



Citation: E. Ansari, M. Khosrowshahli, A. Ashraf Jafari, A. Etminan (2021) Induction of Autotetraploidy and its effects on morphophysiological traits in some annual and perennial medics. *Caryologia* 74(1): 75-82. doi: 10.36253/caryologia-809

Received: January 03, 2020

Accepted: April 26, 2021

Published: July 20, 2021

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Induction of Autotetraploidy and its effects on morphophysiological traits in some annual and perennial medics

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Abstract. In order to study of the effects of polyploidy on morph-physiological traits in some annual and perennial medics, five annual and three perennial diploid medics were subjected to different concentrations of colchicine solution (0.1%, 0.5%, 1% and 2%) in mitosis stage. The induced tetraploids were identified by counting stomata guard cells chloroplasts. The highest survival rate and tetraploidy induction with average values of 80.2% and 74.3% were obtained using 0.1% colchicine concentration. All of the 21 entries (5 annual diploids and their induced tetraploids as *M. lupulina*, *M. radiata*, *M. rijidula*, *M. truncatula*, *M. turbinata*, and 3 populations of perennial diploid of *M. sativa* ssp. *Caerulea* (Karaj1, Karaj2 and Tehran) and their induced tetraploids and 5 commercial tetraploid alfalfa cultivars (Bami, Hamadani, Baghdadi, Ghareyongeh and Renger) grown in pots in a glasshouse experiment using completely randomized design with five replications in 2018 in Ahvaz, Iran. Data were collected for leaf length, leaf width, shoot and root length, seedling weight, shoot weight, root weight, branch number, leaf area index (LAI) and water use efficiency (WUE). Result of mean comparisons between three groups (2x, induced 4x and controls 4x), showed that both groups of induced and natural tetraploids had significantly higher mean values for all the traits except leaf length, shoot length and root length than that for diploids. For the latter traits there were no significant differences between 2x and induced 4x. On the overall, the induced 4x had 60%, 62%, 68%, 65%, 22%, 109% and 47% higher mean values than their parental 2x for seedling weight, shoot weight, root weight, LAI, WUE and branch number, respectively. It was concluded that increasing ploidy level provides plant breeders with a promising tool in the breeding improve new varieties suited for future climate scenarios.

Keywords: ploidy level, colchicine, morphological traits, annual *Medicago*, perennial *Medicago*.

INTRODUCTION

Alfalfa (*Medicago sativa*) is a strategic and important forage species for animal feeding. A limited number of *Medicago* species are cultivated for animal uses or for breeding improved varieties. Among these species, *Medicago sativa* is widespread in the most parts of the world and its wild forms are rarely available. Commercial varieties of *M. sativa* are perennial cross-pollinated autotetraploid ($2n=4x=32$), with Tetrasomic inheritance. *M. sativa* has both diploid and tetraploid forms (Lapina *et al.*, 2011). The sub species of *Caerulea* is a perennial and diploid form ($2n=2x=16$). Theoretically, conventional alfalfa has been evolved by sexual polyploidy, which is equivalent to crossing non-reduced ($2x$) gametes present in diploid species (Bauchan and Hossain, 1997; Rosellini *et al.*, 2016; Pfeiffer and Bingham, 1983). *Caerulea*'s germplasm has the unused potential of being selected as a perennial, sustainable, drought-resistant and soil improving and pasture rehabilitation (Li *et al.*, 2010).

Annual alfalfas are more resistant to plant pests than perennial alfalfa (Bauchan and Azhar, 1998) and can be used in breeding programs as a genetic source to fungal and pest diseases resistance (Yaeger and Stutville, 2000), cold resistance and resistance to adverse and acidic soil conditions (Gillespie, 1989). Annual alfalfas through fertility and improved physical quality, nitrogen fixation, increased organic carbon of soils (Dalal *et al.*, 1995) have a positive effect on grain yield of further grain crops. In addition, with the development of annual alfalfa cultivation, significant amounts of forage can be obtained. Annual alfalfa can grow in summer and fall and planted in rotation with wheat. They also prevent soil erosion (Biederbeck *et al.*, 1993; Badaroddin and Meyer, 1990; Mirzaei Nodoushan, 2001). For many years, common alfalfa has been improved based on the classic plant breeding program. Alfalfa varieties are usually synthetic varieties; Developed by crossing of selected heterozygous parents and their offspring have advanced over three or four generations of seed proliferation (Rowe and Hill, 1999).

