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In Vitro Induction of Polyploidy in *Citrus reticulata* Blanco

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Abstract

Possibility of polyploidy induction by colchicine in "Balady" mandarins was investigated *in vitro*. Seeds were immersed in different concentrations of colchicine solutions (0.01%, 0.05%, 0.1% and 0.2%) for different durations (12, 24 and 48 hr), then cultured *in vitro* on MS medium at half strength. Seed survival percentage decreased by increasing colchicine concentration and duration of treatment showing the lowest percentage at 0.2% for 48 hr. The highest DNA content was recorded at 0.2% for 24 hr. Stomata No. per unit area was decreased by colchicine treatments; moreover stomata length and width were studied. The results indicated that colchicine treatment at 0.1% for 48 hr had the highest tetraploid induction efficiency percentage.

Keywords

Colchicine, DNA, Mandarin, Polyploidy, Tetraploid

1. Introduction

The phenomenon of polyploidy has played a vital role in the evolution of many crops. Some of the economically important plants whose triploids are in commercial use include several varieties of apple, bananas, mulberry, sugar beets, tea and watermelon [1]. In citrus and its relatives, there are a few known tetraploid and triploid types. The great majority of the species of Citrus, Fortunella and Poncirus are diploid, having 18 chromosomes [2]. Doubling of an entire chromosome complement may result in an increase of cell volume and consequently in an increase of plant parts. This can be a useful tool in breeding and selecting for larger fruit size [3].

Balady mandarin (*Citrus reticulata* Blanco) is one of the major citrus cultivars grown in Egypt. Besides its several merits, there are certain demerits like alternate bearing, loose skin and high number of seeds. Seedlessness, which is a desirable characteristic for the fresh fruit market [4], can be induced in citrus by crossing

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tetraploid and diploid strains [5]-[7]. However, desirable autotetraploid that can be used in such crosses is still limited. In this regard, induction of more autotetraploids will facilitate recovery of triploid via interploid crosses. Stomata density and size are used as markers in differentiation of diploids and tetraploids [8]-[11]. Diploid citrus had numerous and densely arranged stomata while tetraploids had larger and widely spaced stomata [12].

Since colchicine being discovered in 1930s, it has been widely used for chromosome doubling in a variety of plant species leading to the production of novel germplasm that can be used as bridging materials or as direct commercial cultivars [13]. However, the responsiveness of cells is genotype dependant and influenced, to a varying degree, by numerous biological, environmental and chemical factors [14].

The objective of this study was to investigate the possibility of inducing polyploidy in Balady mandarin *in vitro* by colchicine in order to improve this variety.

2. Materials and Methods

2.1. Plant Materials and Colchicine Application

Seeds of "Balady" mandarins were collected from mature fruits, washed up with tap water and left to dry. The seeds were divided into 13 groups, twelve of which were immersed in different concentrations of colchicine solutions (0.01%, 0.05%, 0.1% and 0.2%) for different time durations (12, 24 and 48 hrs) and one group (Control) was immersed in distilled water for 12 hrs. After treating the seeds, it were surface sterilized under aseptic conditions by immersing it in 70% ethanol for 30 sec., followed by 50% Clorox (5.25% sodium hypochlorite, NaOCl) for 20 min. and rinsed three times in sterile distilled water for 5 min. per each.

The seeds were cultured *in vitro* on Murashige & Skoog (MS) medium [15] at half strength plus 15 g/l sucrose and 7 g/l agar for solidification. All cultures were incubated in a growth chamber at 27˚C ± 2˚C with a 16 hrs. photoperiod in 2200 - 2400 Lux light intensity.

Germination rate was monitored weekly and seed survival percentage was calculated after one month of the culture.

2.2. Stomata Properties

To study the number and size of stomata, the lower epidermis of the leaves was covered with a thin layer of clear nail polish and left to dry for few minutes to conform to the shape of leaf surface, then it was peeled off, placed on a microscope slide and a drop of safranine was added to stain the stomata, then a cover slip was placed over the peel. Each slide was examined under a light microscope (Leica DM 1000) equipped with a digital camera. Photos were taken under magnifying power of 400× and the photos were further processed for obtaining stomata number, length and diameter using Leica Image Manger software.

