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INDUCTION OF POLYPLOIDY IN WATERMELON GENOTYPE WITH POWDERY MILDEW RESISTANCE (*Podosphaera xanthii*)[1]

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ABSTRACT - Triploid watermelon is highly appreciated by the most demanding markets, and due to its small size, it is ideal for consumption by small families. With the growth in areas cultivated with seedless watermelon worldwide, there is a demand for the development of tetraploid germplasm to obtain triploid hybrids with better agronomic performance. This study performed two tests to induce polyploidy in the powdery mildew–resistant line developed by Embrapa Semi-Arid, LDRO, under different colchicine concentrations and application methods. In Experiment 1, the seeds were treated with colchicine (0.0%, 0.1%, and 0.2%) for 24 h and 48 h. In Experiment 2, 0.2% colchicine was applied by different methods: (a) directly on the seed (MDS) with and without scarification, (b) on seeds with radicle emission (MER), (c) at the insertion point between the hypocotyl and the root (MIHR), (d) at the seedling apex (MAP), and (e) in the inverted hypocotyl (MHI). Chromosome count (cytogenetic analysis), number of chloroplasts per pair of guard cells, number of stomata, seedling height, and hypocotyl diameter were measured. In the LDRO line, chromosomal duplication occurred in some plant cells, but it was not possible to obtain 100% tetraploid plants. Colchicine (0.2%) for 48 h without mechanical scarification induced chromosomal duplication in watermelon. The analysis of the number of chloroplasts identified the level of ploidy early, reducing the number of plants needed to be evaluated by cytogenetics, which allowed us to more accurately identify the different levels of ploidy of the plant.

Keywords: Chromosomal duplication. Tetraploidy. Cytogenetics. *Citrullus lanatus.*

INDUÇÃO DE POLIPLOIDIA EM GENÓTIPOS DE MELANCIA COM RESISTÊNCIA AO OÍDIO (*Podosphaera xanthii*)

RESUMO - A melancia triploide é muito apreciada pelos mercados mais exigentes, e, devido ao seu tamanho reduzido, torna-se ideal para consumo em famílias pequenas. Com o incremento de áreas cultivadas com melancia sem sementes no mundo, há demanda pelo desenvolvimento de germoplasma tetraploides para obtenção de híbridos triploides de melhor desempenho agronômico. Foram realizados dois ensaios com o objetivo de induzir poliploidia na Linhagem resistente ao oídio desenvolvida pela Embrapa Semiárido, LDRO, sob diferentes concentrações de colchicina e métodos de aplicação. As sementes foram tratadas com colchicina (0,0%; 0,1% e 0,2%) por 24h e 48h (Experimento 1) e sob diferentes métodos de aplicação de colchicina a 0,2% a) direto na semente (MDS) com e sem escarificação, b) sementes com emissão da radícula (MER), c) no ponto de inserção entre o hipocótilo e a raiz (MIHR), d) no ápice da plântula (MAP) e e) no hipocótilo invertido (MHI) (Experimento 2). Foi realizada a análise citogenética (contagem do número cromossômico), do número de cloroplastos por pares de células-guardas, número de estômatos, altura da plântula e diâmetro do hipocótilo. Na Linhagem LDRO, a duplicação cromossômica ocorreu em algumas células das plantas, mas não foi possível obter plantas 100% tetraploides. A colchicina a 0,2% por 48h sem escarificação mecânica induz a duplicação cromossômica em melancia. A análise do número de cloroplastos identifica os níveis de ploidia, de forma precoce, reduzindo o número de plantas, que serão avaliadas em citogenética, sendo que esta permite identificar com maior precisão os diferentes níveis de ploidia da planta.

Palavras-chave: Duplicação cromossômica. Tetraploidia. Citogenética. *Citrullus lanatus.*

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INTRODUCTION

Watermelon is one of the most representative fruits in Brazil. In 2019, its production was 2,278,186 t of fruit, and the harvested area was 98,489 ha, with an average yield of 23,131 kg/ha (IBGE, 2019). In general, the cultivars most planted in Brazil are very similar in appearance to the 'Crimson Sweet' variety. These cultivars have large, round fruits, weighing more than 10 kg, with a dark-green and striped peel, red pulp, and high soluble solid content (11-13 °Brix) (LIMA et al., 2018; DIAS; SANTOS, 2019). The main exported variety from Brazil is the seedless mini watermelon, whose main destinations are the European Union (Germany, Spain, Italy, France), Mercosur (Argentina, Bolivia, Uruguay) and Russia (MDIC/SECEX, 2018), and the demand for this fruit is growing. This type of watermelon is highly appreciated by more demanding markets, and due to its small size, it is ideal for consumption by small families. With the high demand for seedless watermelon in different countries, there is a need to develop tetraploid germplasms to obtain triploid hybrids with better agronomic performance.