Duplication of the plant genomes, or polyploidy induction, which leads to changes in some traits, especially in horticultural, pharmaceutical and agronomic plant species, is based on the application of different drugs such as colchicine, new dinitroanilines, phosphorothioamidates oryzaline, and triflurarin etc. (Melnychuk *et al.*, 2020; Niazian *et al.*, 2020; Su-Jin *et al.*, 2020; Touchell *et al.*, 2020). Colchicine still remains the most efficient and clearly the most preferred and the most used anti mitotic agents because of its widely successful mitosis inhibition ability (Touchell *et al.*, 2020), and for

its advantages such as high percentage of viability (Melnychuk *et al.*, 2020) and because of its widely successful mitosis inhibition ability, high solubility in water and ethyl alcohol and heat stability, ability to be autoclaved and easily applied to plant tissues solutions. Therefore reduce the use of additional solvents, it is heat-stable, and can be autoclaved and easily applied to plant tissues (Touchell *et al.*, 2020).

In the context of successful sexual autopolyploidy induction (by crossing (in alfalfa species, a comprehensive research was conducted by Rosellini *et al.* (2016). Osborn *et al.*, (2003) in their review concluded that a novel variation in polyploids could involve changes in gene expression through increased variation in dosage-regulated gene expression, altered regulatory interactions, and rapid genetic and epigenetic changes. Mirzaei Nodoushan, (2001) reported that induction of polyploidy in self-pollinated and annual species is likely to improve their morphological and physiological properties and probably can facilitate the adequate gene transfer.

There are some reports on phylogeny of domestic and foreign populations of *Medicago* genus in Iran. Ghanavati *et al.* (2005) in study of Genetic diversity of 22 species of genus *Medicago* collected from Iranian natural habitat using RAPD marker generated a phylogenetic tree with 5 main cluster. Populations of *M. aculeala*, *M.constricta*, *M.rigiduloides* and *M. rigidula* with hard pod walls and spongy texture were classified in one cluster. Populations of *M. sauvagei*, *M. laciniata* and *M. polymorpha* which had soft and flexible pod walls were classified together in a separate cluster. In another experiment Ghanavat (2010) in phylogenetic analysis of 23 species of *Medicago* based on 90 morphological characteristics by maximum parsimony approach, observed the most relationships between *M. rugosa* and *M. scutellata*, *M. sativa* and *M. lupulina*, *M. coronata* and *M. minima*, *M. rigidula* and *M. rigiduloides*, *M. polymorpha* and *M. arabica*, *M. tornata* and *M. turbinata*. Salimpour (2012) in phylogenetic analysis of 23 species of alfalfa, the most phylogenetic relationships was observed between *M. rugosa* and *M. scutellata*, *M. sativa* and *M. lupulina*, *M. coronata* and *M. minima*, *M. rigidula* and *M. rigiduloides*, *M. polymorpha* and *M. arabica*, *M. tornata* and *M. turbinata*. This information can be used in determining the degree of success of inter-specific hybridization between different species in *Medicago* genus.

There are many comparative cytogenetic analyses between diploid and tetraploid perennial medics (Yu *et al.* 2017). But there are few published reports of comparative cytogenetic analysis between perennial and annual diploid medics and their induced tetraploids. The aim of this study was to generate the best populations of annual and

perennial diploid medics using polyploidy breeding after induction by colchicine treatment and to compare changes of morpho-physiological traits between diploids 2x and induced 4x species and commercial 4x alfalfa cultivars.

