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A Comparative Study on Colchicine Application Methods in Obtaining Doubled Haploids of Tobacco (*Nicotiana tabacum* L.)

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Abstract: In order to obtain androgenic doubled haploids of tobacco (*Nicotiana tabacum* cv. Karabağlar 6265) colchicine was applied at 3 different stages of anther culture. Before culture, anthers were treated with 0.4% aqueous solution of colchicine for 0, 2, 4, 6, 8, 10, and 12 h. Culture response of anthers decreased as the treatment duration increased (except 12 h) and the highest diploidization of 29.7% was obtained with 6 h. During culture, when macroscopic embryoids appeared in dehisced anthers, they were wrapt up in sterile cotton saturated with 0.2% colchicine for 3 days and transferred to fresh medium. This application resulted in 60% diploidization, but plant recovery from treated embryoids was low and only 5 plants could survive. When plantlets with 4 to 8 leaves immersed in 0.2% colchicine for 0, 7, 24 and 48 h on a shaker, besides 4.3%, 42.3%, 37.8% and 33.3% doubled haploids, respectively, haploids, tetraploids, aneuploids, and mixedploids were also found among the treated plants. The main advantage of this method is that treated plantlets can be transplanted directly into pots in order to grow androgenic plants.

When chromosome doubling rate and viability are taken into consideration, among the 3 methods tested, plantlet treatment with 0.2% colchicine for 7 h appeared to be more efficient with 42.3% dihaploids. Durations shorter than 7 h must be tested in order to optimize the application.

Key Words: Colchicine, doubled haploid, *Nicotiana tabacum* L.

Tütün (*Nicotiana tabacum* L.) Dihaploidlerinin Elde Edilmesinde Kullanılan Kolkisin Uygulama Metotları Üzerinde Karşılaştırmalı Bir Çalışma

Özet: Androjenik tütün (*Nicotiana tabacum* cv. Karabağlar 6265) dihaploidlerini elde etmek için anter kültürünün üç farklı döneminde kolkisin uygulaması yapılmıştır. Anterler, kültür öncesi % 0,4 kolkisin çözeltisi ile 0, 2, 4, 6, 8, 10 ve 12 saat muamele edilmiştir. Anterlerin kültüre cevabı, uygulama süresinin artması ile (12 saat uygulama dışında) azalmıştır ve en yüksek diploidleşme % 27,9 olarak 6 saat uygulamada elde edilmiştir. Kültür boyunca embriyoidlerin gözle görüldüğü açılmış olan anterler % 0,2 kolkisin çözeltisi ile ıslatılmış pamuk arasında üç gün bırakılmış ve sonra anterler taze besin ortamına aktarılmıştır. Bu uygulamada % 60 diploidleşme elde edilmiştir fakat, muamele edilen embriyoidlerden bitki oluşumları düşük olmuştur ve sadece 5 bitki elde edilmiştir. Dört ile sekiz yapraklı bitkicikler % 0,2 kolkisin çözeltisi ile çalkalayıcıda 0, 7, 24 ve 48 saat muamele edildiğinde sırasıyla % 4,3, % 42,3, % 37,8 ve % 33,3 diploidleşme görülmüş, muamele edilen bitkiler arasında haploid, tetraploid, aneuploid, ve miksoploidler de bulunmuştur. Bu metodun başlıca avantajı muamele edilen bitkilerin doğrudan saksılara aktarılabilmesidir.

Kromozom katlanma oranları ve bitkiciklerin yaşatılması dikkate alındığında, denenen üç metot arasında, bitkicik döneminde 7 saat % 0,2 kolkisin uygulamasının daha avantajlı olduğu görülmüştür (% 42,3 dihaploid). Uygulamayı optimize etmek üzere 7 saatten daha kısa sürelerin etkisinin denenmesi gereklidir.

Anahtar Sözcükler: Kolkisin, katlanmış haploid (dihaploid), *Nicotiana tabacum* L.

Introduction

Colchicine, an alkaloid found in the seeds and corms of autumn crocus (*Colchicum automnale*), is an antimitotic agent and has been used for chromosome doubling to induce experimental polyploids since 1937 after Blakeslee and Avery, and Nebel (1). Besides producing polyploid forms of some crop plants, chromosome doubling also paves the way for fertility restoration of some sterile interspecific and intergeneric hybrids of plants and also of andro-and gynogenic haploids.