The pests and diseases that occur in the cultivation of watermelon with frequency and severity can make its production unfeasible. For example, anthracnose, fusariosis, and powdery mildew are diseases caused by fungi that cause high production losses (CARVALHO, 2005). Powdery mildew (*Podosphaera xanthii*) is an important leaf disease of watermelon and other cultivated or wild cucurbits in Brazil and worldwide (STADNIK; RIVERA, 2001). In the culture of watermelon in a hot and dry environment, such as in semiarid regions, a complete cycle of the disease takes in 7 days; thus, epidemics can occur quickly because it is a polycyclic disease. Powdery mildew is favored by average temperatures between 20-27 °C and relative humidity between 50-70% (DAUGHTREY et al., 2017). Symptoms appear throughout the shoot, but the leaves are the most affected. The disease is difficult to control by conventional methods, and one of the most efficient ways to prevent infection is the use of tolerant or resistant cultivars. The search for watermelon cultivars resistant to its main diseases is constant in breeding programs, as is the effort to develop seedless hybrids and cultivars with smaller fruits, which is also a trend in the current market, especially for export. In the watermelon breeding program developed by Embrapa Semi-Arid, there is a diploid lineage with resistance to powdery mildew, ES 17.31620.001 (LDRO), with a fruit mass of approximately 4 kg, which can be subjected to chromosomal duplication to yield aggregates in the same tetraploid genotype with resistance to powdery mildew and smaller fruit.

Polyploidy induction technology is one of the tools widely used in genetic improvement programs to obtain plants with better development. In addition, fertility restoration can be provided in interspecific and intergeneric hybrids and as a bridge for gene transfer between different ploidy levels (CHEN; COATE; MERU, 2020). In watermelon culture, this technology is applied in the process of obtaining tetraploid plants (4n = 44) that are used for the production of triploid hybrids or seedless watermelons (NOH et al., 2012). However, methods aimed at obtaining tetraploid plants have low inefficiency Souza et al. (2001). In addition, the low number of tetraploid lines and their lack of diversity hinder breeding programs associated with the low percentage of fully tetraploid plants generated, seed germination problems, low seedling recovery during growth, and too few seeds for the development of triploid cultivars (SHEIKH et al., 2013).

The method for obtaining tetraploid plants was initially established by Kihara (1951) by using colchicine at different concentrations. However, other studies were performed with this antimitotic to obtain tetraploid plants to establish the dosage, application time, the different methods of application of the product, and the different parts of the plant it could be applied to (JASKANI et al., 2005, NOH et al., 2012, SHEIKH et al., 2013). In Brazil, Souza et al. (2001) developed watermelon triploid hybrids by applying 0.2% colchicine for 24 h to the seeds of diploid lineages and identified them by counting chloroplasts, guard cells of the leaf stomata, and the morphology of their progenies. After this, there are no more records in the literature of triploid development in watermelon.

Therefore, this study aimed to evaluate the induction of artificially polyploid watermelon plants using different concentrations of colchicine at two exposure times and different application methods.

MATERIALS AND METHODS

The experiments were conducted at Embrapa Semiárido in the municipality of Petrolina, PE, Brazil, from December 2016 to November 2017. Polyploidy induction was performed with colchicine (Lot # SLBQ8131 V-Sigma®, manufactured on 04.05.2016), applied to the diploid watermelon line ES 17.31620.001 (LDRO) developed by Embrapa Semiárido, which have powdery mildew resistance. Two experiments were performed to establish the best dosage and exposure time to colchicine for the LDRO line (Experiment 1) and the best method of application of colchicine (Experiment 2).

In Experiment 1, the design was completely randomized in a 3×2 factorial arrangement (three concentrations of colchicine: 0.0%, 0.1%, and 0.2%; and two exposures to antimitotic agents: 24 h and 48 h), totaling six treatments, with 14 replicates, and the experimental unit was one plant. The watermelon seeds were subjected to treatments with colchicine

and kept in the dark at room temperature. Then, the seeds were washed three consecutive times and placed in sown plastic containers (0.5 L) perforated in the bottom base, containing commercial substrate for vegetables.