MATERIALS AND METHODS:

In this study, seeds of five annual diploid alfalfa were provided from natural resource gene bank of the Institute of Forests and Rangelands, Tehran, Iran (Table 1). At the same time, seeds of three perennial diploid were provided from College of Agriculture, Tehran University and seeds of five commercial varieties were obtained from Pakan Seed Company, Isfahan, Iran.

Prior to seed sowing, seeds were scarified mechanically with sand paper and then sterilized with a benomyl fungicide. Seeds of five annual diploid medics (*M. lupulina*, *M. radiata*, *M. rigidula*, *M. truncatula*, *M. turbinata*) and three perennial diploid *Medicago sativa* ssp. *Caerulea* populations (Karaj1, Karaj2 and Tehran) were sown in a mixture of peat moss and field soil at a ratio of 2: 1 in plastic pots with 17 cm diameter. Pots were kept in 20-25°C glasshouses and were irrigated regularly. About three months after sowing, 20 rooted single node cuttings from each of the young plants of diploid seedlings in mitosis stage subjected to three concentrations of colchicine solution (0.1, 0.5, and 1%) using dropper method. A drop of colchicine in the lateral bud was put and was repeated for four days after drying or being absorbed and after 15 days, the viability of cuttings was evaluated. The survival rate of the plants was calculated based on the number of well-established plants on the total number of cuttings treated in all the species studied. The induced tetraploids were identified using the usual method of chromosomes counting in the metaphase cells of root tip meristems with having a minimum of 10 metaphase plate mitosis for each species/populations. The highest induced tetraploidy (80.2%) was obtained using 0.1% colchicine concentration. Then, all of the 21 entries: 5 annual diploids and 5 induced tetraploids, 3 perennial diploids and 3 induced tetraploids

of *Medicago sativa* ssp. *Caerulea* and 5 commercial tetraploid alfalfa cultivars as control (Baghdadi, Bami, Hmamadani, Renger, Ghareyounje) grown in pots in a glasshouse using completely randomized design with five replications in 2018 in Ahvaz, Iran.

In diploid and induced tetraploids (using 0.1% colchicine) and commercial tetraploids, data collected for leaf length (mm), leaf width (mm), shoot length (cm), root length (cm), shoot weight per plant (g), root weight (g), seedling weight (g) and, branches number per seedling, leaf area index (LAI) and water use efficiency (WUE). After about two months after growth of seedlings and induction polyploidy plants, middle leaves of seedlings were selected for measurements. LAI measurements were performed manually using leaf plot and shadow measurements using mm checkerboard paper. The WUE was estimated as Y/W , where: Y= water beneficially used (seedling dry weight) and W=total amount of water that was estimated for each plant during the growth period. The data were analyzed by completely randomized design experiment and means were compared using Tukey's test. The SAS9.1 software was used for analysis of variance and mean comparisons.

RESULTS

In the present study the effects of polyploidy induction on morph-physiological traits in five annual and three perennial diploid medics were assessed and they were subjected to different concentrations of colchicine solution (0.1%, 0.5%, 1% and 2%) in mitosis stage. The highest survival rate (80.2%) and tetraploidy induction (74.3%) were obtained in 0.1% colchicine concentration.

Result of analysis of variance showed a significant difference between all of the 21 entries for all of traits ($P<0.01$). Such differences were predictable due to the origin and genome differences and ploidy levels between populations (Table 2). The Maximum of coefficient of variation (CV%) was 21.77% for root length and minimum was 11.87% for seedling weight, indicating that good accuracy of the experiment in evaluating traits.

Table 1. Name, Specifications and Origin of Annual Diploid alfalfa.