The tobacco species *N. tabacum* L. is of amphidiploid origin (2n = 48) (2) and meiotic behaviour in its

androgenic haploids was shown to be similar to monoploids (3). Thus, haploids are sterile and in order to get seed their chromosomes have to be doubled. The successful application of haploid technology to plant breeding depends on both reliable methods for production of haploids in large number and achieving a high frequency of chromosome doubling. For dihaploid production, effective colchicine treatments have been reported for several crop plants like wheat (4, 5), maize (6), rapeseed (7), Indian mustard (8), and watermelon (9). Colchicine treated plantlets can be multiplied by cutting them into modal microcutting as done by Sarı and Abak (9) with 3-4-week-old parthenogenetic haploid watermelon plantlets.

Several methods have been used for chromosome doubling in haploids of tobacco using colchicine. When anther derived haploid plantlets were treated with different concentrations of colchicine, diploidizations ranging from 6.4% to 70% have been reported (10-14); the rates are higher for younger ones with 2 to 3 leaves (10,13). Besides doubled haploids, tetraploids, aneuploids, and chimeric forms have also been observed (12,13).

By dipping anthers excised from flower buds directly into colchicine solution, Takashima et al. (15) obtained the highest frequency of diploidization (66.7%) in the treatment with 0.4% colchicine for 8 h. Colchicine can also be applied to embryoids and Dai et al. (16) reported 60% diploidization following minimum 72 h treatment of embryoids with 0.2% colchicine solution and a success of 30% by putting drops at the same concentration on opened anther thecae containing macroscopic embryoids. Chromosome doubling has also been achieved by adding colchicine to culture medium (4,5,17-19).

Like colchicine, acenaphthene vapour also causes chromosome doubling, and it has been used to produce tobacco dihaploids, by applying during culture (20-22), haploid plantlets (23), and plants (24). Callus (25) and leaf-midvein cultures (26,27) and treatment with an antimitotic herbicide trifluralin (28) have been used in tobacco for the same purpose.

In some projects on Turkish tobaccos, colchicine has been applied to double the chromosome number. Emiroğlu (29) used this chemical to overcome interspecific hybrid sterility, and Emiroğlu et al. (18), besides acenaphthene, also used colchicine in a breeding project based on the haploid technique. In the former study (29), treatment of seeds in an aqueous solution of colchicine has been reported to be more effective compared to germinated seed and axillary bud treatments. In the latter (18), colchicine was applied to growing points of haploid plantlets in pots and resulted in low chromosome doubling (11.97%).

Tobacco is an industrial crop and also an important model plant for molecular genetics and transformation studies. To make use of the advantages provided by haploid technique and dihaploidy, efficient chromosome doubling methods have to be used. The objective of the present work was to investigate the chromosome doubling efficiencies of colchicine treatments at 3 different stages of anther culture in Aegean tobacco Karabağlar 6265. Plants obtained from colchicine treated plantlets were especially checked for chromosome number aberrations in order to find the ratio of 2n = 48 dihaploids.

Materials and Methods

Plant material: For obtaining haploid material needed, the anthers of *Nicotiana tabacum* L. cv. Karabağlar 6265 (2n = 48) containing microspores at about first pollen mitosis stage were surface sterilized and in vitro cultured.

Culture medium: Sterilized anthers were cultured on Nitsch and Nitsch (30) medium with 40 g dm^{-3} sucrose and 8 g dm^{-3} agar, and without the addition of folic acid and biotin (22).

Culture conditions: Culture tubes containing about 15 ml of medium and anthers (colchicine treated and untreated) were placed in a growth chamber at 23-25 °C with 16 h 3500 lux illumination.

Colchicine Treatments

Treatment of anthers before culture: Anthers were dipped in 0.4% aqueous solution of colchicine for 0, 2, 4, 6, 8, 10, and 12 h, then were rinsed in sterile distilled water and cultured.

Treatment of embryoids: During culture when macroscopic embryoids appeared in dehisced anthers, they were wrapped up and kept in sterile cotton saturated with 0.2% colchicine for 3 days, and transferred to fresh medium after rinsing with sterile distilled water.

Treatment of plantlets: Androgenic plantlets with 4 to 8 leaves were treated in 0.2% colchicine solution on a shaker (62 rpm) for 0, 7, 24 and 48 h. After rinsing with sterile distilled water, treated plantlets and the controls were planted directly into 6.5 cm diameter pots in a greenhouse.

Ploidy level determinations

Ploidy level of androgenic plants was determined by chromosome countings using leaf squashes (20,31); and stoma guard cell size (3,20,32), pollen size (20,29), and seed setting ability were also considered.

For leaf squashes, early in the morning leaves smaller than 1 cm in length were collected from the plants grown in greenhouse and placed in 2 mM 8-hydroxychinoline for 3.5 to 4 h. After rinsing 5 to 6 times with tap water, they were hydrolyzed in a mixture of 1 part conc. HCl + 2 parts 96% ethyl alcohol for 6 min. Following rinsing again 4 to 5 times with tap water, the leaves were stored in distilled water in a refrigerator until use. For chromosome countings, small lamina pieces were taken from the base of the leaves and squashed in 2% aceto-orcein (20).

