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Autotetraploid plant production in endemic *Onobrychis elata* with colchicine treatments

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Summary

This study aimed to induce autotetraploidy in endemic *Onobrychis elata* plants by colchicine treatment of seeds or seedlings. Colchicine was applied to *O. elata* directly on germinated seeds, pre-germinated seeds (root length of 3-8 mm), and apical regions (using cotton) under in vivo conditions. Out of a total of 1,210 colchicine-treated seeds that were evaluated, only 203 survived. There was an inverse relationship between the number of surviving plants and colchicine concentration and exposure time. The highest percentage of tetraploidy in surviving plants (50%) was obtained by applying 0.2% colchicine for 6 hours to pre-germinated seeds. No significant tetraploidy was achieved by colchicine application to seedlings. Flow cytometry observations indicated that DNA content varied between 0.99 and 1.06 pg in diploid plants (controls), while DNA content varied between 2.22 and 2.48 pg in tetraploid plants. It was concluded that tetraploid plants were induced successfully only in seedlings obtained from pre-germinated seeds, with their ploidy level confirmed via flow cytometry analysis.

Key words: *Onobrychis*, colchicine, flow cytometry, polyploidy

Introduction

Sainfoin (*Onobrychis viciifolia* Scop.) is an important forage legume for hay and pasture in calcareous and arid lands because of its well-developed root system and adaptability to water limitations (Jefferson et al., 1994; Borreani et al., 2003). The nutritive values of hay are similar to alfalfa, and because hay contains appreciable amounts of condensed tannins, animals grazed in pure pasture are not at risk of bloat (Wang et al., 2015; Bhattarai et al., 2016).

There are approximately 170 species of the genus *Onobrychis* around the world, located mainly in southwestern Asia, the Mediterranean, and temperate regions of Europe and Asia (Cronquist, 1981; Zohary, 1987; Aktoklu, 2001). Wild *Onobrychis* species constitute important genetic resources for breeding sainfoin and improving grassland (Avci et al., 2013; Özaslan Parlak and Parlak, 2008).

In Turkey, there are 55 *Onobrychis* species with two subgenera (*Onobrychis* and *Sisyrosema*) and five sections (*Dendobrychis*, *Laphobrychis*, *Onobrychis*, *Hymenobrychis*, and *Heliobrychis*); 28 of them are endemic (Aktoklu, 2001; Avci et al., 2013). *Onobrychis elata* Boiss. et Bal. is a valuable native species that is widely scattered due to its morphological similarity to cultivated *O. viciifolia* (Avci et al., 2013). *O. elata* is diploid (2n = 14) and has a basic chromosome number of x = 7 (Cartier, 1976). It has been considered an important potential wild genetic source for hybridization with *O. viciifolia* to help further forage production under biotic and abiotic stresses; therefore, it would be advantageous to double its chromosome number.

Autopolyploids originate from the combination of unreduced gametes in nature and can also sometimes be artificially stimulated (Chen, 2010). Chromosome doubling is used to more efficiently breed superior varieties with high yields of forage crops (Acquaah, 2007; Meru, 2012). Moreover, polyploidy plays an important role in the fertility of interspecific hybridization between cultivated species and their wild relatives (Falcinelli, 1999; Aversano et al., 2012).

Colchicine treatment is the most effective and widely used method of producing polyploidy in forage legumes. Anderson et al. (1991) obtained polyploidy in *Trifolium* plants and their hybrids by treating seeds, seedlings, and growing shoot apices with colchicine. Similar results were achieved by Dibyendu (2010) in *Lathyrus sativus* L., Zeinab et al. (2012) in *Trifolium alexandrinum* L., and Wu et al. (2015) in *Stylosanthes guianensis* (Aublet) Sw.

However, research on polyploidy in *Onobrychis* species has not been found in the literature. This study aimed to produce autotetraploid plants from diploid *O. elata* by colchicine treatment under *in vivo* conditions.