Scientific name	Code	Origin of seed	Province	Altitude m	Latitude	Longitude
<i>Medicago turbinata</i>	24646	Kermanshah	Kermanshah	1200	34°08'00"	46°10'00"
<i>Medicago rigidula</i>	45078	Dallahou	Kermanshah	1213	34°23'34"	46°03'31"
<i>Medicago radiata</i>	44137	Izzeh	Khozestan	767	35°36'07"	39°03'78"
<i>Medicago lupulina</i>	20307	Unknown				
<i>Medicago truncatula</i>	20587	Unknown				

Mean comparisons were made between average over three groups of 2x, induced 4x and controls cv. 4x (Table 3). Result showed that the higher values of all traits were observed in control (cv. 4x) followed by induced tetraploid in terms of leaf width, shoot length, shoot weight, seedling weight, WUE and branches number. In the other word, there were no significant differences between control cv. 4x and induced tetraploids for the latter traits. However, for leaf length, root length, root weight and LAI, the means of induced tetraploids were significantly lower than that for controls (cv. 4x). The overall means of diploids were ranked as third place, however, for leaf length and root length. There was no significant differences between 2x and induced 4x. for other traits as: leaf width, shoot weight, root weight, seedling weight, LAI, WUE and branch number, the means of induced tetraploids were significantly higher than diploids (Table 3).

A separate mean comparisons were made among only naturally tetraploids varieties (Table 4). Result showed Baghdadi cv. had higher mean values for all of traits except root length and root weight. For these two traits, the higher values were observed in Ghareyounja cv. and for other traits, the Hamadani cv. ranked in the third place (Table 4).

Mean comparison were made between populations separately for diploids and induced tetraploids (Tables 5 and 6). For diploid populations, the higher values of WUE, LAI, root and shoot weight, seedling weight, root and shoot length, leaf size were obtained in *M. truncatula* 2x followed by *M. lupulina* 2x as the second place. Both species are annual (Table 6).

In comparisons between perennial diploids, there was no significant differences between Tehran Karaj1 and Karaj2 2x for WUE, seedling, shoot weight, root and shoot length and leaf width. For branch number the higher value was obtained in Tehran 2x (Table 5). Similarly, in comparisons between perennial induced tetraploids Tehran 4x, ranked in the first class than two other induced perennial populations in terms of the morphological traits (Table 5 and 6).

In comparisons between annual induced tetraploids, the highest values of all of traits except of LAI and branches number were observed in *M. truncatula* 4x. Similarly, *M. lupulina* 4x in terms of leaf width, root weight, LAI, branches number was ranked in the first class and for the shoot and root length, shoot weight and seedling weight it was ranked as the second class.

In comparisons of total average of 2x and total average of induced 4x groups, result showed in *M. radiata* by increasing ploidy level, leaf length and leaf width decreased by 12% and 15%, respectively. In contrast, for *M. rigidula* the leaf length and leaf width of the 4x, increased by 15 and 42% than 2x, respectively. In the other diploid species, the increase in ploidy level showed no significant effect on leaf size, but a decrease in root and shoot length was observed in all 2x species ranging from 7 to 30% for shoot length and between 15 and 51% for roots length. Thus, root weight and WUE value in 4x were higher than twice that of 2x. In contrast, low increase was observed for LAI (Table 5 and 6).

In overall, there were no significant differences between 2x and 4x for leaf size and LAI. The induced 4x had 25% and 38% lower values than 2x for Shoot and

Table 2. Analysis of variance studied traits in 23 medic species/populations.

SOV	DF	Leaf length	Leaf width	Shoot length	Root length	Seedling weight	Shoot weight	Root weight	Leaf area index	Water use efficiency	Branch number
Populations	20	30.17**	39.07**	1074.9**	966.5**	0.476**	0.712**	0.039**	3.26**	0.384**	583.7**
Error	79	4.75	3.73	8.63	13.30	0.006	0.012	0.002	0.11	0.010	13.08
CV%		15.49	17.49	14.01	21.77	11.87	12.51	21.07	14.87	16.52	15.99

** =Significant at the 1% probability level.

Table 3. Means Comparison of morpho-physiological traits based on total ploidy levels in all studied alfalfa species.

