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Research Article

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20 Years Experiences on Paprika (Capsicum annuum L.) Doubled Haploid Production

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ABSTRACT

In laboratory of Medimat Ltd. over the past twenty years have been tested more than 3600 different genotypes originating from Hungarian sweet pepper types (Cecei, tomato-shaped, apple-shaped, white blocky, light green and dark green blocky, green spice), Hungarian spice pepper genotypes, Dutch blocky types, Spanish types (Dolce Italiano, Lamuyo, red blocky, yellow blocky), Chinese types (hot lamuyo and different spice types) and Turkish types (e.g. Dolma, Charliston, Sivri) using in vitro anther and microspore culture to produce new homozygote breeding lines for the private companies.

We improved a very efficient genome doubling method by treating the haploid paprika regenerants with colchicine (500 mg/l) in vitro for six days. The ratio of induced doubled haploids reached to 95.13 %. Survival rate of treated haploid plants was 99.4 %. Since diploidised plants developed similarly to their untreated, spontaneous diploid counterparts and since no fertility problems were recorded in the past fifteen years.

When haploid method has been combined with marker-based pre-selection it provided an exceptionally efficient method for paprika resistance breeding (TMV, TSWV, powdery mildew, Xanthomonas, nematodes).

Keywords: Anther Culture, Haploids, Flow Cytometry, Genome Doubling, Colchicine

Introduction

The production of doubled haploid plants has become a key tool in advanced plant breeding. Plant breeders are increasingly using this system in their mainstream pure-line programs to reduce the number of years needed from crosses to commercial variety registration [1]. Haploid technology offers a shortcut to perfectly homozygous lines and provided an attractive solution for production huge number of pure-lines and new paprika candidates for registration [2,3].

Last year's we have been taking an integrated research approach the effectiveness of fertile pepper doubled haploid (DH) production by optimizing in vitro and in vivo culture parameters in anther and microspore culture as well [4-7].

In another culture we are using the protocol described by Dumas de Vaulx et al. with a little modification proved to be efficient, but it results in high amount of haploid paprika regenerants [8,4]. The flow cytometer analysis of the ploidy level of paprika plants regenerated from anther culture proved the existence of haploid, diploid and a negligible amount tetraploid plant. The level of spontaneous diploidisation depended on the genotypes and the methods have been applied [9,10]. Generally, endoreduplication or nuclear fusion can result spontaneous chromosome duplication in the early phase of in vitro culture.

In case of pepper, we have to improve an efficient chromosome doubling protocol. An efficient doubled haploid protocol involves evaluating factors which influence induction of embryogenesis, the regeneration of those embryos to plants and fertile doubled haploid (DH) induction from haploid plants [11].

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Colchicine is still the most effective chromosome doubling agent, although the frequency of diploidization varies with the concentration used, plant parts treated and developmental stage of the tissue [12]. Colchicine inhibits the polimerization of tubulin so the chromosomes arrest at methaphase. Colchicin can be applied in situ or in vitro in various concentrations and exposures [12]. In the case of paprika Dolcet-Sanjuan et al. and Gyulai et al. have used colchicine in situ after removal of the primary axillary eye and then treated secondary eyes [10,13].

In vitro colchicine treatment applied in eary induction phase of anther and microspore culture. Colchicine treatment before the first microspore division can efficiently arrest mitosis and results in homozygous doubled-haploid microspores [14-16]. Chromosome doubling could be obtained young in vitro plantlets has been cultured in many species [17-20].

Material and Methods

Another Culture

Prior to the initiation of in vitro cultures, the closed flower buds were sterilized. Due to the bacterial infection observed in some experiments, the traditional sterilization procedure was modified, as the usual treatment with 20% bleach was unable to provide complete protection against the bacterium strains attacking the buds. The ratio of infection was particularly high in the hot summer months. The best sterilization technique proved to be the use of a 1% concentration of the disinfectant Dodenal (Merck/Arcana, Chem. Ph. Fabrik, GmbH, Austria), which gave approx. 100 times greater protection against saprophytic bacteria than the chemical previously applied [21]. The multiplication of bacteria which survived even this form of was inhibited by adding Cefotaxim (200 mg/l) to the induction medium. The use of antibiotics did not have any notable influence on embryo induction.

