which has 8 introns and 9 exons. They all code for between 213. Therefore, the two genetic structures have almost similar stability in gel electrophoresis field (Table 2).

Effects of 0.1% colchicine on comet parameters patterns and values

The pattern and values of the three comet parameters did not show any significant difference between the induced tetraploids resulted from annual diploid species and the tetraploids resulted from the perennial diploid populations treated with 0.1% colchicine concentration (Table 3, Fig. 1b, 2b, and 3b).

Table 2. Comet parameters in 3 populations of perennial and 5 diploid annual medic species.

Growth type	Diploids	Tail Length (µ)	Tail Intensity (%)	Tail Moment (µ)
Perennials (2n=2x=16)	Karaj 1	10.38	7.21	0.84
	Karaj 2	11.29	7.49	0.94
	Tehran	10.76	7.88	0.81
Mean values		10.81±0.26	7.52±0.19	0.86±0.04
Annuals (2n=2x=16)	*M. truncatula*	10.60	7.75	0.88
	M. lupulina	10.51	7.27	0.82
	M. rigidula	10.92	7.55	0.87
	M. radiata	10.67	7.19	0.86
	M. turbinata	11.22	7.59	0.84
Mean values		10.78±0.29	7.47±0.23	0.85±0.024

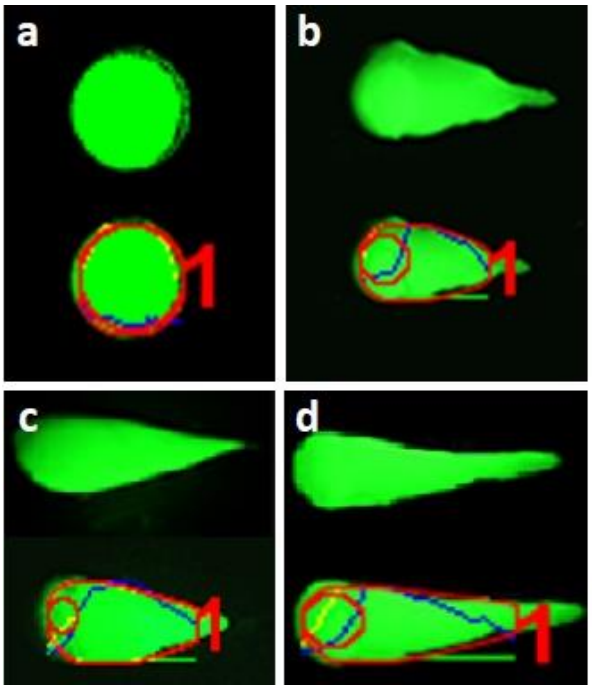

Figure 1. Comet assay in *M. truncatula* at diploidy level (a) and induced tetraploids at different concentrations of colchicine, 0.1% (b), 0.5% (c) and 1.0% (d).

Table 3. Comet parameters in the induced tetraploids from perennial and annual diploid species at 0.1% colchicine concentration.

Growth habit	Induced tetraploids	0.1% colchicine		
		Tail length (μ)	Tail intensity (%)	Tail moment (μ)
Perennials *M. sativa ssp. caerulea*	Karaj 1	15.17	12.94	1.96
	Karaj 2	15.26	13.03	1.98
	Tehran	15.28	13.39	2.08
Mean values		15.24 ± 0.03	13.12 ± 0.14	2.01 ± 0.04
Annuals	*M. truncatula*	15.37	13.56	2.17
	M. lupulina	15.74	12.86	2.02
	M. rigidula	15.51	13.27	2.07
	M. radiata	15.47	13.65	2.16
	M. turbinata	16.10	13.61	2.17
Mean values		15.64 ± 0.13	13.39 ± 0.15	2.12 ± 0.03

This means that the self-pollinated genome structure of the tetraploid from the annual *Medicago* species has shown a behavior similar to the cross-pollinated genome structure of the tetraploid from the perennial *Medicago* species against colchicine damages at the concentration of 0.1%. (Table 3).

