Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: S. Tarkesh Esfahani, G. Karimzadeh, M. Reza Naghavi (2020) *In Vitro* Polyploidy Induction in Persian Poppy (*Papaver bracteatum* Lindl.). *Caryologia* 73(1): 133-144. doi: 10.13128/caryologia-169

Received: February 15, 2019

Accepted: February 23, 2020

Published: May 8, 2020

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.

In Vitro Polyploidy Induction in Persian Poppy (*Papaver bracteatum* Lindl.)

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Abstract. *Papaver bracteatum* Lindl. grows as a wild perennial medicinal plant in Northern Iran and is known mainly for its high amounts of the pharmaceutically valuable alkaloid of thebaine. In vitro production of tetraploid *P. bracteatum* through colchicine treatment of imbibed seeds is reported. Resulted tetraploid and mixoploid plants were effectively identified by chromosome counting and flow cytometry technique. The chromosome number in diploid and successfully induced tetraploids were confirmed to be $2n=2x=14$ and $2n=4x=28$, where their calculated 2C DNA values were 6.15±0.03 and 11.95±0.07 pg, respectively. The highest induction efficiency was obtained by colchicine concentration of 0.05% and the treatment duration of 24 h. The effects of colchicine toxicity on plant survival and growth were proportional mainly to its concentration rather than duration of exposure to colchicine. Tetraploid plants possessed significantly larger and less frequent leaf stomata as well as a larger cell size. These attributes may serve as criteria for preliminary screening of *P. bracteatum* populations for ploidy level.

Keywords. Persian poppy, seed, tetraploid, colchicine, flow cytometry, 2C DNA value.

INTRODUCTION

Persian poppy (*Papaver bracteatum* Lindl.; $2n=2x=14$) is a wild perennial medicinal plant belonging to the Papaveraceae family, section *Oxytona* that grows natively in the Alborz Mountains in the North of Iran in altitudes higher than 1800 m on the slopes facing the Caspian Sea (Sharghi and Lalezari 1967). It is mainly known for the high amounts of the valuable isoquinoline alkaloid thebaine as the main secondary metabolite in different organs particularly in roots and capsules (Nyman and Bruhn 1979; Madam 2011) while some 20 other alkaloids are reported to be present in this species only in trace amounts (Wu and Dobberstein 1977). Persian poppy seeds contain 45-48% oil rich in nutritionally valuable unsaturated fatty acids, so the other important usage of the plant is in the food industry (Madam 2011).

Seddigh *et al*. (1982) reported mean seed yield and seed-oil yield of *P. bracteatum* to be 90 and 40 kg ha^{-1}, respectively.

Successful polyploidy induction has been reported in various medicinal and ornamental plants with the aim of producing plants with improved agronomical, phytochemical or economically important characteristics. Application of anti-mitotic agents such as colchicine (Chen and Gao 2007; Sakhanokho *et al*. 2009; Majdi *et al*. 2010; Omidbaigi *et al*. 2010a, b; Kaensaksiri *et al*. 2011; Wu *et al*. 2011; Marzougui *et al*. 2011; Tavan *et al*. 2015; Javadian *et al*. 2017; Sadat Noori *et al*. 2017), oryzalin (Bouvier *et al*. 1994; Thao *et al*. 2003; Kermani *et al*. 2003; Lehrer *et al*. 2008; Sakhanokho *et al*. 2009), amiprophosmethyl (Rodrigues *et al*. 2011) and trifluralin (Eeckhaut *et al*. 2002) has been reported as the most common procedure for *in vitro* polyploidy induction in plants with colchicine being the most commonly used anti-mitotic agent.

Polyploid plants have been found to be valuable genetic resources due to possession of superior agronomic and phytochemical traits over their diploid progenitors. They have therefore attracted increasing attention in breeding programs, agriculture and medicinal plants industries. Some of the more frequently reported advantages of induced polyploid plants include larger vegetative and reproductive organs such as leaves and flowers (Chen and Gao 2006; Majdi *et al*. 2010; Tang *et al*. 2010; Gantait *et al*. 2011; Miller *et al*. 2012), darker green leaves with a higher chlorophyll content and photosynthesis capacity (Kulkarni and Borse 2010; Gantait *et al*. 2011), increased tolerance to environmental stresses (Natuli and Zobolo 2008), increased production of secondary metabolites (Dhawan and Lavania 1996; Kaensaksiri *et al*. 2011; Xu *et al*. 2013; Tavan *et al*. 2015; Javadian *et al*. 2017), increased expression of important genes and enzymes (Adams *et al*. 2003; Mishra *et al*. 2010; Miller *et al*. 2012; Xu *et al*. 2013) and delayed florescence time (Gu *et al*. 2005). On the other hand, decreased fertility, increased level of mitotic disruptions and pollen sterility (Liu *et al*. 2012) and facilitated biological invasion (Beest *et al*. 2012) are reported as the most important unfavorable consequences of induced polyploidy. *In vitro* polyploidy induction in several Papaveraceae plants has been previously studied mainly with the aim of obtaining an increased content of medicinal alkaloids. Mishra *et al*. (2010) successfully induced tetraploidy in *Papaver somniferum* L. and reported a significant enhancement in the morphine content and increased expression level of important genes involved in alkaloid biosynthesis pathway in tetraploid plants. Milo *et al*. (1987) reported the induction of tetraploidy in *P. bracteatum* through colchicine treatment of apical meristems and subsequent production of triploid plants by crossing induced tetraploids to diploid plants. They suggested the ploidy breeding and tetraploidy induction as the most promising approach for development of thebaine-rich poppy lines. In their studies, they selected the plants with different ploidy levels through chromosome counting and cytological techniques. So an effective, easy and clearly described method for *in vitro* polyploidy induction in *P. bracteatum* and effective discrimination of tetraploid and mixoploid results based on flow cytometric (FCM) technique and 2C DNA value is not reported yet. Consequently, we report for the first time the *in vitro* production of autotetraploid *P. bracteatum* by colchicine treatment of imbibed seeds followed by FCM identification of polyploidy. The differences in the DNA C-value, anatomical and morphological traits between diploid and induced tetraploid plants were also measured and their capability for being employed as reliable indicators of ploidy level in the plant populations was described.