For fertility checks, pollen grains were stained with 1% aceto-orcein. Stoma guard cell length measurements were conducted on slides prepared from lower epidermal tissue of the middle leaves (20).

Results and Discussion

General Features

First growth of colchicine treated plants was slower compared to the controls and they showed a variation in size (Figure 1). In general, haploid flowers were a little smaller compared to the diploids. Stoma guard cells were

(a)

(b)

(c)

(d)

Figure 1. Control (a) and 7h (b), 24 h (c), 48 h colchicine treated plants (d) 1.5 months after transfer to pots.

also smaller, the lengths being 27.35 ± 0.76 μm in haploids and 42.79 ± 0.07 μm in diploids.

Haploids were sterile, did not set seed, but they formed small and shrunken capsules instead of shedding unfertilized flowers. Emiroğlu (3) also observed that among the haploids of 7 Turkish tobacco examined, only the haploids of Karabağlar 6265 formed such capsules. Haploid pollens showed a variation in size, majority being small and undeveloped. However, some stainable pollen grains were also observed, with an average diameter of 24.6 μm. These pollens could be originated from unreduced microspores, because Emiroğlu (3) observed in haploid meiocytes of tobaccos including Karabağlar 6265 that at first anaphase some of the 24 univalents underwent division into their chromatids; and at sporade stage besides tetrads, triads, dyads, and a few monads were present. Stained pollen grain diameter in dihaploids was found to be 34.0 μm in average.

The Effects of Colchicine Treatments

Treatment of anthers before culture: When anthers were treated with colchicine before culture for 0 to 12 h durations, the rate of plantlet producing anthers tended to decrease as the duration increased. In the control group, 33.3% of the anthers produced plantlets, but 2 h treatment reduced the anther culture response to 17.5%; treatments for longer durations resulted in lower rates, changing between 0.0% and 6.94% (Table 1). Takashima et al. (15) also reported a decrease in culture response with the increase in soaking duration.

Two (1.41%) spontaneous diploid fertile (2n = 48) plants were found amoung the plants derived from the untreated anthers (Table 2). For the same oriental tobacco cultivar, i.e. Karabağlar, Emiroğlu (3) reported one spontaneous fertile diploid plant (2.6%). Six hours

Table 1. Culture response of anthers treated with 0.4% colchicine for various durations.

Treatments	No. of cultured anthers (h)	Anther culture response (%)
Control	297	33.3
2	97	17.5
4	80	-
6	245	6.94
8	376	0.53
10	283	0.71
12	158	4.43

colchicine treatment of anthers resulted in 29.7% diploidization but in other treatments the ratios were lower and between 0.0% and 14.3%. Fluctuations in diploidization rates could be explained by the inequality in the number of surviving plants (Table 2).

Treatment of embryoids: After 20 days of culture with 20.7% response, embryoids were observed in the anthers and were treated with 0.2% colchicine for 3 days. Following transfer to fresh medium only 5 plants were generated from 10 treated anthers and 3 of them were found to be fertile diploid. Thus the diploidization was 60%. Dai et al. (16) stated that they obtained 60% diploids in a similar study.

Treatment of androgenic plantlets: After treatment of plantlets with colchicine for 7 to 48 h, they were rinsed and planted into pots. Following transfer, some differences were observed between treated plantlets and untreated controls. Treated ones grew slower and survivors' rate decreased from 81.3% to 14.1% as the treatment duration increased (Table 3).

Chromosome counts revealed the occurrence of spontaneous diploids as high as 4.3% and thus the percentage of haploids in the control group was found to

Table 2. Number of plants grown from colchicine treated anthers and the percentages of fertile diploids.

Treatments (h)	No. of plantlets transferred to pots	No. of plants surviving	No. of plants not flowering	Fertile diploids	
				No.	%
Control	296	166	24	2	1.41
2	51	25	2	2	8.7
4	-	-	-	-	-
6	72	51	14	11	29.7
8	1	1	-	-	0.0
10	3	3	1	-	0.0
12	26	13	6	1	14.3

Table 3. Number of androgenic plantlets treated with 0.2% colchicine for various durations and number and percentages of survivors after transfer to pots.

Treatments (h)	No. of plantlets treated and transferred to pots	Surviving Plants	
		No.	%
Control	64	52	81.3
7	231	121	52.4
24	237	86	36.3
48	269	38	14.1

be 95.7% (Table 4) (Figure 2). Frequencies of diploids were 42.3%, 37.8%, and 33.3% for 7, 24, and 48 h treated plantlets, respectively (Table 4) (Figure 3). However, tetraploids with 2n = 96 chromosomes and/or aneuploids at the tetraploid level were also found among the treated plants (Figure 4). When the total number of tetraploids, i.e. tetraploids plus aneuploids at the tetraploid level, are considered, 7, 24 and 48 h treated groups had 12.1%, 2.7%, and 13.2% tetraploids, respectively. These frequencies are higher than those of the spontaneous diploids observed and imply occasional 2 successive doubling of chromosomes.