Effects of 0.5% Colchicine on Comet parameters pattern

At the 0.5% colchicine concentration, tetraploids were obtained only in three species of annuals, i.e., *M. radiata*, and *M. truncatula*. The study of these tetraploids resulting from the aforementioned self-pollinated diploid species showed a similar comet parameters pattern (Table 4, Fig. 1c and 3c). Given the absence of the tetraploids originating from *M. rigidula* and *M. turbinata* self-pollinated diploid species, one could have imagined that they behaved differently compared to the three other annual species examined in this study at this colchicine concentration. Parameters pattern of tetraploids from three populations of perennial diploid

species, treated with 0.5% colchicine, was similar to each other (Table 3, Fig. 2c) and to the three annual species.

Effects of 1% Colchicine on Comet parameters pattern

Only the genomes of *M. truncatula* and *M. lupulina* have tolerated the damages of colchicine at 1% concentration (Fig. 1d and 3d). Then, genotype-dependent (species-dependent) genotoxic effects were shown. Genotype-depending effects of genotoxic agents were already demonstrated in *V. faba*, which appears to be more sensitive than *A. cepa* to Cd-induced genotoxicity (Arya and Mukherjee, 2014). The tetraploid plants resulting from the two aforementioned diploid species with tail lengths of 32.14 (*M. lupulina*) and 31.03(*M. truncatula*) microns showed greater but tolerable damages (Table 5).

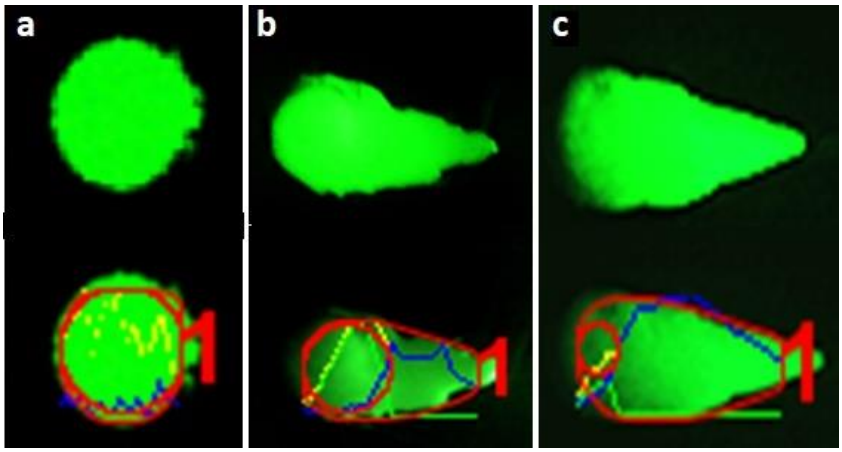

Figure 2. Comet assay in interphasique nucleus of: *M. sativa* cultivar Hamadany (a), and induced tetraploids at different concentrations of colchicine from *M sativa* ssp. caerulea Karaj 1 population at 0.1% (b) and 0.5% (c) colchicine.

Table 4. Comet parameters in the induced tetraploids in 3 annual and perennial species at 0.5% colchicine.

Growth habit	Induced tetraploids	Colchicine 0.5%		
		Tail length (µ)	Tail intensity (%)	Tail moment (µ)
Perennials	Karaj 1	25.03	23.55	5.80
	Karaj 2	25.18	23.84	6.04
	Tehran	25.06	24.37	6.14
Mean value		25.09 ±0.05	23.92 ±0.24	5.99 ±0.10
Annuals	*M. truncatula*	25.21	24.81	6.26
	M. lupulina	26.13	23.66	6.28
	M. radiata	25.22	24.84	6.34
Mean value		25.52 ±0.31	24.44 ±0.39	6.29 ±0.02

Table 5. Comet parameters in the induced tetraploids from two annual species at 1% colchicine concentration.

Annual species	1% colchicine		
	Tail length (µ)	Tail intensity (%)	Tail moment (µ)
M. truncatula	31.03	36.22	11.34
M. lupulina	32.14	34.54	11.18

None of the three populations of the perennial *M. sativa* ssp. *caerulea* could tolerate the damages caused by this concentration of colchicine, and no survivors were observed. According to these results, it might be possible to think about the more stable genomic structure of some self-pollinated species compared to the cross-pollinated species in this genus.