MATERIAL AND METHODS

Plant material

Seeds of mature Persian endemic *Papaver bracteatum* plants were collected in Polour region (Latitude 35° 52' 16.99" N, Longitude 52° 04' 38.62" E, Altitude 2489 ± 50 m) from hillsides of Alborz Mountains in northern Iran. The seeds from each individual plant were collected separately and kept in small plastic bags. Since *P. bracteatum* is a self-incompatible totally cross-pollinating plant (Nyman and Bruhn 1979), the seeds which originated from each individual plant were considered as progenitors for future colchicine-treated plants. The seeds of each maternal plant were collected separately so that the eventual comparisons between different ploidy levels could be conducted between two half-sib plants rather than two plants with completely different genetic backgrounds. For this purpose, all treated seeds in polyploidy induction assay were selected from the seeds of an individual maternal plant.

Polyploidy induction

Seeds obtained from one capsule from an individual *P. bracteatum* plant were sterilized by immersing in ethanol 70% (v/v) for three 30 s times, followed by sodium hypochlorite 5% (v/v) for 7 min. The seeds were then rinsed with distilled water for 5 min and transferred on

two layers of moistened filter papers in glass petri dishes and irrigated regularly with distilled water to allow water imbibition. Germination process progressed up to the radicle emergence. After 8-10 days, the imbibed seeds (swollen seeds without a well-defined radicle apex) were transferred to tubular penicillin vials containing 1000 µl of colchicine solutions (Sigma-Aldrich Corporation, MO, USA) with different concentrations comprising 0.00, 0.025, 0.050, 0.075, 0.10 and 0.20% (w/v). The vials were placed on a shaker with a rotation speed of 95 rpm and shaken for predetermined durations, including 4, 8, 12, 24, 36, 48, 72, and 168 h. At the end of treatment duration, the treated seeds were washed thoroughly with distilled water for 3 × 3-min and transferred to a 250-ml glass baby food jars containing 40 ml of ½ Murashige and Skoog (1962) medium with 1 g l^{-1} charcoal. The latter was used in order to prevent the seed phenolic compounds from interfering with the emergence and growth of new seedlings. Seven treated seeds were cultured in each jar (representing one replication for a given treatment in data analysis). The seedlings were re-cultured once a month on a new medium with the above-mentioned composition and conditions. After three months, the plants with 6-7 true developed leaves were transferred to 500 g pots containing sand, commercial potting soil and vermiculite as the main components mixed with a ratio of 2:2:1 orderly.

Flow cytometry analysis

One cm^2 of young, healthy and fully green developed leaf material from each examined Persian poppy plant together with about 1/3-1/2 in area of leaf material from *Pisum sativum* cv. 'Citrad' (2C DNA = 9.09 pg; Doležel *et al*. 1998) as an internal reference standard, were chopped into small pieces by a sharp razor blade in a 100 mm glass petri dish, containing one ml of Woody Plant Buffer (WPB; Loureiro *et al*. 2007). The resultant nuclear suspension was filtered through a Partec (Partec, Münster, Germany) 30 µm-nylon mesh, followed by treating with 50 µg ml^{-1} RNase (Sigma-Aldrich Corporation, MO, USA) and 50 µg ml^{-1} Propidium Iodide (PI, Fluka) as DNA staining agent, and then incubated for two min at room temperature. To determine nuclear 2C DNA amount, the nuclei suspension was analysed by a BD FACSCanto II flow cytometer (BD Biosciences, Bedford, MA, USA), using BD FACSDiva™ Software. Output data were then transferred to a Flowing Software version 2.5.0 to be editable in Partec FloMax ver. 2.4e (Partec, Münster, Germany). The measurements of relative fluorescence intensity of stained nuclei were performed on a linear scale, analysing at least 5,000 nuclei for each sample. The absolute DNA amount of a sample was calculated based on the values of the G1 peak means (Doležel *et al*. 2003, 2007; Doležel and Bartoš 2005; Mahdavi and Karimzadeh 2010; Karimzadeh *et al*. 2010, 2011; Abedi *et al*. 2015) as follows:

Sample 2C DNA (pg) = (Sample G_1 peak mean/Standard G_1 peak mean) × Standard 2C DNA (pg)

The analysed samples were classified based on the FCM results into diploid (2*x*), tetraploid (4*x*) and mixoploid (2*x* and 4*x*) samples.

Chromosome counting

The ploidy status of the induction results were additionally confirmed by microscopic chromosome, counting in 10 randomly sampled plants from each class of ploidy level. Root tips from confirmed diploid and tetraploid plants were pretreated with α-bromonaphthalene for 1 h at 24 °C, followed by rinsing with distilled water for 3 × 3 min. The pretreated roots were then fixed in Carnoy solution (3:1 ethanol:glacial acetic acid) and stored at 4 °C followed by washing in distilled water, hydrolyzing with 1 N HCl for 8 min at 65 °C and staining with 1% (w/v) aceto-orcein for 1 h. Treated 1-2 mm long root tips were excised and squashed on slide glass, with a drop of 45% (v/v) acetic acid, and protected with a cover slip. Chromosome counts were analysed by observation under a BX51 Olympus light microscope (Olympus Optical Co., Tokyo, Japan), equipped with an Olympus DP12 digital camera (Olympus Optical Co., Tokyo, Japan) using WH10X (FN22) eyepiece and 100x objective lens.