Ploidy levels	Leaf length Mm	Leaf width mm	Shoot length cm	Root length cm	seedling Weight g/plant	shoot weight g/plant	Root weight g/plant	Leaf area index	Water use efficiency	Branch number
Perennial 4x (Control)	15.88 a	11.96 a	26.30 a	25.51 a	1.08 a	0.77 a	0.31 a	2.96 a	0.26 a	26.04 a
Diploids 2x	13.02 b	9.90 b	18.97 b	14.46 b	0.63 b	0.47 b	0.16 c	1.89 c	0.11 b	17.64 b
Induced tetraploids	14.23 b	11.70 a	20.33 ab	14.79 b	1.02 a	0.76 a	0.27 b	2.31 b	0.23 a	25.68 a

Means with similar letters in each column has no significant difference at 5% probability level by Tukey test.

Table 4. Means Comparison of morpho-physiological traits in perennial alfalfa cultivars.

Commercial Varieties 4x	Leaf length mm	Leaf width mm	Shoot length cm	Root length cm	Seedling weight g/plant	Shoot weight g/plant	Root weight g/plant	Leaf area index	Water use efficiency	Branch number
Baghdadi	19.60 a	13.00 a	39.18 a	27.54 b	1.32 ab	1.07 a	0.25 b	3.43 a	0.42 a	37.80 a
Bami	14.10b	10.90 a	18.30 c	18.90 c	0.72 c	0.46 c	0.26 b	2.00 b	0.17 c	22.80 b
Ghareyounje	14.70b	11.50 a	29.18b	32.48 a	1.27 b	0.91 b	0.35 a	3.30 a	0.29 b	18.40 bc
Hamadani	15.60 ab	13.00 a	26.46b	27.68 b	1.45 a	1.07 a	0.38 a	3.25 a	0.31 b	35.00 a
Renger	15.40b	11.40 a	18.38c	20.96 c	0.63 c	0.35 c	0.28 b	2.82 ab	0.13 c	16.20 c

Means with similar letters in each column has no significant difference at 5% probability level by Tukey test

Table 5. Mean comparison of diploid *Medicago* species and their induced tetraploids for Leaf length, leaf width, shoot length, root length and seedling weight.

Populations	Leaf length mm		Leaf width mm		Shoot length cm		Root length cm		Seedling weight g/plant	
	2x	4x	2x	4x	2x	4x	2x	4x	2x	4x
<i>M. sativa</i> (Karaj1)	12.20 b	12.43 c	10.60 bc	10.57 b	14.64 b	11.30b	13.00 b	8.81b	0.59 c	0.75 cd
<i>M. sativa</i> (Karaj2)	17.00 a	16.86 a	15.00 a	14.86 a	15.54 b	10.83b	8.60 b	5.43b	0.53 c	0.88 bc
<i>M. sativa</i> (Tehran)	12.40 b	12.00 c	10.60 bc	10.83 b	16.78 b	12.47 b	9.50 b	5.92 b	0.60 bc	1.01 b
<i>M. lupulina</i>	12.60 b	12.86 c	13.60 ab	13.43 a	14.54 b	10.69b	11.92 b	5.77b	0.68 b	0.96 b
<i>M. radiata</i>	13.60 ab	12.00 c	9.20 cd	7.80 c	16.78 b	11.86 b	11.28 b	7.80 b	0.61 bc	0.90 bc
<i>M. rigidula</i>	11.60 bc	13.40 bc	6.60 de	9.40 bc	15.10 b	11.48 b	12.10 b	8.84 b	0.64 b	0.93 b
<i>M. truncatula</i>	15.00 ab	15.75 ab	12.80 b	13.25 a	54.52 a	50.44 a	51.58 a	40.63 a	1.32 a	1.54 a
<i>M. turbinata</i>	15.80 ab	16.25 a	8.80 d	9.50 bc	14.86 b	12.75 b	11.08 b	9.33 b	0.43 cd	0.61 d
Average	13.78	13.94	10.90	11.21	20.35A	16.48B	16.13A	11.57B	0.68B	0.95A

Means with similar letters in each column has no significant difference at 5% probability level by Tukey test.