In Experiment 2, the experimental design was completely randomized, with eight treatments, 10 replicates, and an experimental of one plant. The watermelon seeds and seedlings of the LDRO line were subjected to 0.2% colchicine using different product application methods, adapted from Medina et al. (1958) and Noh et al. (2012). The treatments consisted of eight colchicine application methods (T1-T8). In the direct application to the seed (MDS), the watermelon seeds of the LDRO line were immersed in distilled water (0.0% colchicine) without scarification (T1) or with scarification (T2) or in 0.2% colchicine solution for 48 h without scarification (T3) or with scarification (T4). For scarification, 150-grit sandpaper was used to open a small hole in the upper region of the tegument (opposite the radicle emission site). In the application on seeds with radicle emission (MER) (T5), the seeds were germinated in Petri dishes, lined with two sheets of moistened paper, in a biochemical oxygen demand incubator at 30 °C, and when the radicle was emitted, they were placed in 0.2% colchicine solution for up to 6 h. In the application at the insertion point between the hypocotyl and the root (MIHR) (T6), the watermelon seedlings with expanded cotyledons were treated with three drops of 0.2% colchicine at the point between the hypocotyl and the root. Then, the seedlings were transferred to glass jars containing cotton and filter paper moistened with distilled water, autoclaved, sealed with aluminum foil and plastic film, and incubated at 30 °C for 15 h in the dark. In the method of application at the apex of the seedling (MAP) (T7) and in the method of immersion of the aerial part of the seedling (MHI) (T8), the seeds of watermelon line LDRO were placed to germinate in 500-mL plastic containers that were perforated and contained substrate for vegetables. When the seedlings had fully expanded cotyledons, they were treated with 0.2% colchicine. In T7, three drops of the antimitotic were applied at the growth point of each seedling, in which the first drop was applied, 9 h later the second drop, and 15 h later the third drop. Thirty minutes after the last application, the plants were put in a greenhouse. In T8, the shoots of all seedlings were immersed in 0.2% colchicine solution two consecutive times. Next, the seedlings were placed in glass jars as described for the MIHR method and then incubated at 30 °C for 15 h in the dark.

In all treatments, after the established time, the seeds and seedlings were washed three times in a row and placed in 500-mL plastic containers that were perforated and contained commercial substrate for vegetables. They remained in a greenhouse as in Experiment 1.

The ploidy level of LDRO plants treated and not treated with colchicine was evaluated by the number of stomata and chloroplasts and cytogenetic analysis. The numbers of stomata and chloroplasts in the two guard cells were counted both in the cotyledons (Experiments 1 and 2) and in the second definitive leaf (Experiment 2). The procedure consisted of removing the cuticle present in the lower epidermis of the leaf. The cuticle was placed on a slide with a drop of distilled water, covered with a coverslip, and observed under a Leica optical microscope. To count the stomata (No./µm) and chloroplasts, the cuticle was observed under 20× and 40× objectives, respectively, with the aid of DinoCapture 2.0 software.

The number of chloroplasts was randomly evaluated in the guard cells of 10 stomata and the mean calculated. The plants were preliminarily classified as diploid (<16), tetraploid (16-20), and with different levels of ploidy (> 20) (COMPTON et al., 1996, MCCUISTION; ELMSTROM, 1993, SOUZA et al., 2001 and NOH et al., 2012). The seedling height was measured with a millimeter ruler, and the diameter was determined using a digital caliper. Both variables were measured 35 days after the treatments.

For the cytogenetic analysis (mitotic), on the 12th day after the application of the different treatments, the root tips (primary and secondary) were collected and immediately pretreated with 8-HQ at 0.002 M, then kept at approximately 8 °C for up to 24 h. Then, they were fixed according to the protocol established by Guerra and Souza (2002). The best cells were captured with a Leica FX-350 camera using Leica QFish software coupled to a Leica DM2000 epifluorescence microscope. The chromosome number was counted in five cells that were clearly in metaphase and had well-scattered chromosomes.

The data obtained were subjected to the Shapiro–Wilk normality test and Bartlett's homogeneity of variances at 5% probability. The variables with normal distribution and homogeneity of variances were subjected to analysis of variance (ANOVA), and the means were compared by Tukey's test at 5% probability. On the variables that did not meet the assumptions of ANOVA, nonparametric statistics were performed: the Kruskal –Wallis test at 5% probability for single comparisons and the Dunn.test package for multiple comparisons (DINNO, 2017). All analyses were performed using the statistical software R version 3.3.2 (R CORE TEAM, 2016).