Anatomical and morphological analysis

To assess any possible relations between anatomical traits and ploidy level in *P. bracteatum*, leaf cell size as well as the dimensions and the frequency of leaf stomata were measured on the lower epidermis of 20 fully developed leaves each taken from an individual plant in each confirmed class of ploidy. Data on stomata dimensions were measured on 60 stomata from leaves of the plants in each class of ploidy level. The stomata were visualized by the impression method (Majdi *et al*. 2010; Tavan *et al*. 2015). The density of the stomata was counted at 200x and the area of stomata were measured at 1000x magnification, using high resolution microscopic digital photographs taken with an Olympus DP12 digital camera (Olympus Optical Co., Tokyo, Japan) interfaced to

an Olympus BX50 (Olympus Optical Co., Tokyo, Japan) microscope. The stomata and the cells in three random microscopic fields per each leaf were counted and measured. The measurements of stomata dimensions, including area and large diameter (length) as well as the cell area were determined, using the captured images and the UTHSCSA ImageTool program (University of Texas Health Science Center at San Antonio, Texas, USA).

Data analysis

The data regarding the effect of time and concentration of the colchicine treatment on survival rate and polyploidy induction were analysed through a Completely Randomized Design (CRD) with three replications. Mean comparison was carried out by using Least Significant Difference (LSD) test at $P<0.05$. Seeds germination percentage was calculated as GP = (number of germinated seeds / total planted seeds) * 100. Tetraploidy induction efficiency was assessed using the method reported by Bouvier *et al*. (1994) and Majdi *et al*. (2010) as follows:

Induction efficiency = % Seedling survival × % Tetraploidy induction

For induction efficiency calculation, a seedling was considered as survived if it persisted for three months after being treated with colchicine and had enough green leaf area to be analysed by FCM. The resultant data were first tested for normality with the Kolmogorov-Smirnov test. The logarithmic transformation was then used for both stomata area and stomata frequency data. Mean comparisons between two different ploidy levels for anatomical traits were conducted, using Student's *t* test. All statistical analysis were conducted, using SPSS 18 (Chicago: SPSS Inc., 2009).

RESULTS

Survival and the growth of colchicine-treated seeds

The toxic effects of colchicine on the treated Persian poppy (*Papaver bracteatum* Lindl.) plants were different in terms of their survival and consecutive growth. Some treated seeds could no longer survive colchicine treatment, as they remained as dark necrotic non-emerging seeds without any root appearance. Whereas some others could survive in the colchicine and remained alive initially at the time of transferring to the medium, but their seedlings could not keep normal growing and turned to dark brown-colored necrotic tissues during later three weeks after being transferred. The degree of mortality caused by different colchicine either concentrations or durations were variable. The toxic effects of colchicine treatment on the survival and on the growth of the seeds were therefore assessed 30 days after treatment induction. The general colchicine-induced mortality was divided and expressed as two different criteria including seed mortality and seedling mortality (Table 1). Seed mortality was calculated based on non-emerging seeds which could not survive in the colchicine treatment, while seedling mortality was reflected by the emerged seedlings with inhibited successive growth. The results showed that increased colchicine concentration significantly increased the level of lethal effects (Table 1, Fig. 1), so that only 4.76±2.38% seed survival but no subsequent growth was observed in the seeds treated with the 0.2% (w/v) colchicine solution. While no seedling growth was identified in the 0.2% colchicine treatment, the survival and the growth in the seeds treated with other concen-

Table 1. Results of the analysis of variance (ANOVA) for the effect of colchicine concentration and treatment duration on the mortality of *Papaver bracteatum* seeds.

Source of Variation	Mean Squares	
	Seed mortality	Seedling mortality
Colchicine concentration (C)	13097.26**	13173.03**
Treatment duration (D)	126.22n.s.	162.97*
C*D interaction	116.51*	70.15n.s.

**, * and n.s. indicate significant at 1%, 5% level and and not significant, respectively.

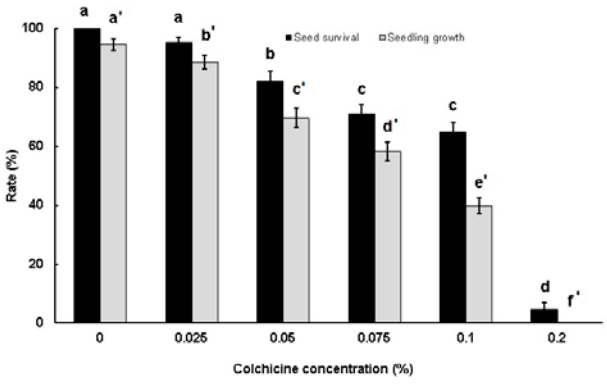

Figure 1. Effect of colchicine concentration on seed survival and seedling growth of treated *Papaver bracteatum* explants. All values are in percentage. Mean values specified by the same latter are not significantly different at $P<0.05$ by LSD test. Letters with a prime symbol designate mean differences in seedling growth.

Table 2. Effect of colchicine concentration and treatment duration on the percentage (mean ± standard error) of seed mortality, seedling mortality, diploid (2*x*), mixoploid (2*x*+4*x*), and tetraploid (4*x*) explants in *Papaver bracteatum*.