Table 6. Mean comparison of diploid *medicago* species and their induced tetraploids for shoot weight, root weight, LAI, WUE and branch number.

Population name	Shoot weight g/plant		Root weight g/plant		Leaf area index		Water use efficiency		Branch number	
	2x	4x	2x	4x	2x	4x	2x	4x	2x	4x
<i>M. sativa</i> (Karaj1)	0.38 cd	0.48 c	0.20 b	0.27 ab	2.10 bc	2.18 d	0.08 de	0.17 c	15.00 c	17.14 c
<i>M. sativa</i> (Karaj2)	0.41 cd	0.63b	0.12 c	0.25 ab	2.21 bc	2.33 c	0.09 d	0.18 c	16.60 c	19.00 c
<i>M. sativa</i> (Tehran)	0.48 bc	0.74 b	0.12 c	0.27 ab	1.53 c	1.65 f	0.11 cd	0.21 bc	16.40 c	18.00 c
<i>M. lupulina</i>	0.55 b	0.71b	0.13 c	0.25 ab	3.22 a	3.41 a	0.17 b	0.23 b	35.60 a	44.00 a
<i>M. radiata</i>	0.47 bc	0.67b	0.14 bc	0.23 ab	1.35 c	1.45 g	0.10 d	0.18 c	13.20 c	18.60 c
<i>M. rigidula</i>	0.46 bc	0.64b	0.18 bc	0.29 ab	1.32 c	1.42 g	0.13 cb	0.18 c	14.40 c	17.60 c
<i>M. truncatula</i>	0.95 a	1.22 a	0.37 a	0.31 a	2.82 ab	2.96 b	0.24 a	0.37 a	28.80 b	37.08 b
<i>M. turbinata</i>	0.29 d	0.40 c	0.14 bc	0.21 b	1.64 c	1.78 e	0.07 de	0.11 d	12.80 c	16.50 c
Average	0.50B	0.69A	0.18B	0.26A	2.02B	2.15A	0.12B	0.20A	19.10B	23.49A

Means with similar letters in each column has no significant difference at 5% probability level by Tukey test.

root length, respectively. In contrast, the induced 4x had 40%, 38%, 44%, 65% and 23% higher values than 2x for seedling weight, shoot weight, root weight, WUE and branch number, respectively (Tables 5 and 6).

DISCUSSION

In this study, we found higher ploidy induction at the lower colchicine concentration (0.1%). Colchicine still remains the most efficient and clearly the most preferred and the most used anti mitotic agents because of its widely successful mitosis inhibition ability (Touchell et al, 2020). The others anti-mitotic agents such as diniroanilines and etc.. may also increase the ploidy levels. In addition, increasing of the ploidy levels can probably be an appropriate solution for crossing of various alfalfa species and increasing their genetic diversity and transferring desirable traits. It seems polyploidy induction in self-pollinated annual medic species are likely to improve the morphological and physiological characteristics in these species.

The result indicated that induced 4x had 25% and 38% lower values for the shoot and root length than 2x, respectively, averaged over all of species. The reduction in root length of *M. truncatula* was higher than that of other species. The results of the present study were in line with results of Pickens (2004) showing shorter shoots of colchicine treated plants compared to not-treated controls.

Overall, the induced 4x had 60%, 62%, 68%, 65%, 22%, 109% and 47% higher mean values than their 2x for seedling weight, shoot weight, root weight, LAI, WUE and branch number, respectively. In *M. truncatula*, the seedling weight of induced 4x was 16% higher than 2x (1.32 vs. 1.54 g/p). This result was in agreement with the result of the polyploidy induction in *Catharanthus roseus* using colchicine solution that significantly increased the seedling weight of tetraploid plants compared to diploid plants (Hosseini et al. 2013).