2.3. Cytological Examination

Root tips were collected from each treatment and washed thoroughly. The root tips were fixed in a fresh Carnoy's solution (1:3-glacial acetic acid: absolute ethanol) for 24 hrs at room temperature. Then roots were washed twice and stored in 70% ethanol in a refrigerator till being used for cytological examination according to Darlington and La cour [16]. At examination, the root tips were squashed in acetocarmine (2%) and cells were screened under a light microscope. Ploidy level of the plants was determined by chromosome counting in root cells**.** Tetraploid induction efficiency (TIE) percentage was calculated according toBouvier *et al*. [17] as follows: TIE% = seed survival% × tetraploid induction%/100.

2.4. DNA Extraction and Quantification

Genomic DNA was extracted from young leaves using the hexadecyltrimethylammonium bromide (CTAB) method according to Doyle and Doyle [18]. DNA concentration was quantified by measuring absorbance at 260 nm wavelength using Ultrospec 1000 UV/Vis spectrophotometer, Pharmacia, Biotech and calculated according to Sumbrook *et al*. [19].

2.5. Data Analysis

The obtained data were statistically analyzed by subjecting to analysis of variance (ANOVA) according to

Snedecor and Cochran [20] using MSTAT program and LSD used to compare among means of treatments according to Duncan [21] at probability of 5%.

3. Results and Discussion

Data in **Table 1** shows that seed survival percentage decreased by increasing colchicine concentration and the exposure time under the same concentration compared to the control. The control recorded the highest seed survival percentage (92.3%) and the lowest percentage (37%) was recorded under 0.2% colchicine for 48 hrs.

Comparing the effect of the concentration regardless the time, the results clear that the survival percentage decreased by increasing the colchicine concentration (**Figure 1**). Germination inhibition caused by colchicine treatments maybe due to the toxic effect of colchicine and this toxicity is proportional to the concentration and exposure duration. Zeng *et al*. [22] reported that colchicine decreased protoplast viability, delayed protoplast division and inhibited callus growth indicating presence of toxicity to cells on kumquat and navel orange. Moreover, Sanford [23] stated that if the solution is too concentrated or the duration of treatment too long, a high portion of the meristems will be killed.

Table 1. Effect of colchicine treatments on the percentage of seed survival, ploidy and tetraploid induction efficiency of Balady mandarin seeds.

Treatment	Seed survival %	Seedling ploidy %		(TIE)Tetraploid induction efficiency %*
		Diploid (2n)	Tetraploid (4n)	
Control	92.3	100	0	0
0.01% for 12 hr	88.6	95.3	4.7	4.16
24 hr	87.26	95.1	4.9	4.27
48 hr	85.86	91.3	8.7	7.46
0.05% for 12 hr	82.92	87.4	12.6	10.44
24 hr	70.25	81.3	18.7	13.13
48 hr	66.40	79	21	13.9
0.1% for 12 hr	65.0	64.1	35.9	23.33
24 hr	62.5	61.3	38.7	24.18
48 hr	61.09	44.7	55.3	33.78
0.2% for 12 hr	54.42	43.2	56.8	30.9
24 hr	52.25	41.1	58.9	30.77
48 hr	37.0	42.3	57.7	21.34

*Tetraploid induction efficiency % = seed survival % × tetraploid induction %/100.

