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RESEARCH ARTICLE

# Morphological, anatomical, physiological, and cytological studies in diploid and tetraploid plants of Ispaghul (*Plantago ovata* Forsk.)

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**Abstract** Ispaghul (*Plantago ovata* Forsk.) as an important medicinal plant has obtained a remarkable reputation due to therapeutic applications of seed mucilage. To determine the effect of in vitro-induced polyploidy on various characteristics of *P. ovata*, the terminal bud of two true leaves seedlings were separately treated with colchicine [0.1, 0.3 and 0.5% (w/v) for 6, 12 and 24 h] and trifluralin [7.5, 15 and 22.5% (w/v) for 24, 48 and 72 h] solutions. The ploidy level of induced tetraploids was determined via chromosome counting of root tip cells, and then confirmed through flow-cytometric analysis. Comparison the morphological, physiological, anatomical features of intact diploids and induced tetraploids revealed that tetraploids had considerable more height, thicker leaf, larger spike and seed, larger pollen grain and more seeds per spike. Moreover, the amount of chlorophyll (a, b, and total) and carotenoids, as well as chloroplast number in guard cells was further in tetraploids than diploids. Unlike density, stomata size in tetraploids was bigger than that one in diploids. It was also observed that seeds of tetraploids had more mucilage than diploids. In summary, we firstly developed the *P. ovata* tetraploids and suggested 0.3% colchicine for 24 h and 22.5% trifluralin for 72 h as the optimum treatments for inducing tetraploidy in *P. ovata*.

**Keywords** *Plantago ovata* · Colchicine · Trifluralin · Genome duplication · Flow cytometry · Chromosome counting

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## Introduction

Changing drug trends has led to an increase in demand for biomass and bioactive compounds in medicinal plants (Sabzehzari and Naghavi 2018, 2019). *Plantago* genus was known as an important medicinal herb in the pharmacy industries (Shahriari et al. 2018). The genus contains several species-especially *P. ovata*-that are important due to agricultural and medicinal values. From an agricultural point of view, domestication and cultivation of *P. ovata* as alternatives of high-water consuming plants-such as corn and wheat-can be adopted for marginal agronomic areas (Koocheki et al. 2007; Bannayan et al. 2008). From a medicinal perspective, *P. ovata* seeds contain mucilage (Karimi et al. 2013), which is mainly utilized in the food and cosmetic industries (Dhar et al. 2005; Ebadi-Almas et al. 2012), and also medicine (Dhar et al. 2002, 2005; Saeedi et al. 2010). The oral use of mucilage helps

decline the level of blood cholesterol (Dhar et al. 2002, 2005). Furthermore, in various countries especially China, India and Iran, *P. ovata* seeds are utilized to treat breathing difficulties, fever, cough, cold, urinary problems, gonorrhea, dysentery and gastrointestinal malfunction as an alternative of chemical medicines such as antibiotics (Bahmani et al. 2016). As a result, *P. ovata* can be considered as an economic crop with good export value and relatively high mucilage yield applicable in medicine industries (Koocheki et al. 2007; Bannayan et al. 2008; Ebadi-Almas et al. 2012). Economic analysis revealed that the USA is the chief importer of *P. ovata* seeds and consumes annually 8000 metric tons, which indicating the importance of *P. ovata* market in the world (Rehana et al. 2015).