Materials and methods

O. elata is a perennial with erect stems measuring approximately 60-120 cm (Fig. 1A). Leaves include 5-7 pairs of narrow elliptical leaflets (Fig. 1B). The raceme is many flowered with a loose structure (Fig. 1C), and the corolla of the flower is rose with deeper striations (Fig. 1D). The fruit is suborbicular and includes long and short spiny teeth on the crest and disk, respectively (Fig. 1E). The seed is roughly kidney-shaped (reniform), and seed color varies from light brown to dark brown (Fig. 1F). Seeds of *O. elata* were collected from Mount Erciyes (1,443 m), Kayseri, Turkey, and stored at 4 °C until used. The seed coats were very hard and impermeable; consequently, they were abraded with sandpaper as described by Avci and Kaya (2013). The seeds were then surface sterilized in 96% alcohol for 2 minutes and rinsed twice with distilled water. Germination was performed in petri dishes (100 mm × 20 mm) on top of two filter papers moistened with distilled water. Each petri dish was then sealed with laboratory film, and the seeds were allowed to germinate in the dark at 20 ± 1 °C.

Colchicine (Sigma catalog no.: C9754) was dissolved in water at room temperature, and the aqueous solution was stored at 4 °C. The colchicine was directly applied to germinated seeds at two concentrations (0.05% and 0.1%) and three exposure times (24, 48, and 72 hours). Pre-germinated seeds with 3-8 mm root lengths were treated with four concentrations (0.1%, 0.2%, 0.3%, and 0.4%) and three exposure times (6, 12, and 24 hours), followed by rinsing. In addition, three concentrations of colchicine (0.05%, 0.1%, and 0.2%) at two exposure times (48 and 72 hours) were applied with cotton to the apical regions of 10-day-old seedlings. The surviving seedlings were transferred into pots containing a mixture of peat and perlite (3:1 v/v) and incubated for a 16-hour photoperiod day of 8,000-10,000 lux, at 20 ± 1 °C and 60% humidity.

Ploidy analysis was performed using flow cytometry, which is the newest, fastest, most accurate, and most economical method for this purpose. First, rice, tomato, common vetch, barley, and safflower plants were analyzed separately with diploid *O. elata*, and common vetch (*Vicia sativa* L.) and safflower (*Carthamus tinctorius* L.) were selected as the most suitable internal standards. Partec kits were used to isolate nuclear DNA from fresh leaf samples derived from both

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Fig. 1: Morphological characteristics of *O. elata*. (A) general view with erect stem; (B) leaf; (C) raceme; (D) corolla with calyx; (E) fruits; (F) seeds.

diploid and colchicine-treated two-month-old plantlets, and DNA content was calculated for each sample using the standard plants. The resulting flow histogram was analyzed with the FloMax packet program, and fluorescence intensity, mean, and CV values were determined for each sample. The nuclear DNA content of each *O. elata* sample was calculated according to the following formula: DNA content = (fluorescence intensity of sample) / (fluorescence intensity of standard) × DNA content of standard.

Results and discussion

Out of a total of 480 germinated seeds treated with colchicine, 113 seedlings (23.5%) survived, and chromosome doubling was not observed (data not shown). In similar studies of legume species, direct treatment of germinated seeds with colchicine did not successfully induce polyploidy. PATIL (1992) reported that germinated seeds of *Crotalaria linifolia* Linn. treated with colchicine at concentrations of 0.10%, 0.15%, 0.20%, 0.25%, and 0.30% for 6, 12, and 24 hours did not produce polyploid plants. ARYA et al. (1988) found that colchicine treatment at a concentration of 0.15% for 10 hours was a lethal dose in germinated seeds of fenugreek, and GANDHI and PATIL (1997) stated that direct treatment of germinated seeds of *Clitoria ternatea* L. was not a practically efficient means of inducing tetraploid tissue. Colchicine treatment of pre-germinated seeds resulted in 75 surviving plantlets (12.5%) out of a total of 596 seedlings. Of those 75, 15 plants (20%) were determined to be tetraploid by flow cytometry (Tab. 1). As the concentration and exposure time increased, the number of surviving plants decreased, and plants did not survive when treated at concentrations of 0.2%, 0.3%, and 0.4% for 24 hours. Tetraploid plants were observed for all exposure times at 0.1% concentration, as well as for 6 hours of exposure time at 0.2% concentration, which was determined to produce the highest chromosome doubling percentage (50%) in surviving plantlets. Similar results were obtained from *Astragalus membranaceus*, with the mortality rate higher than 70% at a concentration of 0.3% colchicine over a 24-hour exposure time; the highest chromosome doubling (50%) among surviving plants was obtained from treatment with 0.2% concentration for 6 hours (CHEN and GAO, 2007). TULAY and UNAL (2010) and JOSHI and VERMA (2004) reported that when seeds of *Vicia villosa* Roth and *Vicia faba* L. were first soaked in water for 20 hours, tetraploid plants were attained with colchicine treatment at lower doses (0.005% concentration for 8 hours). PATHAK et al. (2015) supported the finding that soaking seeds in water prior to colchicine treatment was quite efficient at promoting polyploidy.