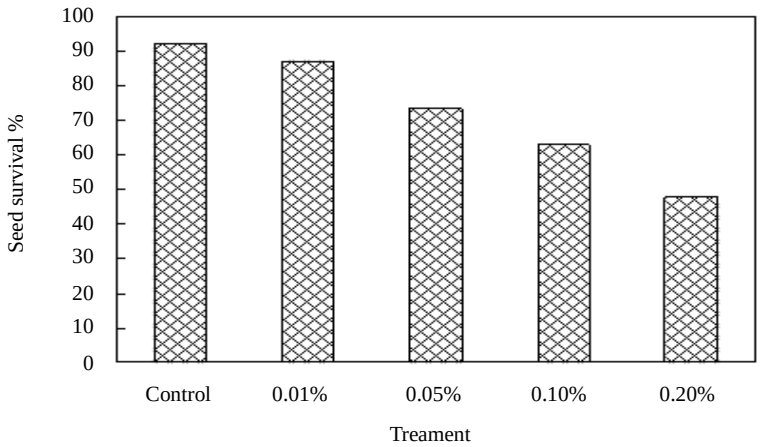


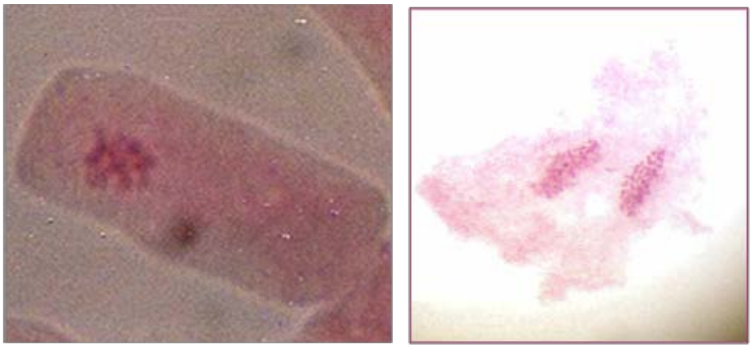
Figure 1. Seed survival percentage as affected by colchicines.

Regarding the effect of colchicine treatments on ploidy induction percentage, the results show a tendency of increasing tetraploid percentage by increasing the concentration and duration of exposure. The highest tetraploid percentage was obtained at 0.2% for 24 hrs (**Table 1**). The most efficient treatment in tetraploid induction was 0.1% for 48 hrs which achieved 33.78%.

The cytological studies of root tips by chromosome counting, which is the only reliable method to confirm the ploidy level [24] revealed the occurrence of tetraploid as shown in **Figure 2**.

DNA content in the leaves generally tended to increase significantly by increasing the colchicine concentration and the time of exposure (**Table 2**). The highest content was recorded at the treatment of 0.2% colchicine for 24 hrs. recording 207.1 µg/ml. However, there was no significant difference among 0.05% for 48 hrs, 0.1% for 24 hrs and 0.2% for 48 hrs and neither between 0.1% for 48 hrs and 0.2% for 48 hrs. Considering the concentration effect regardless the time, DNA highest value was found under 0.2% colchicine and the control was the lowest. Other studies proved that by colchicine treatment, the tetraploid cells contained DNA almost two times more than the diploid ones [22] [25] [26].

Table 3 shows that the stomata number per unit area affected significantly by colchicine treatment. Stomata



2n 4n

Figure 2. Chromosome number in diploid (2n = 18) and tetraploid (4n = 36) of Balady mandarin plants.

Table 2. Effect of colchicine on DNA content (µg/ml) of Balady mandarin leaves.

Treatment duration	Colchicine concentration (%)				
	Cont.	**0.01**	**0.05**	**0.1**	**0.2**
12 hr	111.7 k	121.7 j	137.1 g	196.1 e	203 .0 b
24 hr		125.3 i	168.9 f	198.1 d	207.1 a
48 hr		131.7 h	198.9 d	200.0 c	199.3 cd
Mean	**111.7 e**	**126.2 d**	**168.3 c**	**198.1 b**	**203.1 a**

Means followed by the same letter are not significantly different at 5% level by DMRT.

Table 3. Effect of colchicine on stomata number in 1 mm^2 of Balady mandarin leaves.