Polyploidy is an important approach in improving medicinal plants, because polyploidies exhibit more bioactive compounds (Gao et al. 2002; Berkov and Philipov 2002; Majdi et al. 2010). In general, genome duplication results in larger fruits and flowers, further content of secondary metabolites and finally more yield (Predieri 2001; Roy et al. 2001; Gu et al. 2005; Urwin et al. 2007). Polyploidy also improves starch content as an important nutritional value of the plants, a phenomenon which favored the human selection of cereals during human evolution (Kumar et al. 2018; Denham et al. 2016). For example, the induced polyploidies of *Avena sativa, Saccharum officinarum*, *Solanum tuberosum, Triticosecale, Triticum aestivum*, *Coffea Arabica, Fragaria ananassa*, *Nicotiana tabacum* have demonstrated the remarkable pharmaceutical and agronomic benefits compared with intact diploids (Gao et al. 2002; Zhang et al. 2008). This approach can be induced by using different antimitotic chemicals. Among them, the most common used antimitotic agents are oryzalin, trifluralin, as well as colchicine (Salma et al. 2017). The approach of chromosome doubling by antimitotic agents consists of several steps, including an induction, re-growth, and confirmation phase, which used to identify polyploidies (Salma et al. 2017). Since, induction efficiency depends on various factors-such as antimitotic agents, its different concentrations and exposure durations, and explant types-a range of attempts was carried out in order to find the optimum conditions to polyploidy induction (Lavania 2005). To assess the result of polyploidization, morphological, anatomical and physiological characteristics can be assayed as a rapid technique. However, flow cytometry and chromosome count are used as dominant techniques for absolute confirmation (Doležel and Bartoš 2005; Dhooghe et al. 2011).

Since, genome duplication affects the plant size and its secondary metabolites profile (Adaniya and Shirai 2001; Berkov and Philipov 2002; Jesus-Gonzalez and Weathers 2003; Majdi et al. 2010), polyploidy can be a promising approach to achieve the pharmaceutical and agronomic advantages in tetraploid of *P. ovata* (Dhooghe et al. 2011). Based on the mentioned advantages of polyploidy and on our previous study on *P. Psyllium* (Sabzehzari et al. 2019), present study was made to evaluate the effect of trifluralin and colchicine-induced polyploidy on morphological, physiological, anatomical, and cytological features of *P. ovata* medicinal plant and compare the induced tetraploids with their diploids.

## Materials and methods

### Plant material

*Plantago ovata* (2n = 2× = 8) seeds were provided from Pakanbazr Co. (http://www.pakanbazr.com/). In the following, chromosome doubling was made by colchicine and trifluralin on terminal buds, as discussed below.

### Tetraploidy induction

For chromosome doubling, first a total of 100 seeds were planted in a culture tray with cocopeat content. Then, terminal buds of seedlings containing two true leaves, 30 days old, were treated by colchicine [0.1, 0.3 and 0.5% (w/v) for 6, 12 and 24 h] and trifluralin [7.5, 15 and 22.5% (w/v) for 24, 48 and 72 h] solutions. The concentrations of chemicals were selected based on LD50. In forth to six true leaves stage, seedlings were transferred to pots containing sand, clay and rotten manure (2:1:1) under normal greenhouse conditions (16 h of light period, 25–26 °C and 65% humidity). The experiment was carried out as a factorial in format of completely randomized design with two factors-including concentrations and durations of exposure-with three replications.

### Identification and confirmation of the actual tetraploids

Three months after tetraploidy induction, the treated plants were compared with the controls in order to decline the workload and also rudimentary isolation of putative tetraploids. For achieving this goal, putative 4 × plants were marked according to the differences which observed in their leaves thickness, shape, color and size, in contrast with the 2 × plants. After marking deformed plants as putative tetraploids, two commonly techniques, flow-cytometry and chromosomal counting, were utilized to identify and validate actual tetraploids.

#### *Chromosome counting*

In order to counting the chromosome number, the root tips (0.5–1 cm long) were cut off at 11:00 a.m., and were pretreated with 4 °C for 12 h under refrigerator (physical pretreatment) and 0.002 g/mL $C_9H_7NO$ solution for 3 h at 4 °C (chemical pretreatment), respectively. Carnoy's I solution containing glacial-acetic acid and 95% ethanol (1:3) was used to fix the pretreated samples for 20 h and laid in 70% alcohol at 4 °C. Fixed samples were hydrolyzed in 1 N hydrochloric acid for 5 min at 60 °C, and then were washed with distilled water for 10–15 min and were stained with Aceto-orcein 2% in darkness at 25 °C for 60 min. In finally, stained samples with nearly 1–2 mm in length were squashed on a glass slide and their metaphase chromosomes were observed using Olympus microscope equipped with a camera.