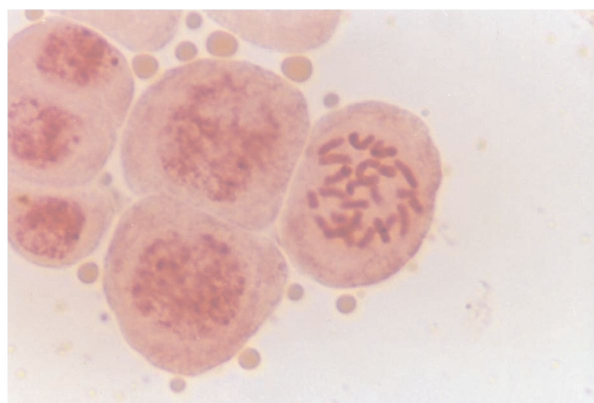

Figure 2. 2n = 24 chromosomes in a leaf cell from a haploid plant. (×1000)

The occurrence of mixedploid plants among 24 and 48 h treated ones were 5.4% and 6.6%, respectively. Different ploidy levels, aneuploids, mixedploids, and chimeras have been reported for colchicine (13), acenaphthene (22), and trifluralin (28) treated androgenetic haploid tobacco plantlets. These results underline the necessity of the selection following treatments with antimitotic agents, including colchicine.

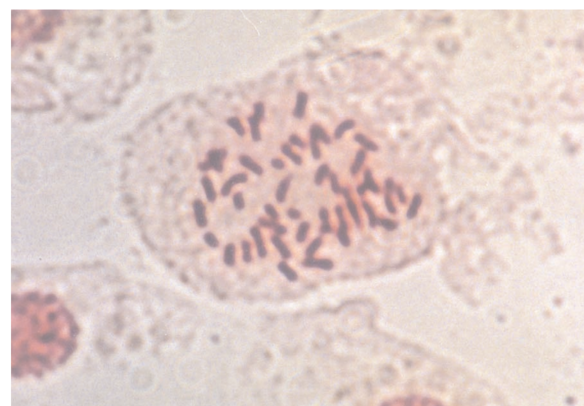

Figure 3. 2n = 48 chromosomes in a leaf cell from a dihaploid plant (×1000)

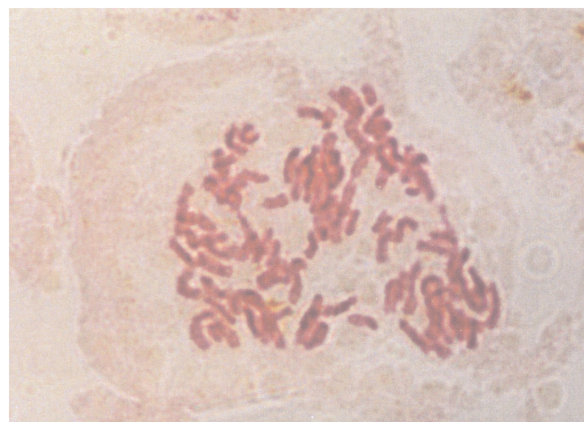

Figure 4. 2n = 96 chromosomes in a leaf cell from a tetraploid plant (×1000)

Table 4. Ploidy levels of plants generated from androgenic plantlets treated with colchicine for various durations.

Treatments (h)	Haploid (%)	Diploid (%)	Tetraploid (%)	Aneuploid *(%)	Mixedploid (%)
Control	95.7	4.3	-	-	-
7	27.3	42.3	-	18.2(d)-12.1(t)	-
24	32.4	37.8	-	8.1(h)-13.5(d)-2.7(t)	5.4
48	20.0	33.3	6.6	26.6(d)-6.6(t)	6.6

*(h) haploid
*(d) diploid
*(t) tetraploid

In conclusion, colchicine treatments tested exhibited toxicity to plant cells by reducing anther culture response, plant generation from embryoids, and survival of plantlets. When chromosome doubling rate and viability are taken into consideration, among the 3 methods tested, plantlet treatment with 0.2% colchicine for 7 h appeared to be more efficient; therefore, durations shorter than 7 h must be tested in order to optimize the application. Because of the occurrence of tetraploids, aneuploids, and mixedploids among treated plants, dihaploids with 2n = 48 chromosomes have to be selected by chromosome counting, and progeny tests are necessary prior to including into research programs.

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