RESULTS AND DISCUSSION

In Experiment 1, the exposure time to colchicine did not influence the number of

chloroplasts in the plants, but the concentration did: there were significantly more chloroplasts in the cotyledons, by 24.4% and 48.2%, under colchicine concentrations of 0.1% and 0.2%, respectively (Table 1). Importantly, the number of chloroplasts in diploid and mixoploid plants may vary depending on the genotype. Jaskani et al. (2005) found, in different watermelon lines, five to seven chloroplasts in each guard cell in diploids and 10 to 12 in tetraploids. Koh (2002) reported in watermelon 'Moodeungson' 12 (diploids) to 22.8 (tetraploids) chloroplasts per stomata and, in some cases, values lower than eight or 13-15, in plants considered mixoploids, which was confirmed by flow cytometry.

Table 1. Number of chloroplasts in the two guard cells (NCL) and number of stomata in LDRO watermelon seedlings subjected to polyploidy induction with different concentrations and immersion times of colchicine (Experiment 1) and different colchicine application methods (Experiment 2).

[1]Experiment 1

Colchicine (%)	** NCL			*Number of stomata (No./µm)		
	Exposure time (h)			Exposure time (h)		
	24	48	Mean	24	48	Mean
0.0	11.7	11.9	11.8 b	42.6	42.9	42.7 a
0.1	13.9	15.5	14.7 a	42.8	35.5	38.9 a
0.2	15.3	19.8	17.5 a	41.2	36.9	39.0 a
Mean	13.5 A	15.7 A		42.2 A	38.4 A	
CV (%)	-	-		21.6		

[2]Experiment 2

IT method	NCL		*Number of stomata (No./µm)	
	** Cotyledons	*2nd. Final leaf	Cotyledons	2nd. Final leaf
0.0 MDS SE	12.7 de	10.0 cd	42.4 ab	21.6 ab
0.0 MDS CE	12.4 e	9.6 d	42.6 ab	24.6 ab
0.2% MDS SE	21.5 b	14.4 ab	23.2 d	33.1 a
0.2% MDS CE	22.3 b	15.4 a	19.8 d	23.9 ab
0.2% MER	26.1 ab	16.6 a	26.6 cd	28.8 ab
0.2% MIHR	13.7 cd	10.6 cd	51.8 a	31.0 ab
0.2% MAP	13.0 cde	12.5 bc	42.1 ab	16.5 b
0.2% MHI	14.9 c	16.2 a	37.9 bc	31.2 ab
CV (%)	-	13.5	22.3	35.7

Means followed by the same lowercase letter in a column do not differ by *Tukey's test or **Dunn's test at 5% significance. [1]Characteristics evaluated in cotyledons, [2]characteristics evaluated in the 2nd definitive leaf. IT = induction of tetraploidy, MDS SE = direct application to seed without scarification, MDS CE = direct application to seed with scarification, MER = application to seeds with radicle emission, MIRH = application at the insertion point between the hypocotyl and the root, MAP = application at the apex of the seedling, MHI = application by immersion of the shoot or inverted hypocotyl.

In Experiment 2, the analyses on the cotyledons and on the second definitive leaf revealed a significant effect between the methods of colchicine application on the number of chloroplasts and stomata (Table 1). The application of 0.2% colchicine to seeds with radicle emission (MER) resulted in an increase of 105.5% in the number of chloroplasts. When 0.2% colchicine was directly to the seed with or without scarification, the number of chloroplasts increased 79.7% and 69.4%, respectively, in tetraploids (16 and 20 chloroplasts/ guard cells). The treatments with the highest number of chloroplasts in the final leaf were those given 0.2% colchicine on the seed (SE and CE), in the inverted hypocotyl, and on seeds with radicle emission (Table 1). The latter group most closely approached the number of chloroplasts it contained in the guard cells of tetraploid plants (16.0 to 20.0).

The number of chloroplasts is a simple and efficient indicator of chromosome duplication during the initial screening phase. According to Jaskani et al. (2005) and Noh et al. (2012), it must be accompanied by other, more efficient methods of determining the level of ploidy, such as cytogenetics and flow cytometry, but has been used by several authors to identify tetraploid watermelons. Souza et al. (2001) and Compton et al. (1996) reported that counting the number of chloroplasts helped them obtain tetraploid plants, which were crossed with diploid pollinators, thus generating triploid hybrids.