#### *Flow cytometry*

In accordance with Doležel and Bartoš (2005), nearly 25 mg fresh leaf was chopped with a sharp blade in a petri-dish which consists of 1 mL modified WPB buffer (containing 10 mM $Na_2S_2O_5$, 4 mM $MgCl_2$-$6H_2O$, 0.2 M Tris–HCl, 20 mM $Na_2EDTA$, 86 mM NaCl, 0.1% (w/v) Triton X-100, 1% (w/v) Polyvinylpyrrolidone-10, pH = 7.5). Then, suspension was filtered via mesh screen with pore size of 30 and 50 µm and centrifuged at 2000 rpm for 5 min. Prepared samples were stained by 2 mL staining solution of Propidum Iodide (50 µg/mL) along RNase (1 µg/mL), in darkness for 30 min on ice. In final, stained samples were analyzed with a flow cytometer (BD FACSCanto$^{TM}$) and FloMax software. Leaf samples belong to 2 × plants were used as diploid reference. As a point, a total of 30 nuclei were studied in order to confirmation the ploidy level.

### Differences between diploids and tetraploids

After identification of actual tetraploids through chromosome counting and flow cytometry techniques, the morphological characteristics including leaf thickness, spike and seed length, as well as seeds per spike were compared between tetraploid and diploid plants. To measure leaf thickness, a digital caliper was used, which own an acceptable accuracy. It is necessary to mention, the greenhouse conditions were considered similar for intact diploids and induced tetraploids.

In the physiological evaluation, each sample about 1.0 g of fresh leaves was homogenized in 10 mL of 80% acetone and kept in the dark for 8 h. The supernatant was made up to 25 mL by the addition of 80% acetone, and absorbance was determined at 470, 646 and 663 nm wavelengths for measuring the content of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid. These values were calculated through the equations of Lichtenthaler and Wellburn (1983).

In the anatomical evaluation, to avoid the outcomes related to the position of stomatal openness, all of leaves were accumulated between 12:00 and 1:00 p.m. Carnoy solution, as well as sterilized water were used to discoloring and washing the samples for 10 h and 15 min, respectively. Afterwards, the bottom epidermis was flake off and hereafter superposed on glass slide. It was employed a photomicroscope with 100 × objective lens to observe stomata, chloroplasts, and pollen grains. It is worth to mention, three vision fields were randomly sampled to determine chloroplast and stomata indicators.

### Seed mucilage measuring

Mucilage of *P. ovata* seeds was ectracted by Sharma and Koul's method (1986). In this method, three 25 g seed samples were used to measure the mucilage content in seeds.

Statistical analysis

The survival rate was measured via dividing the survived seedlings on treated seeds. The induction rate was also calculated through dividing the induced tetraploids on survived seedlings. Eventually, induction efficiency was estimated as survival rate × induction rate (Bouvier et al. 1994). ANOVA and Student's *t* test at 5% probability level were conducted through SPSS V. 25 software. Duncan's multiple-range test was also made to determine the significant differences among the means via the statistical program.

## Results and discussion

In this study, we successfully induced tetraploidy in *P. ovata* by using colchicine and trifluralin and then revealed the differences between intact diploid and tetraploid plants (Fig. 1a–f; Tables 1–3).

Induction efficiency

Our results documented a more decline in survival rate by higher concentration and longer exposure time in all of treatments (Tables 1, 2). The similar findings were reported in *Rosa* (Khosravi et al. 2008); *Tanacetum parthenium* (Majdi et al. 2010); *Lagerstroemia indica* (Zhang et al. 2010); *Paulownia tomentosa* (Tang et al. 2010); *Gerbera jamesonii* (Gantait et al. 2011); *Echinacea purpurea* (Abdoli et al. 2013); *Vitis vinifera* (Acanda et al. 2015); *Trachyspermum ammi* (Sadat-Noori et al. 2017); *Bletilla striata* (Pan-pan et al. 2018). This toxicity of genome doubling agents could result from damaging to cells protoplast (Zeng et al. 2006).