Colchicine concentration (%)	Exposure duration (h)	Seed mortality (%)	Seedling mortality (%)	2*x* (%)	2*x* + 4*x* (%)	4*x* (%)
0.000	4	0.00 ± 0.00	4.76 ± 4.76	95.24 ± 4.76	0.00 ± 0.00	0.00 ± 0.00
	8	0.00 ± 0.00	0.00 ± 0.00	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	0.00 ± 0.00	4.76 ± 4.76	95.24 ± 4.76	0.00 ± 0.00	0.00 ± 0.00
	24	0.00 ± 0.00	4.76 ± 4.76	95.24 ± 4.76	0.00 ± 0.00	0.00 ± 0.00
	36	0.00 ± 0.00	9.52 ± 9.52	90.48 ± 9.52	0.00 ± 0.00	0.00 ± 0.00
	48	0.00 ± 0.00	0.00 ± 0.00	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	72	0.00 ± 0.00	4.76 ± 4.76	95.24 ± 4.76	0.00 ± 0.00	0.00 ± 0.00
	168	0.00 ± 0.00	14.29 ± 8.25	85.71 ± 8.25	0.00 ± 0.00	0.00 ± 0.00
0.025	4	4.76 ± 4.76	4.76 ± 4.76	90.48 ± 4.76	0.00 ± 0.00	0.00 ± 0.00
	8	14.29 ± 8.25	0.00 ± 0.00	85.71 ± 8.25	0.00 ± 0.00	0.00 ± 0.00
	12	0.00 ± 0.00	9.52 ± 9.52	90.48 ± 9.52	0.00 ± 0.00	0.00 ± 0.00
	24	14.29 ± 8.25	4.76 ± 4.76	80.95 ± 4.76	0.00 ± 0.00	0.00 ± 0.00
	36	0.00 ± 0.00	4.76 ± 4.76	95.24 ± 4.76	0.00 ± 0.00	0.00 ± 0.00
	48	0.00 ± 0.00	14.29 ± 8.25	85.71 ± 8.25	0.00 ± 0.00	0.00 ± 0.00
	72	0.00 ± 0.00	4.76 ± 4.76	95.24 ± 4.76	0.00 ± 0.00	0.00 ± 0.00
	168	4.76 ± 4.76	9.52 ± 4.76	85.71 ± 8.25	0.00 ± 0.00	0.00 ± 0.00
0.050	4	9.52 ± 4.76	14.29 ± 8.25	33.33 ± 17.17	19.05 ± 4.76	23.81 ± 12.60
	8	28.57 ± 8.25	4.76 ± 4.76	19.05 ± 4.76	23.81 ± 12.60	23.81 ± 12.60
	12	28.57 ± 14.29	9.52 ± 4.76	0.00 ± 0.00	23.81 ± 4.76	38.10 ± 9.52
	24	4.76 ± 4.76	4.76 ± 4.76	23.81 ± 17.17	33.33 ± 4.76	33.33 ± 17.17
	36	23.81 ± 9.52	9.52 ± 9.52	19.05 ± 12.60	19.05 ± 4.76	28.57 ± 16.50
	48	19.05 ± 9.52	19.05 ± 12.60	38.10 ± 12.60	14.29 ± 0.00	9.52 ± 9.52
	72	0.00 ± 0.00	19.05 ± 4.76	57.14 ± 0.00	23.81 ± 4.76	0.00 ± 0.00
	168	28.57 ± 8.25	19.05 ± 4.76	28.57 ± 16.50	14.29 ± 0.00	9.52 ± 9.52
0.075	4	28.57 ± 14.29	14.29 ± 0.00	9.52 ± 9.52	33.33 ± 4.76	14.29 ± 8.25
	8	9.52 ± 4.76	19.05 ± 4.76	14.29 ± 8.25	23.81 ± 4.76	33.33 ± 4.76
	12	42.86 ± 8.25	4.76 ± 4.76	14.29 ± 0.00	28.57 ± 8.25	9.52 ± 9.52
	24	9.52 ± 4.76	9.52 ± 4.76	23.81 ± 4.76	23.81 ± 4.76	33.33 ± 9.52
	36	38.10 ± 4.76	9.52 ± 4.76	4.76 ± 4.76	23.81 ± 4.76	23.81 ± 12.60
	48	33.33 ± 4.76	14.29 ± 8.25	9.52 ± 4.76	19.05 ± 4.76	23.81 ± 4.76
	72	38.10 ± 4.76	14.29 ± 8.25	19.05 ± 9.52	19.05 ± 4.76	9.52 ± 4.76
	168	33.33 ± 9.52	14.29 ± 0.00	19.05 ± 12.60	9.52 ± 4.76	23.81 ± 4.76
0.100	4	33.33 ± 9.52	19.05 ± 4.76	0.00 ± 0.00	23.81 ± 9.52	23.81 ± 4.76
	8	33.33 ± 4.76	19.05 ± 12.60	9.52 ± 4.76	14.29 ± 0.00	23.81 ± 9.52
	12	47.62 ± 4.73	14.29 ± 4.25	0.00 ± 0.00	14.29 ± 0.00	23.81 ± 4.76
	24	23.81 ± 9.52	28.57 ± 8.25	4.76 ± 4.76	19.05 ± 4.76	23.81 ± 4.76
	36	28.57 ± 8.25	33.33 ± 12.60	9.52 ± 9.52	14.29 ± 0.00	14.29 ± 8.25
	48	28.57 ± 14.29	28.57 ± 8.25	14.29 ± 8.25	14.29 ±0.00	14.29 ± 8.25
	72	38.10 ± 9.52	28.57 ± 8.25	4.76 ± 4.76	9.52 ± 4.76	19.05 ± 4.76
	168	47.62 ± 9.52	28.57 ± 0.00	9.52 ± 4.76	4.76 ± 4.76	9.52 ± 4.76
0.200	4	76.19 ± 12.60	23.81 ± 12.60	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	8	90.48 ± 9.52	9.52 ± 9.52	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	95.24 ± 4.76	4.76 ± 4.76	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	24	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	36	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	48	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	72	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	168	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