However, Noh et al. (2012), working with different methods to obtain tetraploid plants in five

watermelon cultivars, compared the analysis of the number of chloroplasts in leaf stomata with flow cytometry analysis in 649 plants and preliminarily found 28.7% and 10.9% tetraploid plants, respectively. Later flow cytometry confirmed 25.2% tetraploidy. Thus, the analysis of chloroplasts allows fewer samples to be evaluated by more laborious and time-consuming methods, such as cytogenetics and flow cytometry. In addition, chloroplast analysis in cotyledons can anticipate the identification of possible tetraploid plants, reducing the number and area occupied by self-fertilized plants.

The number of chloroplasts in the guard cells of cotyledons and definitive leaves may be similar or different, depending on the genotype. In the present study, the number of chloroplasts in the guard cells of the definitive leaf decreased in almost all treatments evaluated, while the guard cells of the cotyledons showed more chloroplasts, especially when the seeds were immersed in 0.2% colchicine solution for 24 h or 48 h. In these groups, changes in the structure of the stomata (deformation) were also observed. This may have been caused by the presence of colchicine during the initial germination phase, potentiating the reagent effect and thus causing toxicity.

In Experiment 1, stomata number was not influenced by the concentration of or exposure time to colchicine, but in Experiment 2, it varied in both the cotyledons and the definitive leaf (Table 1). In the cotyledons, there were fewer stomata when colchicine was applied to the seeds with radicle emission and to seeds with and without scarification, the latter with reductions of 53.6% and 45.3%, respectively. In the final leaf, the number of stomata of the plants treated with colchicine was similar to that of the control plants (Table 1).

The stomatal density is indicative of polyploidization since polyploid plants usually exhibit a lower density than diploids. However, this is a characteristic that can be influenced by the environment and may be biased by subjectivity in the individual analysis (SOUZA; QUEIRÓZ, 2004). In addition, in the present study, the finding of a similar response of the numbers of chloroplasts and stomata in cotyledons between diploid (control) and polyploid plants in treatments where colchicine was applied to seedlings (MIHR, MAP, and MHI) may be due to the more efficient action of colchicine in tissues that are undergoing active cell division. In this case, the cotyledons that have already formed translocate reserve substances to the growth points of young plants (RAVEN; EVERT; EICHHORN, 1996), such as to the apical meristem. Thus, there was probably not enough colchicine left in the cotyledons to cause changes in the number of stomata and chloroplasts. Such changes were observed in the cotyledons of the treatments where colchicine was applied to the seed (MDS and MER), in which the cotyledons were still in the process of cell division, which is probably the most efficient way to induce polyploidy. However, the drop method in the insertion point between the hypocotyl and the root may not have worked due to the fewer cells actively dividing in this site, in addition to the short time of contact of the product with the tissue.

In the LDRO line in Experiment 1, the variables plant height and hypocotyl diameter were influenced by the concentration of colchicine and the exposure time alone (Table 2). The 0.2% concentration of colchicine and the time of 48 h influenced the seedling diameter, increasing it by 12.7% and 8.3%, respectively. On the other hand, plant height was not influenced by any factor. Souza and Queiróz (2004) also found an increase in the morphological characteristics of tetraploid plants, confirmed later by obtaining triploid fruits.

In Experiment 2, when the LDRO watermelon line was subjected to different 0.2% colchicine application methods, significant differences were found in plant height and hypocotyl diameter (Table 2). For seedling height, it was found that all plants had their development negatively affected by colchicine, regardless of the method of application. The plants in which colchicine was applied to seeds with radicle emission for 6 h and by the inverted hypocotyl method had the lowest heights, by 53.6% and 51.6% compared to untreated plants, respectively.

Regarding the hypocotyl diameter of the seedlings, the MDS CE, MDS SE, and MAP groups stood out, ranging from 2.6 to 2.8 cm (Table 2). Colchicine interferes not only with cell division but also with plant metabolism (DERMEN, 1940) and, as already mentioned, affects the speed of germination and the initial development of seedlings, so it may reduce some growth characteristics, as observed in the present study. Although there were variations in the height and diameter of the hypocotyl of the seedlings subjected to colchicine in both experiments, these evaluations are important to perform when inducing polyploidy. Souza and Queiróz (2004) stated that morphological analysis enables the distinction between polyploid and diploid individuals and allows a considerable reduction in the number of plants subjected to cytogenetic analysis or progeny tests, reducing costs and accelerating the selection process.