When colchicine was applied to the apical regions of 134 seedlings, only 15 (11%) of them survived, and no polyploidic plants were observed (data not shown). Increasing concentration and exposure time negatively affected seedling survival. Treatment with a concentration of 0.2% for 48 and 72 hours led to death of the plantlets. DABKEVICIENE et al. (2016) determined that colchicine treatment to *Trifolium pratense* L. embryos resulted in 3.3 times more polyploid plants than overhead application to cotyledon leaves of six- to eight-day-old seedlings. WU et al. (2015) obtained 10% tetraploid induction by applying colchicine to the apical region of *S. guianensis* at the highest concentration (0.2%) for 48 hours. BEWAL et al. (2009) stated that the highest polyploidy rate obtained by colchicine treatment to the apical region of *Cyamopsis tetragonoloba* L. was observed at 0.2% concentration and 10 hours of exposure time for two days.

In contrast to the current findings, previous studies have demonstrated that polyploid plants were obtained from applications to the apical regions of different legume species, and a reduced survival rate was observed at colchicine concentrations over 0.2%. In the future, new approaches to exposure frequency and times and application type on the apical region should be evaluated.

Colchicine was applied to germinated seeds, pre-germinated seeds, and the apical region of seedlings, and out of a total of 1,210 seedlings, 203 survived. Of the surviving plantlets, 15 of them, including those originating from pre-germinated seeds, were determined to be tetraploid by using flow cytometry (Tab. 2). When safflower (*C. tinctorius*) was used as the internal standard, nuclear DNA peaks of tetraploid (2n = 28) *O. elata* overlapped (Fig. 2), but no overlap was observed in nuclear DNA peaks of diploid (2n = 14) plants (Fig. 3). After analysis of all samples with safflower as the internal standard, those with overlapping nuclear DNA peaks and thus considered to be tetraploid were again analyzed with the nuclear DNA of common vetch (*V. sativa*). As a result of these analyses, tetraploid plants were successfully identified as those without overlap (Fig. 4). The content of nuclear DNA in diploid and tetraploid plants ranged from 0.99 to 1.06 pg and 2.22 to 2.48 pg, respectively (Tab. 2).

In conclusion, tetraploid *O. elata* plants were successfully obtained at a high percentage by treating pre-germinated seeds of diploid plants with colchicine. Increased concentrations of colchicine adversely affected the survival rate of the plants. For unsuccessful treatments of the apical region and germinated seeds, modifications can be made to increase the success of chromosome doubling. Thirteen tetraploid

Tab. 1: Results of colchicine application to germinated seeds (3-8 mm root length)

Colchicine concentration(%)	Application time (hours)	Number of planted seedlings	Number of surviving plantlets	Plantlet survival rate (%)	Number of tetraploid plants in surviving plantlets	Tetraploid plant rate (%)
0.1	6	30	14	47	3	21
	12	50	4	8	1	25
	24	60	10	17	2	20
0.2	6	50	18	36	9	50
	12	50	1	2	0	0
	24	41	0	0	0	0
0.3	6	45	12	27	0	0
	12	50	1	2	0	0
	24	70	0	0	0	0
0.4	6	30	14	47	0	0
	12	50	1	2	0	0
	24	70	0	0	0	0
Total	—	596	75	—	15	—

Tab. 2: Fluorescence intensity, average DNA content, and CV values in samples with and without colchicine treatment