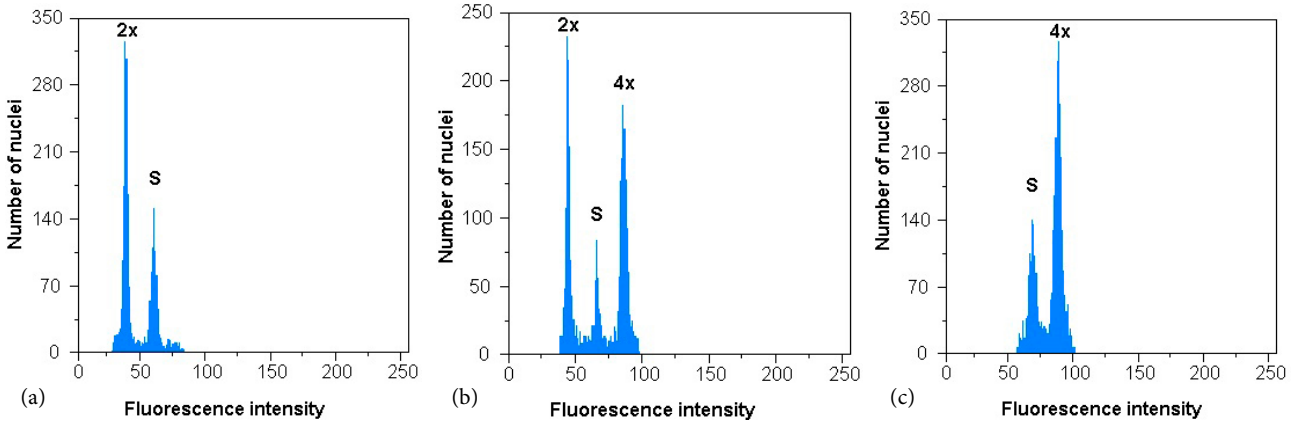

Figure 2. Flow cytometric histogram of the relative fluorescence intensity of nuclei isolated from *Papaver bracteatum* plants. Histograms show the nuclei isolated form diploid (a), mixoploid (b), and induced tetraploid (c) plants. The S in each histogram indicates the peak resulted by the cells of the *Pisum sativum* cv. 'Citrad' (2C DNA=9.09 pg) used as the internal standard.

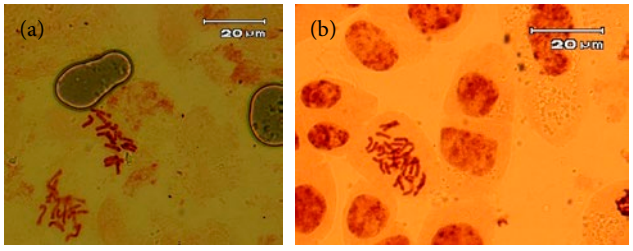

Figure 3. Chromosome numbers of surveyed *Papaver bracteatum* plants. Number of chromosomes in diploid ($2n=2x=14$) (a) and artificially induced tetraploid ($2n=4x=28$) (b) plants are compared.

Table 3. Results of the analysis of variance (ANOVA) for the effect of colchicine concentration and treatment duration on the polyploidy induction rate in *Papaver bracteatum*.

Source of Variation	Mean Squares
Colchicine concentration (C)	16.98**
Treatment duration (D)	2.57*
C*D interaction	1.11n.s.

**, * and n.s. indicate significant at 1%, 5% level and and not significant, respectively.

trations varied widely, where the mean germination percentage and the mean percentage of developed seedlings in the 0.1% treatment were 64.88±3.22 and 39.88±2.72, respectively (Table 2). These values were 95.24 ± 1.86 and 88.69 ± 2.27 in the 0.025% treatment, respectively (Fig. 1).

Flow cytometry analysis of ploidy level

The ploidy level of colchicine-treated plants was determined by FCM analysis. All treated plants with apparently normal growth and development were classified into three main classes including diploids ($2x$), mixoploids ($2x+4x$) and tetraploids ($4x$). As shown in related histograms (Fig. 2), diploid and tetraploid plants revealed a peak at the position of channels 50 and 95 of the relative fluorescent intensity respectively, while mixoploids revealed two peaks with variable heights at both channels 50 and 95.

The 2C DNA contents of the diploid and induced tetraploid plants were estimated as 6.15±0.03 (Fig. 2a) and 11.95±0.07 pg (Fig. 2c), respectively. The ploidy status of the resultant events was additionally confirmed by microscopic chromosome counting in plants with different ploidy levels. It was indicated that all diploid plants had a chromosome number of $2n=2x=14$ (Fig. 3a), whereas all tetraploids had $2n=4x=28$ (Fig. 3b). In the present study, significant differences between induction treatments were seen based on the induction efficiency data. The results showed that both higher concentrations of colchicine and longer durations of exposure to colchicine resulted in a significantly larger percentage of tetraploid plants (Table 3). The highest induction efficiency (31.29) was yielded by 0.05% (w/v) colchicine concentration at exposure duration of 24 h. The next two most efficient treatments were 0.75% (w/v)-24 h and 0.05% (w/v)-12 h with induction efficiency values of 27.89 and 25.85 respectively (Figs. 4, 5).

Anatomical and morphological characteristics

Stomata data measured on the leaves of plants in each class of ploidy showed that the stomata size in

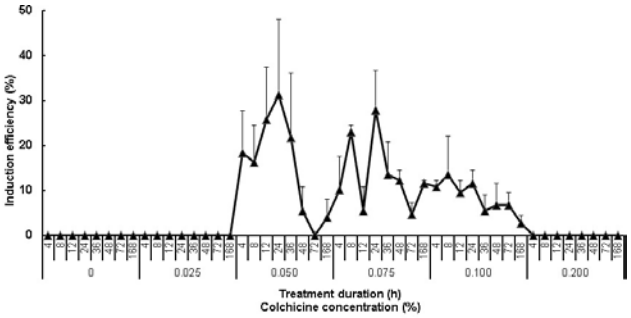

Figure 4. Effect of colchicine concentration and treatment duration on tetraploidy induction efficiency in *Papaver bracteatum*. Bars show standard errors.

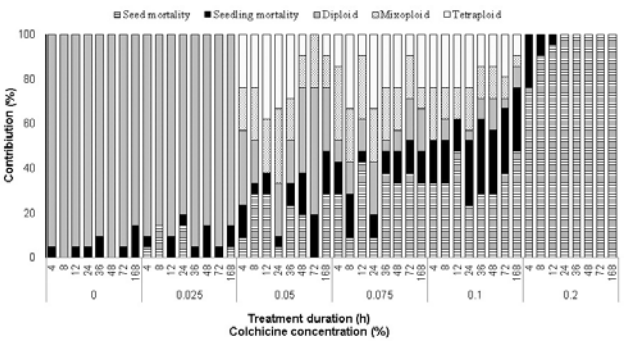

Figure 5. Effect of colchicine concentration and treatment duration on the contribution (%) of seed mortality, seedling mortality, diploid, mixoploid, and tetraploid explants produced during polyploidy induction in *Papaver bracteatum*.

tetraploid plants was larger than that in diploids (P<0.01; Figs. 6a, 6b). The average stomata area for the diploid and tetraploid (Figs. 7a, b, respectively) plants was 531.44±24.02 and 868.98±55.66 μm^2 respectively indicating a 63.51% increase in stomata area of tetraploid plants. It was also included that the stomata length in tetraploid plants (40.46±1.33 μm) was 30.06% larger than that in diploids (31.10±0.89 μm).