Table 2. Height of seedlings and hypocotyl diameter in watermelons of the LDRO line subjected to polyploidy induction with different concentrations, immersion times (Experiment 1), and application methods of colchicine (Experiment 2).

Experiment 1		
Colchicine (%)	* Seedling height (cm)	* Hypocotyl diameter (mm)
0.0	59.0 a	4.8 b
0.1	58.9 a	4.9 b
0.2	62.2 a	5.4 a
Time (h)	- -	- -
24	63.6 a	4.8 b
48	56.6 a	5.2 a
CV (%)	27.5	9.99
Experiment 2		
Methods	* Seedling height (cm)	** Hypocotyl diameter (mm)
0.0 MDS SE	23.9 a	2.30 b
0.0 MDS CE	23.7 a	2.40 ab
0.2% MDS SE	14.3 bc	2.78 a
0.2% MDS CE	14.5 bc	2.73 a
0.2% MER	11.0 c	2.41 b
0.2% MIHR	15.9 b	2.35 bc
0.2% MAP	18.2 b	2.57 ab
0.2% MHI	11.5 c	2.19 c
CV (%)	16.2	23.4

Means followed by the same lowercase letters in a column and uppercase letters in a row do not differ by the *Tukey or **Dunn test at 5% significance. MDS SE = direct application on seeds without scarification, MDS CE = direct application on seeds with scarification, MER = application method on seeds with radicle emission, MIRH = application at the insertion point between the hypocotyl and the root, MAP = application at the apex of the seedling and MHI = application by immersion of the shoot or inverted hypocotyl.

When comparing the ploidy level of the LDRO line, determined by stomatal and cytogenetic analysis, we observed that in Experiment 1, by counting the number of chloroplasts, nine plants were considered to be tetraploids. However, chromosome number count analysis did not confirm these results, as plants with diploid, tetraploid, and mixoploid cells were observed (Table 3). The identification of tetraploid plants (2n = 44 chromosomes) by cytogenetic analysis of the roots is not always an indication that all plant cells' genomes have been duplicated (MEDINA et al., 1958). Several plants may have only one duplicated part, so only by knowing the chromosome number, stomata size, pollen grain size, fruit shape, and seed number and size can we conclude with much certainty that the treatment has had a partial or total effect.

Although cytogenetic analysis did not reveal fully tetraploid plants (Experiment 1), 70% and 50% of plants that were treated with 0.2% colchicine for 48 h and 24 h had tetraploid cells, respectively (Table 3). Souza et al. (2001), when treating seeds of three watermelon lineages with 0.2% colchicine solution for 24 h, deemed 2.7% of the plants tetraploids by morphological analysis and chloroplast count, and when these plants were crossed with diploid lines, they generated triploid hybrids. Handayani, Yusuf and Akmal (2018) also obtained good results in changing the chromosome number of watermelon plants (F_1 hybrid) by applying 0.2% colchicine for 48 h.

In Experiment 1, induction of mixoploid plants was observed, both by analysis of chloroplasts (0.2%/48 h) and by cytogenetics, which revealed mixoploid plants in all colchicine concentration and exposure time groups (Table 3). Inducing polyploidy is easy but obtaining fully tetraploid plants is very difficult. This is because colchicine acts efficiently only on dividing cells, so genome duplication generally does not happen in all cells of the treated material, and the appearance of mixoploids is common. Consequently, there is a problem regarding the total or partial reversal to the diploid condition after some division cycles, mainly due to the proliferation of the remaining diploid cells, which multiply at higher rates than tetraploid cells (ROTH, 1984). Therefore, the form of administration of colchicine has been greatly varied by authors seeking

a more efficient form of treatment. Once it is identified where in the plant the tetraploid cells predominate, by flow cytometry, techniques can be used to make them fully tetraploid, for example, performing successive selection cycles, pollination with male and female flowers from the same branch, and grafting.

Table 3. Classification of ploidy of plants by the number of chloroplasts (NCL) and by cytogenetic analysis, and the frequency of plants with tetraploid cells (FPCT), in the LDRO strain subjected to polyploidy induction with different concentrations, immersion times (Experiment 1), and application methods of colchicine (Experiment 2).

