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COMPARISON OF PROTOCOLS FOR CHROMOSOME DUPLICATION IN HAPLOID MAIZE

Abstract – Different methods of chromosome duplication are used to obtain doubled haploids in maize; however, comparisons of efficiency among protocols have only begun to appear in the literature. This study aimed to compare the efficiency of three protocols of chromosome duplication. A randomized block experimental design was used, with three replications, composed of three treatments of chromosome duplication (duplication by colchicine injection in plants; duplication by immersion of roots in colchicine solution; and duplication by immersion of newly germinated seedlings in colchicine solution). Two hundred seventy haploid seeds were obtained from crossing the genotype source 91500212 with the haploid inducer hybrid Tail P1 × Tail P2, with 90 seeds for each treatment. In the chromosome duplication through injection, a colchicine solution at 0.125% was applied to the stem of the seedlings 15 days after germination. In the chromosome duplication through root immersion, 15 days after germination, roots of the seedlings were immersed in a 1% colchicine solution for six hours, followed by removing colchicine residues and transplanting them in trays with the substrate. In the chromosome duplication of newly germinated seedlings, three days old seedlings were immersed in a 0.06% colchicine solution for 12 hours, followed by removal from the solution and transplanting them in trays with the substrate. Twenty days after treatment, the plants were transplanted in 20-liter pots with fertilized soil in a greenhouse, beginning data collection. Results indicate that the injection protocol showed better survival, better practicality of application, and lower costs and risks.
Keywords: *Zea mays* L., production of lines, doubled haploids.

COMPARAÇÃO DE PROTOCOLOS PARA DUPLICAÇÃO DE CROMOSSOMOS EM MILHO HAPLÓIDE

Resumo - Diferentes métodos de duplicação cromossômica são utilizados para obtenção de duplo-haploides em milho, contudo comparações de eficiência entre protocolos ainda são incipientes na literatura. O objetivo deste trabalho foi comparar a eficiência de três protocolos de duplicação cromossômica. O delineamento experimental utilizado foi o de blocos ao acaso, com três repetições, comparando três tratamentos de duplicação cromossômica (injeção, tratamento via raízes e tratamento em plântula recém-germinada). Foram utilizadas 270 sementes haploides obtidas do cruzamento do genótipo-fonte 91500212 com o híbrido indutor de haploidia Tail P1 x Tail P2 sendo 90 sementes para cada tratamento. No tratamento de injeção, aplicou-se uma solução de colchicina a 0,125% no caule das plântulas, 15 dias após germinação. No tratamento via raízes, aos 15 dias após germinação, raízes das plântulas foram imersas em solução de colchicina 1%, por seis horas, seguindo-se remoção de resíduos de colchicina e transplantio para bandejas com substrato. No tratamento de plântula recém-germinada, plântulas com 3 dias de germinação foram imersas em solução de colchicina 0,06% por 12 horas, seguindo-se a remoção da solução e transplantio para bandejas com substrato. Vinte dias após tratamento, as plantas foram transplantadas para vasos de 20 litros com solo adubado, em casa de vegetação, iniciando-se a coleta dos dados. Os resultados indicam que o protocolo de injeção apresentou melhor sobrevivência, melhor praticidade de aplicação, com também menores custos e riscos.
Palavras chave: *Zea mays* L; Produção de linhagens; Duplo-haploides.

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The development of lines is a vital process in maize breeding, and it requires six to eight generations of self-fertilization to obtain lines with 99% homozygosity (Geiger & Gordillo, 2009). To reduce the time to obtain lines, maize breeding programs have adopted the doubled haploid (DH) technology, which produces homozygous lines in only two generations.

In the maize crop, for the haploid induction process *in vivo*, a haploid inducer genotype and a genotype source are crossed, aiming to obtain haploid seeds (n = 10). After that, these seeds are selected (around 6% to 10% of the seeds in each ear crossed with the inducer) based on phenotype markers, followed by chromosome duplication (Trindade, 2022).

The chromosome duplication (CD) aims to restore the parental chromosome number in haploid cells, mainly to restore fertility in maize genotypes, resulting in a homozygous line. Thus, developing an efficient CD protocol is essential for obtaining DH lines and colchicine protocols have been used most. Colchicine acts as an antimitotic agent, impeding the formation of achromatic spindle fibers during cell division and, consequently, segregation of the sister chromatids; that way, there is duplication of each chromosome previously present in the haploid cell (Prigge & Melchinger, 2012).