In addition, a significant difference was identified between diploid and polyploid plants in the stomatal density (P<0.01), where the average number of stomata per square millimetre in the leaves of diploid and tetraploid (Figs. 7c, d, respectively) plants was 236.18±10.54 and 157.14±3.78, respectively (Fig. 6c). In other words, tetraploidy induction caused a 50.3% decrease in sto-

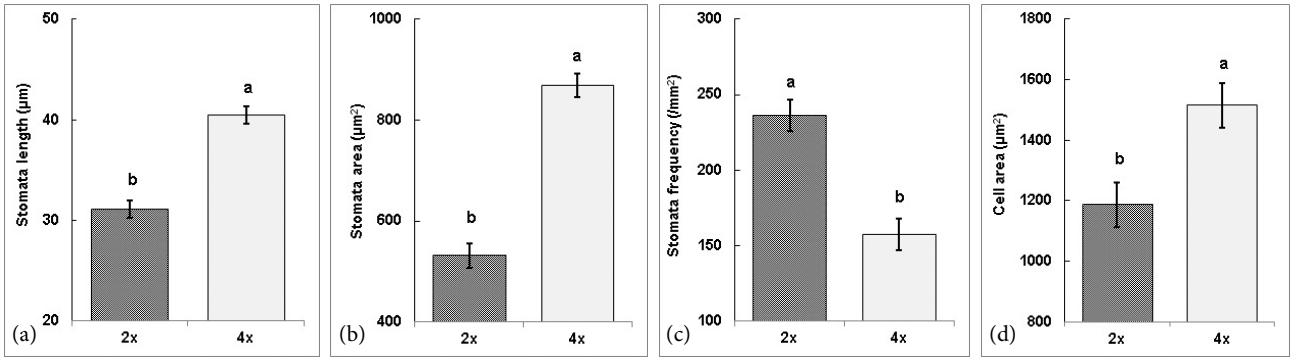

Figure 6. Comparison of anatomical traits between diploid and tetraploid plants of *Papaver bracteatum* at the cellular level. The stomata length (a), stomata area (b), stomata frequency (c), and stomata cell area (d) are significantly changed in induced tetraploid plants. Mean values specified by different letters are significantly different at P<0.05 by Students's *t* test. Bars show standard errors.

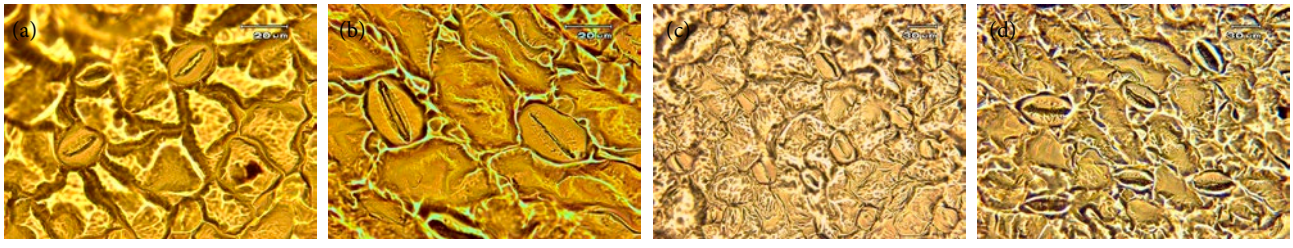

Figure 7. Impressions illustrating the size and density of the stomata in the leaf lower epidermis of *Papaver bracteatum* plants. The smaller stomata size in diploid (a) than in induced tetraploid (b) plants and the lower stomata density in diploid (c) than in tetraploid (d) plants are illustrated.

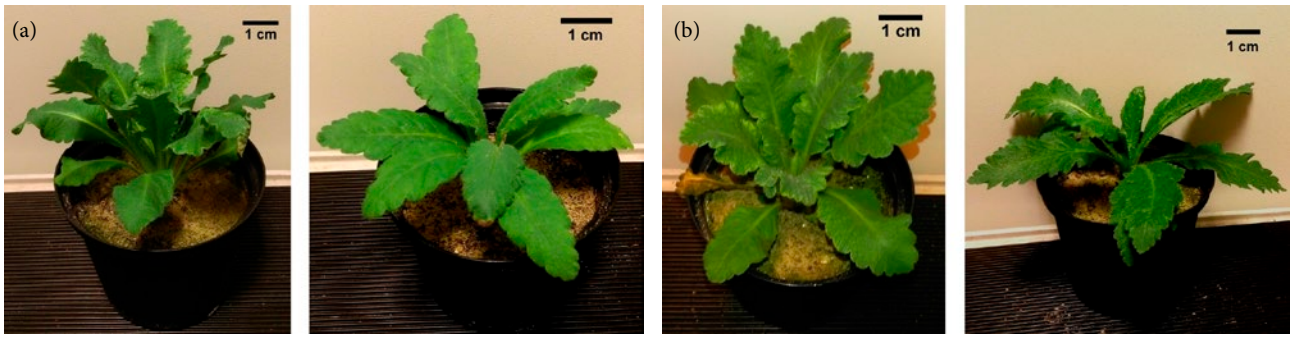

Figure 8. Comparison of visible morphological traits at the whole plant level. The illustrated diploid (a) and tetraploid (b) *Papaver bracteatum* plants are all at the same age (195 d) and acclimated under similar environmental condition.

mata density in studied *P. bracteatum* plants. Data regarding the cell size showed that the average leaf cell area in diploid and tetraploid *P. bracteatum* plants was 1187.20±73.93 and 1515.12±118.31, respectively indicating a 27.62% increase in epidermal cell area of tetraploid plants ($P < 0.05$; Fig. 6d). Unlike in the stomatal morphology, tetraploid plants exhibited no remarkable differences in visible morphological traits such as plant height, shoot or leaf thickness and growth rate compared to their diploid counterparts (Fig. 8).