Experiment 1									
Colchicine (Concentration (%)/Application time)	Plant frequency as a function of ploidy								FPCT (%)
	NCL (cotyledons)				Cytogenetic analysis				
	2x	4x	>4x	4x(%)	2x	4x	>4x	4x(%)	
0.0/24 h	10	0	0	0	10	0	0	0	0
0.1/24 h	10	0	0	0	1	0	9	0	30
0.2/24 h	5	5	0	50	1	0	9	0	50
0.0/48 h	10	0	0	0	10	0	0	0	0
0.1/48 h	6	4	0	40	3	0	7	0	22
0.2/48 h	3	4	3	40	2	0	8	0	70
Total	44	9	3		27	0	33		-
Experiment 2									
*Methods of tetraploidy induction	Plant frequency as a function of ploidy								FPCT (%)
	NCL (Final leaf)				Cytogenetic analysis				
	2x	4x	>4x	4x(%)	2x	4x	>4x	4x(%)	
0.0 MDS SE	10	0	0	0	10	0	0	0	0
0.0 MDS CE	10	0	0	0	10	0	0	0	0
0.2%MDS SE	8	2	0	20	5	0	5	0	0
0.2% MDS CE	9	1	0	10	3	0	6	0	10
0.2% MER	3	2	0	0	2	0	2	0	0
0.2% MIHR	10	0	0	0	5	0	3	0	0
0.2% MAP	10	0	0	0	6	0	2	0	0
0.2% MHI	2	6	0	75	4	0	4	0	25
Total	62	11	0		45	0	22	0	-

*Samples = 10 plants/treatment; MDS SE = direct application on seeds without scarification, MDS CE = direct application on seeds with scarification, MER = application method on seeds with radicle emission; MIRH = application at the insertion point between the hypocotyl and the root, MAP = application at the apex of the seedling and MHI = application by immersion of the shoot or inverted hypocotyl.

When the LDRO line was subjected to different treatment methods with 0.2% colchicine (Experiment 2), we found, by counting the number of chloroplasts in the definitive leaves, that in the treatments with 0.2% MDS (SE and CE), MHI, and MER, a total of 11 plants had mean values of 16 to 20 chloroplasts per stomata, identifying these as possible tetraploids (Table 2). However, by counting the chromosome number, the induction of plants with tetraploid cells was not confirmed, but LDRO plants with diploid cells (2n = 22) and with different chromosomal numbers (mixoploids) were identified (Table 3).

Noh et al. (2012) applied the same methods used in this study to five watermelon genotypes and identified tetraploid plants by counting the number of chloroplasts. They confirmed tetraploidy by flow cytometry. The authors reported that the best method of induction was application to the inverted hypocotyl, which yielded 29.5% tetraploid plants. The different application methods induce different responses of tetraploidy induction in the different evaluated genotypes. In the present study, by the inverted hypocotyl (MHI) method, six plants were identified as possible tetraploids by chloroplasts, but the seedlings were not confirmed as such by counting the chromosome number in root tips (Table 3). However, as the treatment was performed in the shoots of the seedling, the findings suggest that the flow cytometry analysis for this case could confirm the ploidy level one way or the other.

Considering that the efficiency of colchicine treatment to induce tetraploidy varies according to factors such as genotype, method of application, and concentration, these are indispensable requirements in any breeding program for chromosomal

duplication. Thus, it is necessary to evaluate different application methods in the various available genotypes that present characteristics of interest for crop breeding programs. Additionally, we associate the different methods for ploidy analysis. As already mentioned, using only one evaluation method will not predict with much certainty whether a plant is tetraploid, since tetraploidy may not occur in a generalized manner in the plant. The sample collected at the root for cytogenetic analysis may not be representative of the ploidy of the plant as a whole. Confirmation of whether a row is homozygous for tetraploidy can only be achieved by evaluating the result of the cross between the possible tetraploid and the diploid parent, which will result in triploid plants, which will produce seedless fruits.

CONCLUSIONS

In the LDRO line, chromosomal duplication occurred in some plant cells, but it was not possible to obtain 100% tetraploid plants. Treatment with colchicine (0.2%) for 48 h without mechanical scarification induces chromosomal duplication in watermelon. The analysis of the number of chloroplasts identifies the level of ploidy early, reducing the number of plants, which will be evaluated in cytogenetics, and the cytogenetic evaluation allows us to more accurately identify the different levels of ploidy of the plant.

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