In addition to survival rate, induction rate is another factor that determines induction efficiency (Sadat-

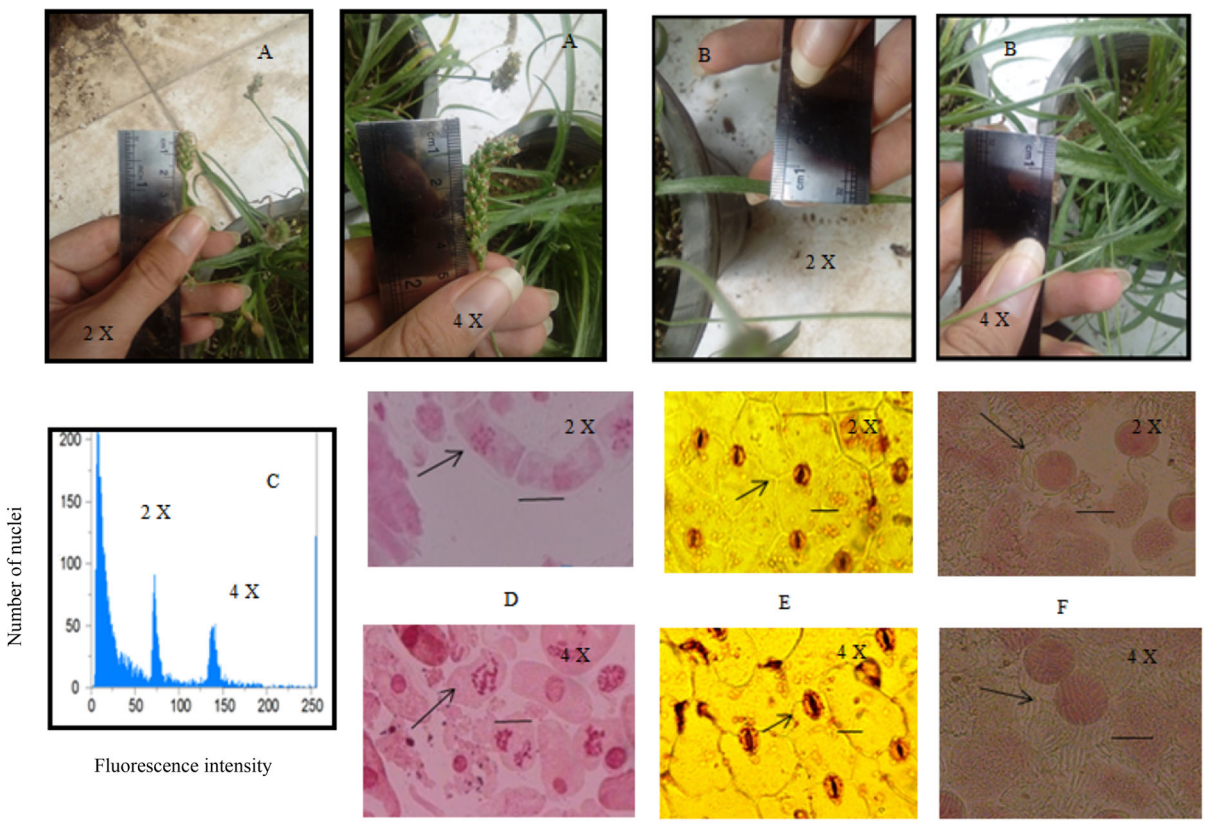


**Fig. 1** The differences between diploid (left) and tetraploid (right) *P. ovata* plants. **a** Spike; **b** leaf; **c** flow-cytometry analysis; **d** chromosomes number (bar 5 μm); **e** stomata size (bar 50 μm); **f** pollen grain size (bar 10 μm). As a point, the only results from the colchicine treatment are given here