Treatment duration	Colchicine concentration (%)				
	Cont.	**0.01**	**0.05**	**0.1**	**0.2**
12 hr	9.00 a	8.50 ab	7.17 c	6.11 d	6.56 cd
24 hr		8.29 b	6.71 cd	6.05 d	5.33 e
48 hr		8.06 b	5.05 e	4.12 f	6.10 d
Mean	**9.00 a**	**8.28 b**	**6.31 c**	**5.43 d**	**5.99 c**

Means followed by the same letter are not significantly different at 5% level by DMRT.

number decreased by increasing colchicine concentration and exposure time to record the lowest value under the treatment of 0.1% for 48 hrs.

The highest stomata length was found at 0.2% for 12 hrs, while the lowest ones were recorded under the control (**Table 4**). The differences among treatments were statistically significant.

Concerning stomata width, data in **Table 5** shows that stomata width recorded the highest significant values under 0.1% for 12 and 48 hrs (128.3 and 128.2 µm, respectively) followed by 0.2% for 12 hrs. whereas, the control and 0.01% for 24 hrs showed the lowest ones. **Figure 3** shows the differences between the diploid and tetraploid in stomata dimension.

Similar results were found on different citrus species and varieties as a result of colchicine treatment [9] [22] [25]-[28], where they found that stomates of diploid citrus appears as numerous, densely arranged, while stomates of tetraploids were larger and spaced more widely.

Table 4. Effect of colchicine on stomata length (µm) of Balady mandarin leaves.

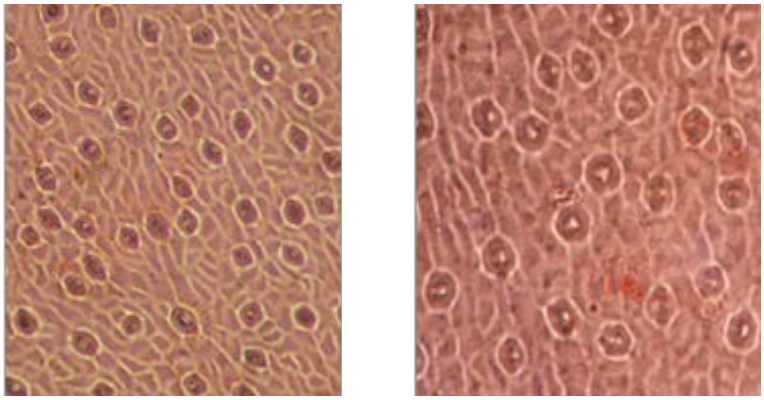
Treatment duration	Colchicine concentration (%)				
	Cont	**0.01**	**0.05**	**0.1**	**0.2**
12 hr	103.2 i	103.7 i	111.8 g	142.2 b	146.0 a
24 hr		107.8 h	138.3 c	130.0 e	133.6 d
48 hr		114.1 f	130.6 e	135.0 d	135.4 d
Mean	**103.2 e**	**108.5 d**	**126.9 c**	**135.7 b**	**138.3 a**

Means followed by the same letter are not significantly different at 5% level by DMRT.

Table 5. Effect of colchicine on stomata width (µm) of Balady mandarin leaves.

Treatment duration	Colchicine concentration (%)				
	Cont	**0.01**	**0.05**	**0.1**	**0.2**
12 hr	96.10 j	88.67 k	99.6 i	128.3 a	125.4 b
24 hr		96.3 j	116.8 f	111.6 g	120.3 d
48 hr		103.7 h	118.2 e	128.2 a	122.3 c
Mean	**96.10 c**	**96.21 c**	**111.5 b**	**122.7 a**	**122.7 a**

Means followed by the same letter are not significantly different at 5% level by DMRT.



2n 4n

Figure 3. A photo shows the difference in stomata density and dimension between the 2n and 4n of Balady mandarin plants. Magnifying times = 400×.

4. Conclusion

In conclusion, treating seeds of Balady mandarin with colchicine at 0.1% for 48 hr had the highest tetraploid induction efficiency percentage. The obtained tetraploid strains are a source of genetic diversity and have a great potential in breeding programs to create seedless/triploid cultivars thereafter.

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Abbreviations

Tetraploid induction efficiency (TIE)
hexadecyltrimethylammonium bromide (CTAB)
Murashige & Skoog medium (MS)