DISCUSSION

The results regarding the colchicine effects on survival of seeds and seedlings are in agreement with those reported in various plant species e.g. *Berberis thunbergii* (Lehrer *et al.* 2008), *Tanacetum parthenium* (Majdi *et al.* 2010) and *Thymus persicus* (Tavan *et al.* 2015), indicating that the toxic effects of colchicine as an anti-mitotic chemical is largely influenced by the colchicine concentration (Table 1). The toxic effects of colchicine on the treated Persian poppy were expressed as two different criteria including seed mortality and seedling mortality. The results showed that increased colchicine concentration significantly increased the level of both lethal effects and decreases the ability of treated plants to survive and grow (Table 2).

The C-value index defined as the DNA content of an unreplicated haploid chromosome complement (i.e. a gamete) is highly useful in systematics, genome size estimation and many other biological fields related to eukaryotic organisms (Doležel and Bartoš 2005). During normal mitosis two daughter cells each with 2C DNA content is formed (Doležel *et al.* 2003). Therefore, the 2C DNA content of a tetraploid cell which is resulted by mitotic arrest is expected to be about two times that of the progenitor diploid cells. In the present study, the calculated 2C DNA contents of the diploid and tetraploid plants were estimated as 6.15±0.03 (Fig. 3a) and 11.95±0.07 pg (Fig. 3b), respectively, indicating the successful induction of tetraploidy. Furthermore, these results suggest the effectiveness of FCM-based analysis as a rapid and reliable strategy for discriminating *P. bracteatum* from other identified or unidentified *Papaver* species with similar morphological traits and different 2C DNA values.

Tetraploid induction efficiency has frequently been used in previous studies (Bouvier *et al.* 1994; Lehrer *et al.* 2008; Majdi *et al.* 2010) as a measure for identifying the most effective treatments capable of inducing complete and stable polyploidy. It is known as reliable index because it takes into account not only the rate of conversion of diploidy tetraploidy, but the survival rate of successful tetraploid events (Lehrer *et al.* 2008). Our results showed that both higher concentrations of colchicine and longer durations of exposure to colchicine resulted in a significantly larger percentage of tetraploid seedlings, but their interaction effect was non-significant (Table 3). There are several reports about the evaluation of the effects by the concentration of the anti-miotic agent and treatment duration as the two main determining factors in polyploidy induction efficiency in different plant types. For instance, Stanys *et al.* (2006) working on *Chaenomeles japonica* reported that with both colchicine and oryzalin as the anti-mitotic agents, the efficiency of ploidy induction was mainly dependent on the concentration of anti-mitotic agent rather than its exposure duration. On the other hand, several authors suggested that the polyploidy induction efficiency is associated with both optimum concentration and the duration of anti-miotic (Gu *et al.* 2005; Tang *et al.* 2010; Tavan *et al.* 2015).

The results of FCM analysis showed that mixoploid plants could be expected to form a large contribution of polyploids sometimes as high as 33% of induc-

tion results obtained by *in vitro* polyploidy induction in *P. bracteatum* (Table 2; Fig. 5). In polyploidy induction works, high percentage of mixoploid results are generally known as a drawback of the procedure because their unstable polyploidy state often reverts partially or totally to the diploid condition after successive cell division cycles. It occurs mainly because the remaining diploid cells proliferate at higher rates than the tetraploid ones (Mergen and Lester 1971). During artificial polyploidy induction, colchicine influences actively dividing cells in the treated tissues and polyploidization therefore occurs unequally among explant cells, leading to the occurrence of mixoploids and chimeras (Wan *et al*. 1989). Accordingly, a low growth rate and intrinsically stunted development in treated plants are expected to aggregate these effects particularly in short exposure durations leading to a higher proportion of mixoploid events among polyploidy induction results. Chakraborti *et al*. (1998) suggested that in *in vitro* induction methods, the occurrence of mixoploids may have been minimized by growing the treated plants under more favorable conditions. They stated that the uniformity of environmental factors like temperature and photoperiod may favor the synchronous division of meristematic cells and help minimize the mixoploid events leading to a high tetraploidy rate (Chakraborti *et al*. 1998).

The ratio of tetraploid to diploid cells based on the analysed FCM data can be calculated as a measure of the contribution of tetraploid cells within a mixoploid event. The values higher than one indicate a higher percentage of tetraploid cells than the diploids. In the present study, the obtained values indicated a high degree of variability in polyploidy induction capability of colchicine in *P. bracteatum* (detailed data not shown because of the large number of hits and lack of a significant interaction). In addition, it was interestingly observed that varying values of the ratio of tetraploid to diploid cells might be obtained by the same concentration-duration combination. For example, both values of 0.19 and 1.75 which indicate a low and a high contribution of tetraploid cells respectively, were seen in the mixoploid events resulted by the 0.05%- 24h treatment combination. So despite of favorable and controlled environmental factors within *in vitro* induction and growth environments, large differences between induction capabilities of certain concentration-duration combinations were revealed by the variable degrees of mixoploidy obtained by the same treatment combination. These results indicate that the effectiveness of anti-mitotic agent in polyploidy induction in *P. bracteatum* can be determined mainly by the explant and cell-related factors rather than those related to the induction environment. However, the rather high percentage of mixoploid events yielded by *in vitro* colchicine treating of *P. bracteatum* explants as well as the wide range of the ratio of tetraploid to diploid cells within mixoploid events can be explained by the incomplete effects of colchicine on meristematic cells in the treated explants. These incomplete effects could be aggravated by the intrinsically stunted growth of *P. bracteatum*. Indeed, short exposure times and lower concentrations of the anti-mitotic agent during polyploidy induction may in turn decrease the chance for complementation of polyploidization process leading to a higher degree of mixoploidy (Wan *et al*. 1989).