Plant number	Sample fluorescence intensity	Standard fluorescence intensity	Sample DNA content (pg)	Standard DNA content (pg)	CV1 (*O. elata*)	CV2 (standard)	Ploidy level
14 (Control)	119.24	238.72	1.00	2.00*	4.75	3.00	Diploid
24 (Control)	115.42	233.24	0.99	2.00	6.15	3.68	Diploid
32 (Control)	128.94	244.13	1.06	2.00	3.95	4.99	Diploid
69 (Control)	133.78	266.69	1.00	2.00	5.24	5.99	Diploid
57	129.28	197.37	2.39	3.65**	5.32	3.43	Tetraploid
59	134.12	199.27	2.46	3.65	5.52	2.75	Tetraploid
64	119.63	186.20	2.35	3.65	3.97	2.54	Tetraploid
71	93.97	150.29	2.28	3.65	6.33	4.13	Tetraploid
77	125.74	187.67	2.45	3.65	5.98	3.02	Tetraploid
79	149.51	234.60	2.33	3.65	3.28	3.88	Tetraploid
82	123.29	196.15	2.29	3.65	6.39	4.09	Tetraploid
85	122.39	201.41	2.22	3.65	7.81	4.25	Tetraploid
89	139.64	211.13	2.41	3.65	3.63	2.47	Tetraploid
90	139.56	205.61	2.48	3.65	3.78	2.56	Tetraploid
91	118.97	182.09	2.38	3.65	3.82	2.89	Tetraploid
92	129.82	202.40	2.34	3.65	3.63	2.82	Tetraploid
94	141.21	218.06	2.36	3.65	3.27	2.74	Tetraploid
96	129.02	200.04	2.35	3.65	4.48	4.36	Tetraploid
97	143.96	218.05	2.41	3.65	3.92	3.16	Tetraploid

* Safflower (*C. tinctorius*) was used as the standard DNA content.
** Common vetch (*V. sativa*) was used as the standard DNA content.

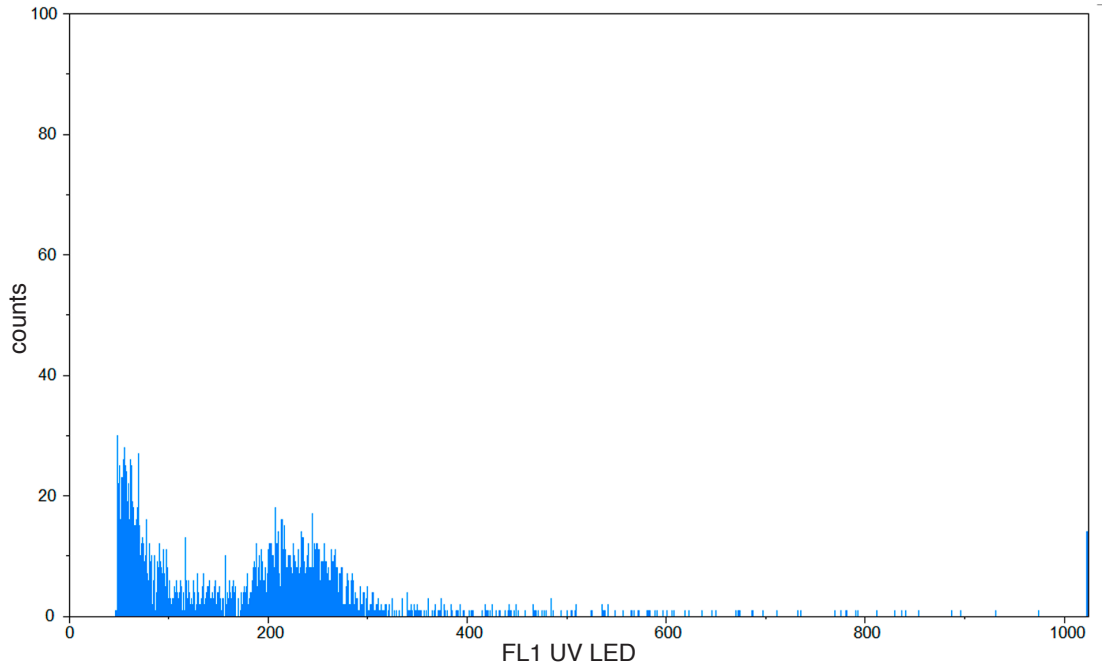

Fig. 2: Overlap of G1 peaks in tetraploid *O. elata* and internal standard (safflower)

plants were transferred to field conditions for further morphological, cytological, and palynological observation, and to hybridization facilities in the sainfoin breeding program.