The induction and regeneration medium was prepared as reported by Dumas de Vaulx et al. [8]. Induction medium supplemented with 3% maltose was applied for six days at 35°C. The use of maltose as a source of sugar in the induction medium led to an improvement in the in vitro androgenesis efficiency [21]. Maltose is hydrolysed more slowly than sucrose in the medium, thus delaying the availability of glucose to the culture. This stress is thought to enhance the induction of androgenesis.

Genome Size Analysis

Before plants originating from another culture can be used for practical breeding purposes it is essential to determine the genome size of the plants, since only diploid (doubled haploid) plants are fertile and suitable for breeding. The genome size of regenerants was first determined in the seedling stage using a PARTEC I flow cytometer (Partec GmbH, Münster, Germany) equipped with a high-pressure mercury vapour lamp (Osram HBO 100W/2). The nuclei were isolated from the samples using the method of Dolezel et al. [22]. Leaf segments measuring approx. 0.5 cm^2 were placed in 1 ml LBO1 buffer and chopped minutely using a razor blade. A pure cell nucleus suspension was obtained by filtration through a 30 µm Ø Cell Trics/TM (Partec GmbH, Münster, Germany) membrane. Samples containing cell nuclei were labelled with 1 ml DAPI solution (Partec High resolution Kit Type P, Solution A). The DNA content of each sample was measured in three replications. Control samples (cell nucleus isolates) were prepared from normal diploid plant leaves.

Colchicine Treatment

Anther derived pepper plants in the 2-3- leaf stage, which were detected by flow cytometric analysis as haploids, were placed on V3 medium containing filter-sterilized 0.05 % colchicines in concentration for two, four or six days [8]. Before the treated plants were potted in greenhouses for seed production their roots were washed for twenty minutes. Two months later the genome change was screened on the new raising of the young leaves developing after the treatment by flow cytometry.

Marker-Based Selection

In the cooperation the laboratory of Medimat Ltd. and Rijk Zwaan Zaadteelt & Zaadhandel B.V. we combined DH technology with marker-based pre-selection, which have provided an exceptionally efficient method for resistance pepper hybrid breeding (Gémesné Juhász et al, 2000, 2002a, 2006) amongst others: Tobacco mosaic virus ™, Tomato spotted wilt virus (TSWV), Potato Y virus (PYV), Phytoptora Capsici (Pc), Xanthomonas campestris (Xcv), Meloidogyne incognita/javanica/arenaria (Mi; Mj; Ma) and Leveillula taurica (Lt).

Results

Colchicine Treatment on Pepper Haploids

Statistical analysis of the results of two- and four-day colchicine treatments on haploids showed significant difference between the treatments. Although the four-day treatment significantly increased the number of dihaploids compared with the two-day treatment, only 24.22 % of the 161 treated plants were diploidised even in this case so two- or four-day treatment with colchicine did not appear to be sufficient for genome duplication Table 1). The high frequency of mixoploids (24.20 %), which contained both haploid and diploid cells, indicated that genome duplication was not yet complete. In order to achieve complete genome duplication, it was thus necessary to increase either the concentration of colchicine or the length of the treatment. In our trial the duration of the colchicine treatment was increased to six days to determine what effect this had on the efficiency of genome duplication.

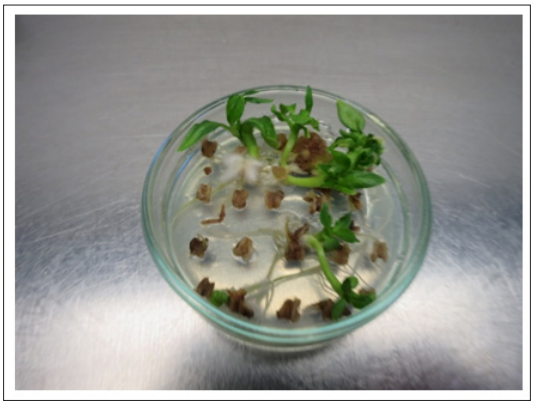


Figure 1: Plants development from another culture of pepper

Comparative statistical analysis of the results of two- and four-day colchicine treatments on haploids showed significant difference between the treatments. Although the four-day treatment significantly increased the number of dihaploids

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Table 1: Results of 2-4-6 Days Treatment with Colchicine (500 Mg/L) On Genome Size in Anther Derived Haploid Paprika Regenerants

No of treated haploid plants	Treatment days	Induced doubled haploids (%)	Induced tetraploids (%)	Induced mixoploids (%)	Rest of haploids (%)
61	2	3.27 (2) a	-	8.19 (5)	88.52 (54)
161	4	24.22 (39)b**	1.24 (2)	24.20 (39)	50.31 (81)
2054	6	95.13 (1954)c**	0.62 (11)	1,32 (10)	4.6 (82)

In our trial the duration of the colchicine treatment was increased to six days to determine what effect this had on the efficiency of genome duplication.