Comet parameters pattern in cultivated alfalfa cultivars

Examination of the comet parameters pattern in alfalfa cultivars without treatment with colchicine (Table 6) showed that the average tail length in these 5 cultivars was 10.64 microns (Fig. 2a and 3a); however, the percentage of DNA in the tail was lower in Hamedany and Qarehyonjeh cultivars (9.19 and 9.31, respectively) versus three others (Table 6). These two Iranian cultivars are generally grown in the cold regions of the country and two others which are also Iranian cultivars are grown in the warm regions of the country, therefore, adaptation of the genome according to the region can be concluded. the cultivar Ranger as a foreign cultivar cultivated in the different regions of the country behaved like the cultivars cultivated in the warm regions and it has shown the percentage of DNA in the tail, like Bamy and baghdady cultivars.

The tail length among the tetraploids derived from the population of the diploid *M. sativa* spp. *Caerulea* at the concentration of 0.1% of colchicine (Fig. 2b) was about 15.24 microns (Table 3), while it was about 25.09 microns at the concentration of 0.5% (Table 4 and Fig. 2c). The tail length of these tetraploids was longer than that of the cultivated cultivars with 10.64 microns (Table 6, Fig. 2a and 3a). Alfalfa cultivars generally have the genotype A1A2A3A4 (tetragenic form), then the full heterozygote form Demarly (1977), while the tetraploids derived from the diploid subspecies of *M. sativa* ssp. *Caerulea* had the genotype A1A1A2A2 or A3A3A4A4, etc (digenic form) because they were originated from Demarly (1977), while the tetraploids are derived from the diploid subspecies of *M.sativa* ssp. *Caerulea* had the genotype A1A1A2A2 or A3A3A4A4, etc (digenic form) because they were originated from A1A2 or A3A4, etc (diploid genotypes), and it has been demonstrated that these induced tetraploids have much lower cultural performance compared to the cultivated cultivar (Khosrowchahli, 1974).

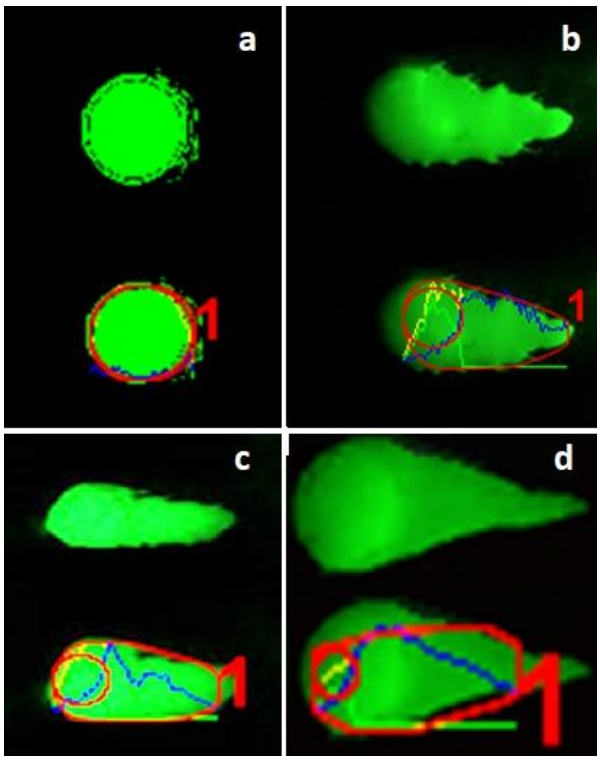

Figure 3. Comet assay in interphasique nucleus of *M. sativa* cultivar ghareyonjeh (a) and tetraploids from *M. lupulina* at 0.1% (b), 0.5% (c) and 1% (d) colchicine.

The high rates of comet parameters in these induced tetraploids (Fig. 2b and 3c) are probably due to the lack of sufficient opportunity and time to repair and rearrange their genome after the doubling process to arrive at the full heterozygote form (or to occur diploidization).