There are different protocols for the use of colchicine aiming at chromosome duplication in maize (Gayen et al., 1994; Zabirova et al., 1996; Deimling et al., 1997; Prigge & Melchinger, 2012; Vanous et al., 2017); however, chromosome duplication efficiency data have not yet been widely reported in the literature. Therefore, this study aimed to evaluate the efficiency of chromosome duplication protocols in obtaining DH lines in maize.

All the experiments were carried out at Embrapa Milho e Sorgo in Sete Lagoas in the central region of Minas Gerais from March to November 2018. The experiment consisted of 270 maize seeds identified as haploids based on phenotypic markers. They were obtained by crossing the genotype source 91500212 derived from a biparental cross between lines of the Flint group with the haploid inducer TailP1 × TailP2.

A completely randomized block experimental design was used with three replications, comparing 3 CD methods. For that purpose, the 270 seeds were divided into three groups of 90 seeds, subdivided into three replications of 30. Each group passed through a CD treatment, as described below.

Chromosome duplication by colchicine injection (Vanous et al., 2017). In this protocol, the seedlings were first sown in 50-cell trays with a commercial substrate, where they were maintained up to the V3 stage (3 fully expanded leaves). Then, 100 µl of a solution of colchicine at 0.125% + 0.5% of dimethyl sulfoxide (DMSO) (1.25 g of colchicine and 5 ml of DMSO) was applied with a disposable syringe of 1 ml containing a hypodermic needle of 8 × 0.30 mm. After the application, the plants were kept in the Doubled Haploid Laboratory of Embrapa Milho e Sorgo outside of direct sunlight and without irrigation for 8 hours for the seedlings to absorb the solution. On the twentieth day after the treatment, the plants were placed in a greenhouse, where they were transplanted definitively in 20-liter pots.

Chromosome duplication through root immersion (Couto et al., 2015). In this protocol, the materials were sown in 50-cell trays with vermiculite as a substrate and conducted up to the V3 stage (three fully expanded leaves). In this stage, the plants were removed from the substrate, their roots were washed in running water, and they were inserted in beakers

containing a 1:1:1 solution of colchicine, DMSO, and Tween 20 (1 g: 1 ml: 1 ml, respectively, per liter of distilled water). The roots of the seedlings were kept for six hours in solution, then washed for 40 minutes in running water, and then transplanted in trays with the commercial substrate. After these steps, the plants were placed in a greenhouse for 20 days, transplanted in 20-liter pots with fertilized soil, and kept in a greenhouse for the entire cycle.

Chromosome duplication by immersion of newly germinated seedlings in colchicine solution (Prigge & Melchinger, 2012). This protocol divided the seeds into groups of 30 and sown in Germitest (germination testing) paper moistened with distilled water. Then the Germitest paper was made into rolls and placed in a seed germinator for 36 hours to obtain seedlings with the emergence of 2 cm of the coleoptile. Afterward, the rolls were removed from the germinator and the seedlings received a cut at 2 mm from the coleoptile tip using a scalpel. Then, the seedlings were inserted in beakers with 400 mL of a colchicine solution at 0.06% + DMSO at 0.5% (600 mg of colchicine and 5 ml of DMSO per liter) until it completely covered all the seedlings. Next, the seedlings were kept in the solution in the dark for 12 hours at 20ºC, and after, they were thoroughly washed in running water for 30 minutes. The seedlings were subsequently transplanted to trays with commercial substrate and placed in a greenhouse, where they remained for acclimatization for 20 days, then transplanted definitively in pots with fertilized soil.

The pots were distributed in a greenhouse according to a randomized block design, where each treatment was allocated in three blocks of 30 seedlings, randomized in each block according to the chromosome duplication treatment. The following data were obtained to evaluate the success of each protocol: i) the total number of plants surviving after the procedure; ii) the total number of possible DHs; iii) the opening of tassels, ranging from fully open tassels with good shedding of pollen, intermediately open tassels with little shedding of pollen, and male-sterile tassels with totally closed anthers; iv) male flowering (MF), in days; v) female flowering (FF), in days; vi) the anthesis-silking interval (ASI), in days; vii) days of shedding of pollen (DSP); viii) length of the central tassel stalk (LCS); ix) number of tassel branches (NTB); x) plant height (PH); xi) first ear height (EH); xii) ear length (EL); xiii) ear diameter (ED); xiv) number of kernels on the ear (NK); xv) number of rows of kernels on the ear (NR); xvi) ear weight (EW); and xvii) seeds weight (SW).