**Table 1** The efficiency of colchicine-induced ploidy in *P. ovata* medicinal plant

| Colchicine treatments | Survival rate (%)[a] | Polyploidy rate (%)[b] | Induction efficiency (%)[c] |
|---|---|---|---|
| Control | $100 \pm 1^{a}$ | $0^{c}$ | $0^{c}$ |
| 0.1% for 6 h | $93 + 7.3^{b}$ | $0^{c}$ | $0^{c}$ |
| 0.1% for 12 h | $87 + 5.4^{c}$ | $0^{c}$ | $0^{c}$ |
| 0.1% for 24 h | $80 + 3.6^{d}$ | $5 \pm 1.6^{c}$ | $4 \pm 1.4^{c}$ |
| 0.3% for 6 h | $87 + 5.6^{c}$ | $0^{c}$ | $0^{c}$ |
| 0.3% for 12 h | $67 + 3.3^{f}$ | $7 \pm 2.3^{c}$ | $4 \pm 1.2^{c}$ |
| 0.3% for 24 h | $67 + 3.7^{f}$ | $57 \pm 6.5^{a}$ | $38 \pm 6.2^{a}$ |
| 0.5% for 6 h | $73 + 4.2^{e}$ | $3 \pm 1.8^{c}$ | $2 \pm 0.7^{c}$ |
| 0.5% for 12 h | $53 + 2.8^{g}$ | $8 \pm 2.7^{c}$ | $4 \pm 1.3^{c}$ |
| 0.5% for 24 h | $53 + 2.4^{g}$ | $37 \pm 4.4^{b}$ | $20 \pm 3.3^{b}$ |

In each column, averages with the same letter are not statistically significant at the level of 5% in the Duncan test

[a]No. of survived plants/treated seeds

[b]No. of induced tetraploids/survived plants

[c]Survival rate × induction rate

**Table 2** The efficiency of trifluralin-induced ploidy in *P. ovata* medicinal plant

| Trifluralin treatments | Survival rate (%)[a] | Polyploidy rate (%)[b] | Induction efficiency (%)[c] |
|---|---|---|---|
| Control | $100 + 1^{a}$ | $0^{c}$ | $0^{c}$ |
| 7.5% for 24 h | $71 \pm 5.1^{b}$ | $0^{c}$ | $0^{c}$ |
| 7.5% for 48 h | $64 \pm 4.4^{dcb}$ | $0^{c}$ | $0^{c}$ |
| 7.5% for 72 h | $76 \pm 4.9^{ab}$ | $7 \pm 2.2^{dbc}$ | $4 \pm 1.3^{bc}$ |
| 15% for 24 h | $67 \pm 3.9^{cb}$ | $0^{c}$ | $0^{c}$ |
| 15% for 48 h | $54 \pm 2.5^{dcb}$ | $5 \pm 1.2^{dc}$ | $2 \pm 0.7^{c}$ |
| 15% for 72 h | $49 \pm 2.3^{dcb}$ | $23 \pm 2.9^{b}$ | $9 \pm 2.1^{b}$ |
| 22.5% for 24 h | $60 \pm 2.9^{dcb}$ | $0^{c}$ | $0^{c}$ |
| 22.5% for 48 h | $40 \pm 1.9^{dc}$ | $19 \pm 1.7^{bc}$ | $9 \pm 2.7^{b}$ |
| 22.5% for 72 h | $38 \pm 1.2^{d}$ | $81 \pm 6.7^{a}$ | $29 \pm 4.1^{a}$ |

In each column, averages with the same letter are not statistically significant at the level of 5% in the Duncan test

[a]No. of survived plants/treated seeds

[b]No. of induced tetraploids/survived plants

[c]Survival rate × induction rate

Noori et al. 2017). Our results showed that induction rate was severely affected by exposure time, and also enough time for the effectiveness of trifluralin and colchicine on explants is an important criterion (Tables 1, 2). Given the survival and induction rate, the maximum of genome doubling efficiency was documented from 22.5% trifluralin for 72 h, and 0.3% colchicine for 24 h. Therefore, these two treatments are optimum for development of tetraploid plants in *P. ovata*.