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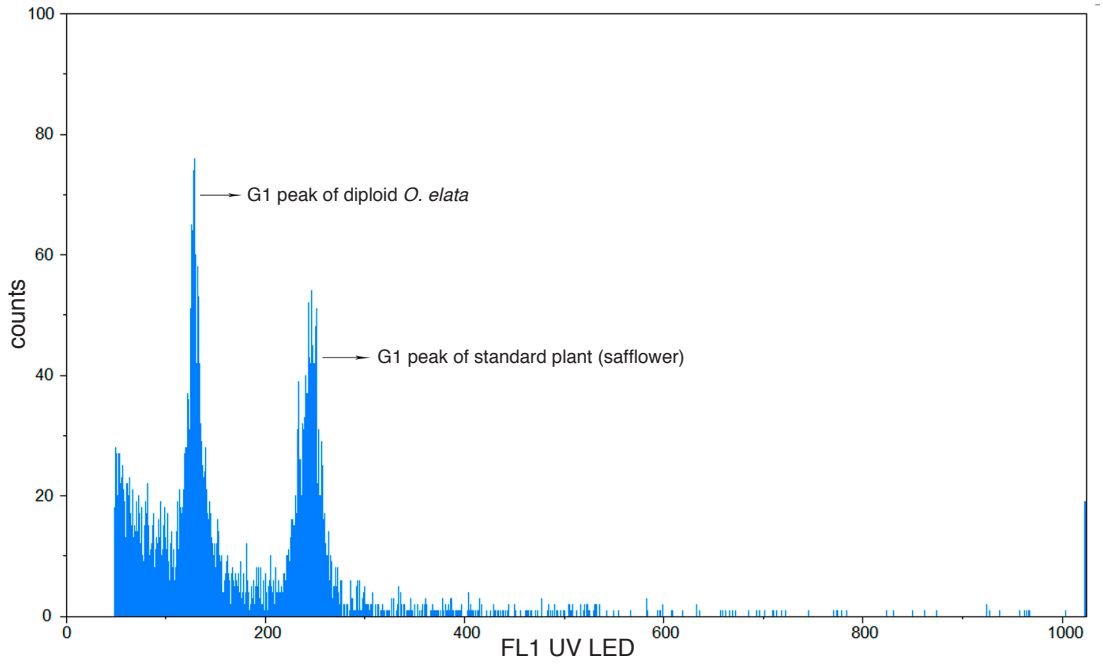

Fig. 3: Positions of G1 peaks belonging to diploid *O. elata* and standard plants (safflower)

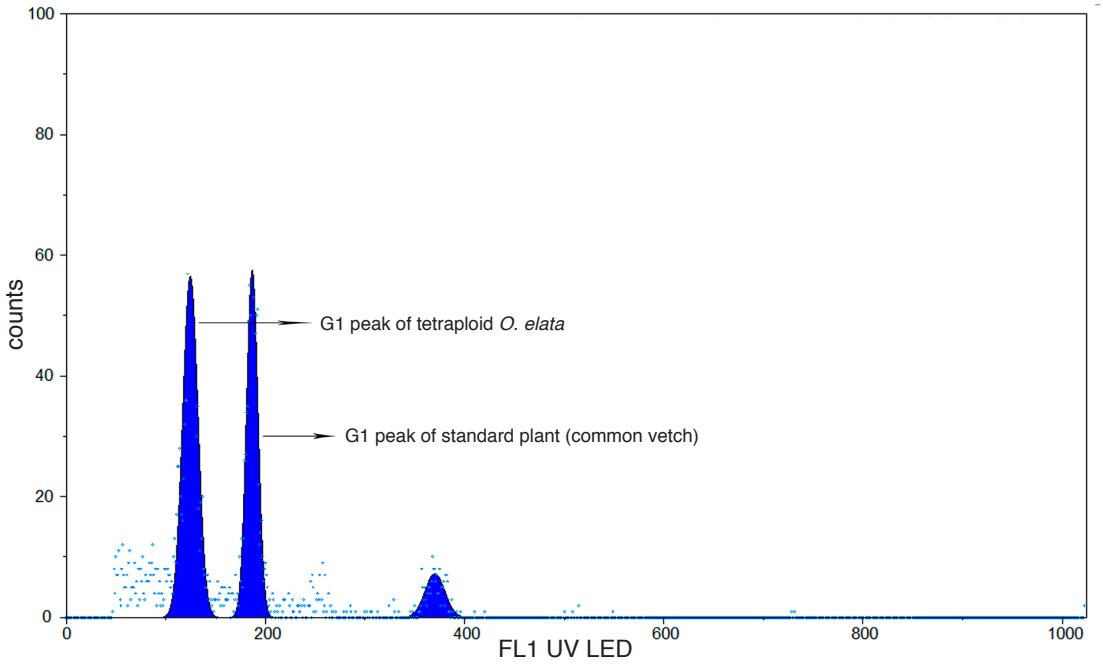

Fig. 4: Positions of G1 peaks belonging to tetraploid *O. elata* and standard plants (common vetch).

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