Comparative statistical analyses on the four- and six-day colchicine treatments also showed a significant difference between the treatments. The six-day treatment led to a significant rise in the ratio of doubled haploid regenerants. Diploidisation was achieved in 95.13 % in average of the 2054 treated plants originated from 20 different genotypes, mainly F1 hybrids. The six-day treatment also led to a substantial reduction in the ratio of haploid-diploid genome mosaics. Few of the mixoploids arising due to the six-day treatment were genome mosaics containing diploid and tetraploid cells. The six-day colchicine treatment had no negative effect on the survival of the plants. During the four-year field experiment no phenotypic mutations have been observed in any of the plants regenerated after colchicine treatment.

The genome size determination allowed haploid plants arising from another culture to be clearly distinguished from those that had undergone spontaneous diploidisation. An efficient method with good reproducibility, requiring less manual work, was elaborated for the in vitro genome duplication of pepper haploids.

Discussion

Over the last twenty years one of the most intensive fields of research in plant biotechnology has been the widespread application of the in vitro haploid methods based on the artificial sporophytic development from gametes. Doubled haploid plants originating from gametes carry the genetic information of only one set of chromosomes, so they can be regarded as genetically homozygotic.

The majority of the plant regenerants arising from in vitro andro- or gynogenesis will be haploid with regard to their genetic background, but plants with other ploidy levels may also develop spontaneously. Due to their sterility, haploid plants are of no value in practical plant breeding, but if their genome is doubled, valuable induced doubled haploids may be obtained for breeding purposes.

The successful utilization of haploids in breeding process depends not only on the production of large numbers of haploids, but also on the efficient doubling of chromosomes to reach homozigosity and genetic stability [12].

Every plant which remained in haploid state means losing a promising genetic combination. Are known antimitotic agents such as colchicin, podophyllin, oryzalin, trifluralin, pronamide, amiphroshosmethyl and nitrous oxide which can induce chromosome doubling. When we choose the proper agent it worth to take in consider the as follows: the methods will be simple, safe, highly efficient and well repetable, we could handle of large material and will result mainly pure 2n product.

Of several chemical agent so far has been used, colchicine proved to be one of the most efficient. Campion et al. reported that three days of colchicine treatment - it was added to medium - at 10 mg/l produced 46% of plants completely diploid in the shoot apex of haploid onion plants [17].

Miyoshi and Asakura (1996) the doubling of chromosomas of 24.2-34.1% gerbera haploids was achived by treatment with 0.05% colchicine for 2-6 days.

In the case of summer squash Caglar and Abak tested the effect of various colchicine concentrations and the treatment times on the doubling process of haploids and found that 0.5% colchicine treatment for 4 hours was the most effective, leading to a doubling of the chromosome number in 60% of the treated plants [19].

Gürel et al. described a method of treating young haploid sugarbeet shoots with colchicine and trifluralin. Their results showed that, although colchicine was more effective (25.3%) than trifluralin (18.2%) the difference was not significant. The treatment duration of 48 h proved to be more effective than 12 h, but it was not different from 24 h or 36 h [20].

In case of pepper nobady described efficient doubling of chromosomes.

The pepper genotypes tested differed from each other in the ratio of spontaneous doubled haploids formed 0–62 %. So far considerable progress has not been made in haploid paprika chromosome doubling as the treatment of decapitated haploid regenerants has not resulted proper solution. Present technique offers an efficient, reproducible and economic method for in vitro genome duplication of pepper haploids. Since survival rate of treated regenerants is about 99 % and colchicine treated plants developed similarly to their untreated, spontaneous diploid counterparts and since no fertility problems were recorded, it would seem that this genome duplication technique will

be suitable for application in a wide field (Gémes Juhász and Kristóf, 2016; Sági and Gémes Juhász, 2020) [23-25].

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