The tetraploids of two annual diploid self-pollinated species, i.e., *M. truncatula* and *M. lupulina* (at 1% colchicine concentration), showed a tail length of 31.01 and 32.14 microns, and a percentage of DNA in the tail (tail intensity) of 36.22 and 34.54%, respectively. Despite serious damage to their genomes (Fig. 1d and 3d), they were able to show a percentage of survival, and tetraploid plants were produced.

According to Table 7, DNA damages increased with increasing colchicine concentrations (changes in the size of comet parameters), which may lead to new and different rearrangements for DNA fragments damaged by each colchicine concentration in the chromosomes. This may also bring about a disruption of the organization of chromatin in the interphase nucleus of newly-produced tetraploids. Dose-dependence of the genotoxic effects of some chemical compounds, such as lead (Pb) in *A. cepa* (Jiang et al., 2014a; Jiang et al., 2014b), cadmium as a soil polluter in *A. cepa*, *V. faba* (Gichner and Plewa, 1998; Arya and Mukherjee, 2014) and *P. sativum* (Hattab et al., 2009) and ethyl methane sulfonate (EMS) as a mutagenic agent in tobacco (Gichner and Plewa, 1998) was already confirmed through the comet assay.

According to Table 7, the comparison of the three parameters of the comet assay between cultivated alfalfa cultivars and annual and perennial diploid species did not show significant differences. However, a significant difference was observed between the aforementioned species and all the tetraploids induced from the diploid species. Therefore, genome instability after treatment with colchicine, as well as the chromosomal doubling process, changes the genome so much that a performance similar to that of natural tetraploids cannot be expected in the short run.

Table 6. Comet parameters in cultivated alfalfa cultivars.

Cultivar	Tail length (μ)	Tail intensity (%)	Tail moment (μ)
Hamadani	10.84	9.19	1.04
Bamy	10.62	10.47	1.21
Ghareyongeh	10.77	9.31	1.07
Baghdady	10.45	10.36	1.53
Ranger	10.53	10.48	1.22
Mean value	10.64± 0.07	9.96 ± 0.29	1.21 ± 0.09

Table 7. Comet assay parameters in cultivated cultivars alfalfa, annual and perennial diploids and tetraploids from perennial and annual diploid species under different concentrations of colchicine.

Growth type of medic species	Colchicine %	Tail length (μ)	Tail intensity (%)	Tail moment (μ)
Cultivated cultivars *M. sativa* L.	Control	10.64± 0.07 d	9.96 ± 0.29 d	1.21± 0.09 d
Perennials diploids(2x)	Control	10.81±0.26 d	7.52±0.19 d	0.86±0.04 d
Annuals diploids(2x)	Control	10.94±0.15 d	7.50±0.08 d	0.87±0.01 d
Perennial diploids(2x)	0.1%	15.24 ± 0.03 c	13.12 ± 0.14 c	2.01 ± 0.04 c
Annual diploids(2x)	0.1%	15.64 ± 0.13 c	13.39 ± 0.15 c	2.12 ± 0.03 c
Tetraploids(4x) from perennial diploids(2x)	0.5%	25.09 ± 0.05 b	23.92 ± 0.24 b	5.99 ± 0.10 b
Tetraploids(4x) from annual diploids(2x)	0.5%	25.52 ± 0.31 b	24.44 ± 0.39 b	6.29 ± 0.02 b
Tetraploids(4x) from annual diploids(2x)	1.0%	31.59 ± 0.56 a	35.38 ± 0.84 a	11.26 ± 0.08 a

Means of columns followed by same letters have no significant difference based on Tukey mean comparison.