## Cytological differences

The findings of chromosome counting of root cells confirmed that chromosome number of diploid was $2n = 2\times = 8$, whereas that of the tetraploid was doubled ($2n = 4\times = 16$) (Fig. 1d). The flow

**Table 3** The effect of colchicine and trifluralin-induced ploidy on morphological, physiological and anatomical characteristics in *P. ovata*

| Characteristics | Intact diploid | Colchicine-induced tetraploid | Trifluralin-induced tetraploid |
|---|---|---|---|
| Leaf thickness (mm) | $0.62 \pm 0.02^{b}$ | $0.90 \pm 0.02^{a}$ | $0.9 \pm 0.03^{a}$ |
| Spike length (mm) | $13 \pm 0.37^{b}$ | $22.66 \pm 0.68^{a}$ | $22.67 \pm 0.53^{a}$ |
| Seed length (mm) | $1.86 \pm 0.3^{b}$ | $2.54 \pm 1.5^{a}$ | $2.55 \pm 1.4^{a}$ |
| Seeds per spike (n) | $20 \pm 1.2^{b}$ | $25 \pm 1.5^{a}$ | $24 \pm 1.2^{a}$ |
| Chlorophyll a (mg $g^{-1}$) | $1.198 \pm 0.145^{b}$ | $2.056 \pm 0.342^{a}$ | $2.048 \pm 0.293^{a}$ |
| Chlorophyll b (mg $g^{-1}$) | $0.392 \pm 0.043^{b}$ | $0.679 \pm 0.101^{a}$ | $0.682 \pm 0.102^{a}$ |
| Chlorophyll total (mg $g^{-1}$) | $1.590 \pm 0.176^{b}$ | $2.735 \pm 0.365^{a}$ | $2.730 \pm 0.348^{a}$ |
| Carotenoid (mg $g^{-1}$) | $0.394 \pm 0.073^{b}$ | $0.722 \pm 0.089^{a}$ | $0.723 \pm 0.076^{a}$ |
| Chloroplast number (n per $mm^2$) | $10 \pm 0.45^{b}$ | $16 \pm 0.54^{a}$ | $16 \pm 0.55^{a}$ |
| Stomata length (μm) | $37 \pm 0.62^{b}$ | $56 \pm 0.83^{a}$ | $55.5 \pm 0.71^{a}$ |
| Stomata wide (μm) | $21.9 \pm 0.44^{b}$ | $28.8 \pm 0.37^{a}$ | $28.3 \pm 0.35^{a}$ |
| Stomata density (n per $mm^2$) | $30 \pm 3.2^{a}$ | $14 \pm 1.5^{b}$ | $14 \pm 1.5^{b}$ |
| Mucilage yield (g $plant^{-1}$) | $0.6 \pm 0.01^{b}$ | $1.2 \pm 0.06^{a}$ | $1.2 \pm 0.08^{a}$ |
| Mucilage content (%) | $14 \pm 0.45^{b}$ | $38 \pm 0.78^{a}$ | $38 \pm 0.83^{a}$ |

In each row, averages with the same letter are not statistically significant at the level of 5% in the Duncan test

cytometry showed that diploids and tetraploids have single peak at ~ 75 and ~ 150 channels, respectively (Fig. 1c). Flow cytometry confirmed the tetraploid plants that already identified by chromosome counting. DNA content in tetraploids was recorded nearly twice than diploids, which suggested genome duplication was successfully achieved by using trifluralin and colchicine. The DNA ratio of tetraploids to diploids was not exactly two, probably due to the fact that tetraploids have the more content of DNA and thereby need the more staining time (Luo et al. 2018).