Shoot weight was higher in all induced species than the diploids. The higher values of shoot weight among all of population were obtained in *M. truncatula*. In this species, the value of induced 4x was 28% higher than its parental 2x. This result was in line with the Staji et al. (2017) that found polyploidy induction significantly increased shoot weight in *Salvia leriifolia*. Tavan (2014) in some endemic species of Thyme genus in Iran found that increase in ploidy level was associated with an increase in shoot weight. Similarly, Bagheri and Mansouri (2015) in cannabis (*Cannabis sativa* L.) found that the root and seedling weight of polyploid plants were

significantly increased than diploid plants. In our study, the higher values of LAI were obtained in *M. lupulina*. LAI was higher in all induced species than the diploids. In the overall average over all species, the LAI of induced 4x was 7% higher than 2x (2.02 vs. 2.15).

As the ploidy levels increased, the average value of WUE in all studied species increased up to 65%. Contributing factors to WUE are related to climate factors that are essential for water use (evapotranspiration) and water supply (atmospheric rain), management factors for cultivation and operations lead to evaporation reduction from the soil surface (Kafi and Damghani 2001). It seems that *M. truncatula* has probably been the best choice for polyploidy induction in annual species.

Ploidy changes in cell size are due to an increase in the number of copies of genes and thus an increase in the amount of protein produced (Tsukaya 2013). Nowadays, it has been found that control of morphological traits at the molecular level by ploidy level alteration via genetic (Tsukaya 2013), transcriptomic (Li et al. 2012) and epigenetic (Zeng et al. 2012, Aversano et al. 2012) modification occurs. For example, changes in the genomic dose of polyploids lead to changes in the expression of genes involved in cell cycle, photosynthesis, and cell metabolism (Shi et al. 2015). As a result of autotetraploidy, alterations in the expression of genes related to stress response, hormonal signaling actions, and response to phytohormones are applied Which may lead to a flexible and rapid response to external and internal stimuli (Del Pozo and Ramirez 2014). Slight but pervasive changes in gene expression are probably correlated with large phenotypic differences in autotetraploids (Allario et al. 2013). In some reports, autotetraploidy has caused epigenetic alteration by DNA methylation at the functional genes sites, encoding proteins and at the sequences involved in DNA replication, electron transport chain, and transcriptional regulation (Zeng et al. 2012). In some cases, methylation has been the result of cytosine methylation at the CG and CHG sites (Aversano et al. 2012). probably a combination of chromosome duplication, along with genetic, epigenetic, and transcriptomic effects, can produce morphophysiological differences due to polyploidy induction, as in our experiment (Yang et al. 2011).

CONCLUSION

In addition to the highest survival rate and most successful induction after colchicine treatment, the induced tetraploid of *M. truncatula*, among all of evaluated species, showed the highest levels of morpho-

logical indices. It seems that if the goal of breeding is to increase in economical properties such as seedling weight and WUE etc. It seems that *M. truncatula* probably is the best choice for polyploidy induction in annual species.

All perennial diploids did not show any un-uniformity by increasing ploidy level in this study, the increasing of the ploidy level of agronomic “perennial” tetraploids may be a more effective step to achieve superior new cultivars.

In comparing diploid alfalfas, the annual species, showed higher mean values for morphological traits than perennial diploid alfalfas. In comparisons among the three perennial “diploid” populations, Tehran had higher mean values for shoot length, shoot weight, seedling weight and WUE. Therefore, Tehran population is advisable for breeding improve new variety of perennial tetraploid via induce polyploidy. As a result, increasing the ploidy level 2x to 4x is a successful way to obtain superior morphological and physiological indices in the studied species and subspecies.

In overall, there were no significant differences between 2x and 4x for leaf size and LAI. The induced 4x had 25% and 38% lower values than 2x for Shoot and root length, respectively. In contrast, the induced 4x had 40%, 38%, 44%, 65% and 23% higher values than 2x for seedling weight, shoot weight, root weight, WUE and branch number, respectively.

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