4. Discussion

While polyploidy induction has been an important technique in plant breeding for several decades (Dhooghe et al., 2011), it's significance particularly pronounced in horticulture, vegetative-propagated crops, and some forages, such as alfalfa, where it plays a pivotal role in refining the quality and enhancing the biomass. Polyploidy was first discovered in 1907, resulting in an increase in the number of chromosomes (Alam and Razaq, 2015). Polyploidy in nature can develop through cytological mechanisms, such as the crossing of unreduced gametes (Pereira et al., 2014), or by the doubling of the number of chromosome at the zygotic or somatic levels due to undesirable environmental conditions (Alam and Razaq, 2015). However, the natural processes to create an adequate and profitable species are slow. Colchicine is an antimitotic agent which has speed up this process. Since the discovery of colchicine in the 1930s as a mitoclasic agent, the induction of polyploidy has experienced a great boom (Marzougui et al., 2011). In addition, the polyploidization effects of colchicine and its mutagenic effects on plants have also been shown (El-Nashar and Ammar, 2016). Moreover, its genotoxic effects have been confirmed using the comet assay, where it is shown to induce DNA damages (Kiffe et al., 2003).

The current study has compared the pattern of comet parameters in non-treated and treated self and cross pollinated diploid medic species with colchicine using the comet assay. In the polyploidization of two types of diploid species, two types of induced tetraploids were obtained (from annual self-pollinated and perennial cross-pollinated species). At a concentration of 0.1% to 0.5% of colchicine, a significant increase was observed in the three comet parameters in the tetraploids from those two types of *Medicago* species compared to each other and to the cultivated cultivars and diploid species which the tetraploids were obtained. However, the comet parameters pattern (evaluating the damages caused by colchicine) at the tetraploids treated with 0.1% and 0.5% concentration of colchicine, did not show a significant difference between the genetic structures of self-pollinated and cross-pollinated *Medicago*. In the concentration of 1%, no survivors were observed in perennial cross-pollinated species. However, in the two annual self-pollinated *Medicago* species (i.e.,*M. lupulina* and *M. truncatula*), tetraploid survivors were observed, showing that these two annual diploid *Medicago* species were more tolerant against the genotoxic effects of colchicine.

Due to the heterozygote genetic structure of the diploid perennial *Medicago* populations like A1A2, A2A3, A1A4 (*M. sativa* spp. *Caerulea*), the tetraploids induced from this perennial *Medicago* diploid species will be heterozygous as well like A1A1A2A2, A2A2A3A3 and so on, therefore the members of the resulting tetraploid population will be different from each other and in the digenetic or duplex state. Consequently, in each colchicine concentration different structural damages and different repair systems may have occurred resulting in a different genome rearrangement in each member of the resulting tetraploid population because the diploid parents had been genetically different from each other from the start (Khosrowchahli, 1974). Tetraploids obtained at the concentrations of 0.1% and 0.5% will also differ from each other due to different damages and restructurings inflicted on the genome at each concentration, as shown in the values of the comet parameters. This can be true for tetraploids obtained from the cross-pollinated perennial species of *M. sativa* spp. *caerulea* and the self-pollinated annual *Medicago* species. Of course, due to the limited number of polyploid plants obtained during the polyploidy induction, these issues are not usually taken into account. The comparison of comet assay parameters between the cultivated alfalfa cultivars which are fully heterozygous, that means in the tetragenetic or quadruplex state (Demarly, 1977) and the tetraploids obtained from the diploid species has shown that the pattern of the three comet parameters in the induced tetraploids were different and their values were much higher than those of the cultivated tetraploids. Therefore, the induced tetraploids may have an unstable and unfixed genetic structure caused by the colchicine treatment. Obtaining, stability in the genome and performance similar to cultivated alfalfa in the induced tetraploids from cross-pollinated species will probably be possible by the establishment of the new rearrangement of chromosomal structures and territory reorganization in the interphase nucleus after the damages inflicted by colchicine and arriving at the state of complete heterozygosity. This is probably not the case of self-pollinated species because these treated

genetic structures are homozygous and the tetraploids derived, can acquire appropriate performance after the damages is repaired. Investigating on the pattern of the three parameters of the comet assay in the two diploid categories of medic species and the auto-tetraploids obtained from them showed that the pattern and their values were significantly different and this difference increased by colchicine concentrations, it was demonstrated that the induced auto-tetraploids despite the damages inflicted by colchicine have revealed increases in the performance of the many characters studied (Ansari et al., 2021).