## Morphological differences

A number of researches have reported that tetraploids have valuable farming characteristics such as the longer leaves, thicker stems and roots, as well as vigorous growth (Shao et al. 2003). Our results also demonstrated that leaf thickness, spike length, seed length and seeds per spike increased in tetraploids achieved from colchicine and trifluralin treatments (Table 3; Fig. 1a). These findings are in agreement with Abdoli et al. (2013) on *E. purpurea,* Tavan et al. (2015) on *Thymus persicus,* Pan-pan et al. (2018) on *Bletilla striata,* as well as Luo et al. (2018) on *Taraxacum kok-saghyz*.

## Physiological differences

Our results uncovered the fact that amount of chlorophylls and carotenoid significantly increased in the leaves of tetraploids (Table 3). The effect of genome doubling on the chlorophyll extent has been documented as greener leaves in others plants such as *Datura stramonium* (Amiri et al. 2010), *E. purpurea* (Abdoli et al. 2013), *H. reticulatus* (Madani et al. 2015), *Taraxacum koksaghyz* (Luo et al. 2018), as well as *Eclipta alba* (Salma et al. 2018). However, there is a report that declared polyploidy induction can't change the chlorophyll content in *Cannabis sativa* tetraploids (Bagheri and Mansouri 2015).

## Anatomical differences

The results of anatomical evaluation revealed that chloroplast number in tetraploids was significantly more than that one in diploids (Table 3). Thus, it can be concluded the chloroplast number is associated with the level of ploidy, and can be used as a simple and effective parameter to distinguish the different ploidy levels. Similar findings have been reported on *E. purpurea* (Abdoli et al. 2013), and *E. alba* (Salma et al. 2018). In addition to chloroplast number in guard cells, it found that tetraploids stomata own more width

and length than diploids (Table 3; Fig. 1e). However, the stomata density in diploids was more than that one in tetraploids. The similar documents have been also reported for *Astragalus membranaceus* (Chen and Gao 2007), *L. indica* (Zhang et al. 2010), *T. parthenium* (Majdi et al. 2010), *Miscanthus* (Głowacka et al. 2010), *Centella asiatica* (Kaensaksiri et al. 2011), *E. purpurea* (Abdoli et al. 2013), *Crocosmia aurea* (Hannweg et al. 2013), *Mitracarpus hirtus* (Pansuksan et al. 2014), *Linum album* (Javadian et al. 2017), as well as *E. alba* (Salma et al. 2018). The pollen grain size was also observed larger in tetraploids than diploids (Fig. 1f).

Seed mucilage content

Since mucilage is produced in the seeds (Gupta et al. 2018), it seems that larger seeds in tetraploids can be resulted in the more content of seed mucilage. To test this assumption, mucilage content of diploids and tetraploids seeds was measured.In the intact diploids, the mucilage yield and content were 0.6 g plant$^{-1}$ and 14%, respectively. However, the mucilage yield and content in the induced tetraploids were 1.2 g plant$^{-1}$ and 38%, respectively (Table 3).

## Conclusion

Given to valuable pharmaceutical properties of *P. ovata* (Shahriari et al. 2018) and also the positive effect of polyploidy on its biomass and bioactive components, it needs to find the optimum conditions for producing tetraploids in *P. ovata* medicinal plant. Thus, we firstly in vitro induced *P. ovata* tetraploid plants via trifluralin and colchicine agents. Our findings indicated that 22.5% trifluralin for 72 h, as well as 0.3% colchicine for 24 h can be used as the optimum treatments for development of *P. ovata* tetraploids. We observed that tetraploids were larger than their intact diploids for height, leaf thickness, spike, seed, pollen grain, seeds per spike. Furthermore, chlorophyll (a, b, and total), carotenoid and chloroplast number in guard cells of tetraploids were more than diploids. Unlike density, stomata size in tetraploids was bigger than that one in diploids. In summary, for the first time we established an in vitro procedure for genome doubling in *P. ovata* medicinal plant. And also, we showed the procedure can increase seed mucilage content-as commercial product for food, cosmetic and pharmacy industries-in *P. ovata* tetraploids.

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**Author contributions** All authors equally participated into this work.

**Compliance with ethical standards**

**Conflict of interest** Authors have nothing to disclose with regard to commercial support.

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