A recent study by Tavan *et al*. (2015) on *Thymus persicus* has showed that the mixoploid events which were produced during polyploidy induction in *Thymus persicus* had a considerable capability for producing pharmaceutically important compounds. They reported significantly increased production of medicinal triterpenoids in both tetraploid and mixoploid results as compared to their diploid progenitors, where mixoploid plants interestingly yielded significantly higher contents of Betulinic acid, Oleanolic acid and Ursolic acid even than successfully induced tetraploids (Tavan *et al*. 2015). Additionally, successful generation of tetraploid plants from mixoploid progenitors using tetraploid cells of leaf callus through callus-based techniques and tissue culture strategies has been previously reported in various plant species such as *Humulus lupulus* L. (Roy *et al*. 2001), *Astragalus membranaceus* (Chen and Gao 2007) and *Echinacea purpurea* L. (Dahanayake *et al*. 2010). Likewise Shao *et al*. (2003) reported that further subculture of mixoploid events resulted by *in vitro* colchicine treatment of shoots in *Punica granatum* L. resulted in their separation to tetraploid and diploid progenies. This strategy has been recommended to be employed when an anti-mitotic agent generates a high degree of mixoploid events during tetraploidy induction. In general, the mixoploids can therefore be considered as valuable sources of genetic material in ploidy breeding programs. They particularly can be employed for certain plant species which are expected to produce high numbers of mixoploid events during polyploidy induction works. Application of tissue culture techniques in *P. bracteatum* has been well established, where successful *in vitro* regeneration pf this species using callus derived from seedlings (Ilahi and Ghauri 1994), roots, seeds and cotyledons (Rostampour *et al*. 2010) as well as through cell suspension culture (Farjaminezhad *et al*. 2013) and hairy roots (Sharafi *et al*. 2013) have previously been reported. Therefore, it allows combination polyploidy induction strategies and tissue culture techniques to achieve higher goals in *P. bracteatum* breeding programs.

These obtained results indicating increased stomata size (Fig. 6a) and decreased stomata frequency (Fig. 6c) in tetraploid plants as compared to their diploid progenitors are in agreement with those of several previous reports in different plant types. Furthermore, differences in stomata size and density are frequently reported to successfully discriminate plants with different ploidy levels, where polyploid plants are often known to have, on average, a lower stomata number per leaf area unit and increased size of the stomata and guard cells (de Carvalho 2005; Tang *et al*. 2010; Gantait *et al*. 2011; Aina *et al*. 2012) as well as an increased number of chloroplasts in stomatal guard cells (Ewald *et al*. 2009). Gantait *et al*. (2011) suggested that the lower density of stomata in polyploid plants was due to the larger stomata and epidermal cell size, as well as reduced stomata differentiation.

The cell size is also reported to be related to polyploidy level and to be significantly different between induced tetraploid plants and their diploid progenitors (Melaragno *et al*. 1993). It is suggested as a potential anatomical indicator of ploidy level being capable of discriminating plants in a mixed population of tetraploid and diploids (Zeng *et al*. 2006). The results regarding the cell size in the plants with different ploidy levels showed that the tetraploid *P. bracteatum* plants had significantly larger leaf epidermal cells than diploid plants (Figs. 6b, 6c). These results confirm previous reports where larger cell dimensions were reported for tetraploid plants as compared to their diploid progenitors in various plant species such as *Fortunella crassifolia*, *Citrus sinensis* (Zeng *et al*. 2006), *Tanacetum parthenium* (Majdi *et al*. 2010) and *Thymus persicus* (Tavan *et al*. 2015).

The results obtained in this study indicated that certain anatomical traits such as leaf epidermal cell and stomata size and frequency may serve as reliable criteria for screening of *P. bracteatum* plants for ploidy level. However, other routinely suggested anatomical, morphological or physiological charachteristics such as chlorophyll florescence, flower size and morphology, pollen grain size, chloroplast density, enzymatic activity, etc. have to be evaluated precisely before being employed as potential indicators of ploidy level in quick evaluation and successful screening of large numbers of *P. bracteatum* plants. Because in the present study, unlike in the stomatal morphology, the colchicine-treated plants did not exhibit any remarkable differences in visible morphological traits such as plant height, shoot or leaf thickness and growth rate (Fig. 8). These observations were in agreement with those reported by Milo *et al*. (1987) who stated that there were no significant differences between *P. bracteatum* plants with different ploidy levels in the morphological traits such as plant height, flower size or in the height of the flowering stem. However, the artificially induced tetraploid plants of *P. bracteatum* were reported to flower later than diploid plants. Their capsules also matured significantly later than their diploid plants (Milo *et al*. 1987).

The lack of visible morphological distinctions between diploid and tetraploid *P. bracteatum* plants might be attributed to the high morphological variation observed in this species particularly in its natural habitat. Wild *P. bracteatum* plants naturally exhibit high variation in plant size, growth rate and other visible characteristics. So, the new polyploid variants are likely to still fall within the wide variation range that already exists in wild *P. bracteatum* populations. Consequently, like their diploid progenitors, tetraploid plants are expected to reveal a wide range of morphological variation. Hence, ploidy statuses of the induction results need to be confirmed by quick, easy and reliable criteria such as flow cytometry techniques rather than morphological measures.

In conclusion, polyploidy was successfully induced in diploid Persian poppy (*Papaver bracteatum* Lindl.) through colchicine treatment of newly germinated seeds. Tetraploid and mixoploid progenies were quickly and effectively recognized by FCM technique and the 2C DNA content. Both colchicine concentration and exposure duration were known as determining factors in success of *in vitro* tetraploidy induction. Morphological traits like stomata size and frequency and cell size were significantly associated with ploidy level in Persian poppy and were known as reliable criteria for preliminary screening of mixed populations based on ploidy level. Further research works are in progress to study the ploidy level dependent secondary metabolites and potentially major candidate gene expressions particularly of those of great importance in biosynthesis and production of pharmaceutically valuable alkaloids.

ACKNOWLEDGMENT

The authors gratefully acknowledge the support provided for this project by the Tarbiat Modares University (TMU).

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