The contrasts between mean values were carried out to analyze the data: (A) chromosome duplication through injection vs. chromosome duplication through root immersion; (B) chromosome duplication through injection vs. chromosome duplication through the application in newly germinated seedlings; and (C) chromosome duplication through root immersion vs. chromosome duplication through the application in newly germinated seedlings. In addition, the T-test was used for the following variables: seedling survival, percentage of haploids and false positives, and chromosome duplication efficiency for tassel fertility. All analyses were done using the SAS statistical software (SAS Institute, 2000).

The T-test did not indicate significant differences between CD through injection and CD through root immersion or between CD through root immersion and CD through the application in newly germinated seedlings to obtain DHs (Table 1). In absolute values, the most remarkable survival of seedlings was observed in CD through injection, in which 89 of the 90 seedlings in treatment survived (98.99% -

Figure 1). For CD through root immersion, 73 plants (81%) survived, while in CD through the application application in newly germinated seedlings, 57 plants (63%) survived. The better results for the treatment with injection of colchicine can be explained by its less aggressive method for the seedling, as it requires fewer transplanting operations and is performed with the seedling in a more advanced stage of development.

Evaluation of probably doubled haploids, that is, how many plants were doubled haploids from the set of seeds selected, was based on morpho-agronomic traits of the plant and ears (Figure 2). In absolute values, there was a more significant number of DHs in chromosome duplication through injection (17 DH plants); however, the highest percentage of surviving DH seedlings was identified in the treatment with newly germinated seedlings (22.81%).

The most significant number of fully open tassels was found in the CD injection treatment (Table 1). This may be related to seedling survival, resulting in lower loss of possible maize doubled haploids. In addition, the CD root and by newly germinated seedling treatments had a low number of plants with male sterility, showing the efficiency of the two treatments in reversing the male sterility in the tassel in the surviving plants.

Concerning the production of ears with kernels among the DHs obtained in the CD newly germinated seedling treatment, five ears had kernels, while eight did not show kernel formation (Table 1). In the CD root treatment, seven ears had kernels, and

Table 1. Total of double haploid and false positive plants; fertile tassels and means of ears with seeds after treatment of haploid seedlings with three chromosome duplication protocols.

Total of doubled-haploid plants			
Chromosome duplication protocol	Doubled-haploid	False positive	
Newly germinated seedlings	13 (22.81%) a	21 a	
Root immersion	15 (20.54%) a	31 a	
Stem injection	17 (19.10%) a	42 a	
Fertile Tassels			
Chromosome duplication protocol	Total opening	Parcial opening	Male sterility
Newly germinated seedlings	27	23	1
Root immersion	39	25	3
Stem injection	43	29	14
Total of ears with seeds after doubled-haploid treatment			
Chromosome duplication protocol	Ears with seeds	Ears without seeds	
Newly germinated seedlings	5 a	8 a	
Root immersion	7 a	8 a	
Stem injection	11 a	6 a	

Number in parentheses represents the success rate in obtaining the double haploid. Means followed by the same letter do not differ from each other by the T test at 5% probability.

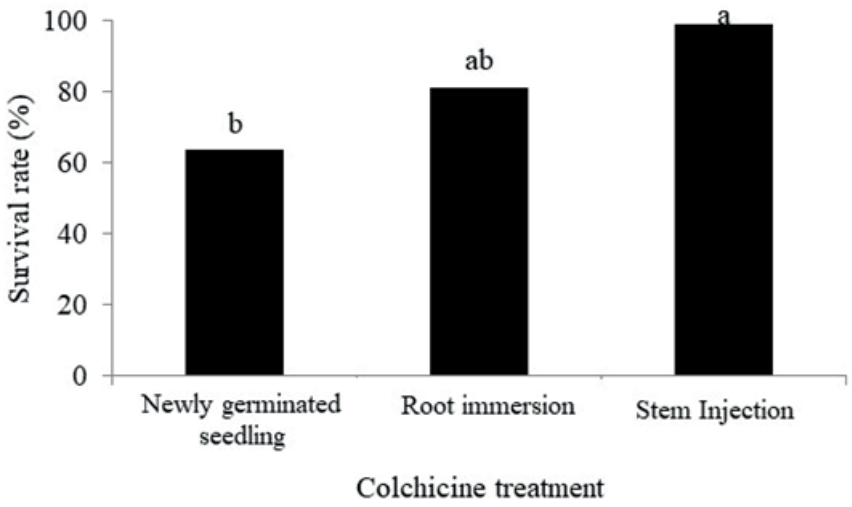

Figure 1. The survival rate of plants after treatment with colchicine by different protocols. Means followed by the same letter do not differ from each other by the T-test at 5% probability.