Conclusion

Owing to the variations in genetic structure of the diploid cross-pollinated species populations, such as *M. sativa* spp. *caerulea* populations, where plants may exhibit the heterozygous nature (A1A2, A1A3 or A2A3, A1A4, etc,) of genetic structures, the disparities observed in morphological and other characteristics of the induced autotetraploids can be attributed partly to these inherent structural genetic differences and partly to the pattern of genotoxic effects of colchicine at each level of concentration. At each concentration level of colchicine, the genetic structure of treated plants will give rise to distinct plant variations. Another source of variability in the resulting autotetraploids in this cross-pollinated species must be acknowledged. This variability arises from the varying concentration of colchicine, each exerting a distinct genotoxic effect. Consequently, different genotypes will respond differently to different colchicine concentrations, leading to additional variability. While this variability is valuable for breeding programs, its significance may be overlooked due to the limited number of autotetraploid plants obtained. This holds particular significance for the alfalfa breeding program, given the current narrowness of the alfalfa gene pool. Due to the homozygous structure inherent in self-pollinated diploid species, such as the various annual diploid species of *Medicago*, a diverse range of tetraploids will only emerge when colchicine concentrations change. Interestingly, comparable patterns and comet parameter values were observed for the cross-pollinated and self-pollinated Medicago species at 0.1% and 0.5% colchicine concentration. This suggests a parallel behavior of these distinct genetic structures in response to these two levels of colchicine concentrations.

Supplementary Materials:

No supplementary material is available for this article.

Author Contributions:

The authors confirm contribution to the paper as follows: conceptualization, M.Kh.; methodology, E.A.; formal analysis: A.A.J.; writing—review: M. Kh., A.E. All authors have read and agreed to the published version of the manuscript.

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مقاله پژوهشی

مقایسه الگوهای پارامترهای سنجش دنباله‌دار بین گونه‌های *Medicago* دیپلوئید خودگرده‌افشان، دگرگرده‌افشان و تتراپلوئیدهای حاصل از آنها و ارقام زراعی

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چکیده: سه پارامتر آزمون دنباله دار (طول دم، شدت دم و گشتاور دم) بین اتوتتراپلوئیدهای تولید شده از سه جمعیت گونه *Medicago sativa* زیر گونه *caerulea* دگر گرده افشان، پنج گونه دیپلوئید خود گرده افشان از جنس *Medicago* تیمار شده با سه غلظت کلشیسین (۰/۱ ، ۰/۵ و ۱٪) و پنج رقم یونجه زراعی مقایسه شد. در غلظت‌های ۰/۱ و ۰/۵ درصد، افزایش و الگوی مشابهی در دو گروه اتوتتراپلویدالقا شده مشاهده شد. همچنین افزایش غلظت از ۰/۱٪ و ۰/۵٪ منجر به افزایش بارزتر پارامترهای دنباله‌دار شد. اتوتتراپلوئیدهای القا شده از دو غلظت کلشی سین مذکور در مقایسه با ارقام کشت شده و دو رده یونجه در سطح دیپلوئید، افزایش در ارزش و الگوی پارامترهای آزمون دنباله دار نشان دادند. در غلظت ۱ درصد کلشیسین، تنها دو گونه یونجه یکساله اتوتتراپلوئید در مقایسه با غلظت های ۰/۱ و ۰/۵ درصد کلشیسین و افزایش واضح تر پارامترهای آزمون دنباله دار را نشان دادند. تغییرات در الگوها و مقادیر هر سه پارامتر در تتراپلوئیدهای القایی در مقایسه با یونجه کشت شده و گونه‌های دیپلوئید یونجه، اثرات متمایز آسیب‌های کلشیسین را از غلظتی به غلظت دیگر نشان داد، در نتیجه تغییرات جدیدی در هر تغییر غلظت مورد انتظار است.

کلمات کلیدی: آزمون دنباله‌دار، اتوتتراپلوییدی، یونجه، کلشیسین، گونه‌های یونجه.

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