Figure 2. Type of ears obtained in the doubled-haploid process: (A) Ears of haploid plants, with few unpigmented seeds; (B) False positives with purple pigmentation and white seeds after harvest; (C) Ears with seeds, indicating efficiency in the reversal of male sterility; (D) Ears without seeds, indicating inefficiency in the treatment of chromosome duplication.

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eight ears did not. In the CD injection treatment, 11 ears had kernels, and four ears did not form kernels. Thus, the injection treatment resulted in the most significant number of plants with viable pollen for crossing, directly reflected in the number of ears with kernels.

The data from the orthogonal contrasts for the 14 agronomic traits evaluated indicate significant differences among treatments for male flowering, female flowering, days of pollen shedding, plant height, and ear height (Table 2). The MF and FF were the lowest for the injection treatment (64 and 67 days, respectively). In comparison the root treatment exhibited a mean of 72 days for MF and FF, and the newly germinated seedling treatment exhibited a mean value of 78 and 79 days for MF and FF, respectively. The plants under the injection treatment showed a mean of two days of pollen shedding, four days for plants with root treatment, and five days for the newly-germinated-seedling treatment, with tremendous variation in this last group. Significant differences were observed only for comparing injection vs. root and injection vs. seedling application.

Table 2. Means and orthogonal contrasts for the 14 agronomic traits evaluated in double haploid maize plants under three distinct chromosome duplication protocols with colchicine application.

Agronomic traits	Protocols of chromosome duplication			F orthogonal contrasts test		
	Stem injection (A)	Root immersion (B)	newly germinated seedlings (C)	A versus B	A versus C	B versus C
MF (days)	64	72	78	15.99**	62.34**	17.65**
FF (days)	67	72	79	8.89**	96.06**	48.77**
ASI	3	1	2	3.59	0.04	2.26
DSP	1	3	4	12.70**	20.56**	1.64
LCS (cm)	26	24	27	0.88	0.31	1.91
NTB	6	4	4	2.17	0.78	0.2
PH (cm)	96	87	88	3.89*	0.17	1.85
EH (cm)	54	35	41	36.78*	8.43**	6.53**
EL (mm)	69	66	59	0.16	1.42	0.66
ED (mm)	29	28	24	0.19	1.99	3.16
NK	83	64	46	1.49	3.45	0.54
NR	9	9	8	1.43	3.3	0.52
EW (g)	30	21	15	0.63	3.53	1.3
SW (g)	27	20	15	0.76	3.3	1.01

* or ** Significance of 5 and 1% for the F values, respectively. Male flowering (MF), in days; v) female flowering (FF), in days; vi) the anthesis-silking interval (ASI), in days; vii) days of shedding of pollen (DSP); viii) length of the central tassel stalk (LCS); ix) number of tassel branches (NTB); x) plant height (PH); xi) first ear height (EH); xii) ear length (EL); xiii) ear diameter (ED); xiv) number of kernels on the ear (NK); xv) number of rows of kernels on the ear (NR); xvi) ear weight (EW); and xvii) seeds weight (SW).

The PH trait showed a significant difference only in comparison of the injection and root CD protocols, where the mean height of the injection treatment was 96 cm, and the mean height of the root treatment was 87 cm. The EH value was 54 for the injection treatment, 35 for the root treatment, and 41 for the seedling treatment. Although the differences were insignificant, there was more outstanding seed production in the injection treatment.

Each treatment has its particular aspects in being carried out, which affects how easy it is to perform the treatment and the residues generated. The root treatment is the easiest to carry out since it only requires the removal of the seedling from the substrate and placing the root in the colchicine solution. However, this treatment requires a large quantity of the reagent, generates many residues at the end of its application, and requires subsequent transplanting, which can cause stress and loss of seedlings. The newly germinated seedling treatment requires germination in Germitest paper, followed by cutting the coleoptile of each seedling and insertion in colchicine solution. However, as in the root treatment, there is a large quantity of reagents, generation of residues, and possible stress and loss of seedlings at the time of transplanting. For its part, the injection treatment requires a smaller quantity of reagent and does not generate subsequent residue; however, performing this method depends on the ability of the one who applies the injection of the colchicine solution, requiring previous training to prevent accidents.

Based on the data obtained, we conclude that the injection protocol showed the most outstanding efficiency for DH production, resulting in more remarkable survival, more excellent production of doubled haploids with viable pollen and ears, and more excellent seed production. In addition, this protocol has the advantages of being more economical in terms of using substrate and reagents, reducing the number of transplant operations, and not generating additional residues after application, which results in lower environmental impact.

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