DIPLOIDIZATION OF CUCUMBER (*CUCUMIS SATIVUS* L.) HAPLOIDS BY COLCHICINE TREATMENT

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ABSTRACT

Haploid cucumber plants are totally infertile and do not undergo spontaneous diploidization. The use of haploids depends on the possibility of doubling the chromosome number and the obtaining of stable doubled haploids (DH). Four haploids of different genotypes propagated vegetatively were treated with colchicine in order to obtain DH. The following procedures were used: 1) apical shoot meristem treatment, 2) soaking of shoot explants, 3) placing of shoot explants on medium with colchicine. Plants of the C1 generation were evaluated in respect to morphological and cytological characters and fertility.

The best result of 20.9% DH was obtained after repeated treatment of the meristem with colchicine. A large group of chimeras (28.5%) was also distinguished as were haploids and tetraploids. DH plants were fertile and gave uniform progeny. Chimeras had a decreased fertility and showed disturbances in meiotic divisions.

KEY WORDS: haploids, cucumber, colchicine treatment, doubled haploids, chimeras.

INTRODUCTION

It was not possible to obtain haploid plants in cucurbits for a long time. The fertilisation of muskmelon flowers with gamma rays irradiated pollen and there subsequent isolation and transfer young haploid embryos to medium allowed the obtaining of the first haploid plants (Sauton and Dumas de Vaulx 1987).

The application of this method permitted the isolation of haploids from cucumber (Niemirowicz-Szczytt and Dumas de Vaulx 1989, Przyborowski and Niemirowicz-Szczytt 1994).

Cucumber haploids are infertile and do not undergo spontaneous diploidization (Przyborowski and Niemirowicz-Szczytt 1994). Haploid plants are capable of prolonged vegetative growth and occasionally form fruit but do not have seeds. An annual plant such as the cucumber dies after a few months without producing seeds.

No assessment of the performance of DH cucumber plants has previously been reported. It was therefore necessary to elaborate an effective method of doubling the chromosome number to get fertile diploid plants which could be used for obtaining homozygous lines.

Examples of effective in vitro and in vivo colchicine treatment of plants are known in doubled haploid development. In the case of haploids from the Poaceae above-ground parts of the plant are treated (excluding the roots) or else the whole plant is immersed in an aqueous colchicine solution. In the

case of haploid rice this method makes it possible to obtain 83% of diploid plants (Oh et al. 1991).

The effectiveness of the method can be improved by in vitro cultures of haploid tissues. Often early treatment of cells which have not yet differentiated into haploid plants is used. Up to 90% diploid embryos of *Brassica napus* were obtained after a 24 h treatment of microspores with 50 mg/l colchicine (Moellers et al. 1994). Colchicine treatment of gametophyte cells which have been stimulated to divide in a sugar beet ovule may lead to formation of 62% of diploid plants (Hansen et al. 1994). The in vitro treatment of shoot segments of potato dihaploids and monohaploids allowed the obtaining up to 70% of doubled haploids (Yu et al. 1991).

Taira et al. (1991) have indicated that in addition to such factors as colchicine concentration, duration of the treatment, type of tissue subjected to colchicine treatment and other factors optimising the obtained effects, the pH of the aqueous colchicine solution may also affect the result of diploidization. Detailed investigations of plants treated with colchicine (C1) indicate that except doubled haploids some other ploidy plants can be of obtained (Kweon et al. 1993).

The lines of doubled haploids may show a large variability and most of the variation detected could be attributed to the process following chromosome doubling, possibly the colchicine treatment (Suenaga and Nakajima 1993).

The aim of the work was to check various methods of colchicine treatment of cucumber haploids in vitro and to determine the most effective method. In addition an evaluation of morphological and cytological traits as well as fertility of C1 and C2 plants was performed.

MATERIAL AND METHODS

The plant material consisted of four different haploid genotypes (individuals). These plants were multiplied vegetatively by cutting the main stem and branch shoots into segments and rooting on MS medium containing one half concentration of macro- and micro nutrient salts (Murashige and Skoog 1962).

Young shoots in the 2-3 leaf stage in the initial phase of rooting were chosen. Thirty shoots or explants were treated simultaneously in each combination.

Single, double and multiple (3-5) treatment of apical shoot meristems (one droplet on each meristem) with 0.1% colchicine solution was applied. Additionally shoot segments (with the bud) were immersed in 0.1% aqueous colchicine solution for 2 h and were placed on medium after washing in sterile water.

In the last combination colchicine was added to the medium (0.1%) and shoot segments were placed on medium for 48 h.

Shoots growing from treated meristems were transferred to fresh medium and after rooting were potted into soil and maintained until maturity in the greenhouse.

The plants were evaluated in respect to the following characters: a) morphological – plant shape, dentation of the leaves, shape of flower petals, b) cytological – number of chromosomes in the PMC (pollen mother cell) of the meiotic division, chromosome associations in diakinesis and MI, number and type of sporads, pollen stainability, c) fertility – ability to set seeds and seed germination ability.

Selected progeny (C2) of colchicine treated plants (C1) was also analysed. On the basis of shape and characteristic morphological traits (spine colour, colour and shape of fruit, plant sex as well as the stainability and size of pollen grains and ability to set seeds) the uniformity of the progeny and its fertility were evaluated. Plants were divided into four classes depending on their fertility: 0 – infertile, 1 – low fertility (1-10 normal seeds), 2 – medium fertility (11-30 seeds), 4 – fertile (over 30 seeds).

Cytological investigations were also performed on material (small male buds) fixed in (1:3) acetic alcohol for 24 hours. The anthers were squashed in 4% acetocarmine. Sporads and pollen grains were also stained with acetocarmine and glycerine (1:1).

RESULTS

Effects of colchicine treatment on cucumber haploid plants

The choice of the four tested haploids (genotypes) was linked to their viability and ease of multiplication in vitro. In Table 1 the results of using colchicine in 3 different combinations are presented. The immersion of shoots in colchicine as well as culturing on medium with colchicine did not yield the best results. The highest percentage of changed plants, was obtained by multiple treatment of apical meristems (up to 50% of changed plants and 20.9% doubled haploids). Among the changed plants chimeras, doubled haploids and tetraploids were present.

The observation of shoots selected for colchicine treatment indicates that the result depends on the condition of the treated plants. Only very viable and strongly growing shoots survive effective (change-inducing) colchicine treatment.

The shape and dentation of leaves were the first characters made possible to distinguish plants of different ploidy. Leaves

TABLE 1. Effect of colchicine on ploidy changes in cucumber haploids.

Haploid symbol	No of plants obtained	Plants (%)			
		Without changes	Chimeras	Doubled haploids	Tetraploids
Meristem treatment					
One time					
16	21	85.6	4.8	9.6	0.0
One time					
16	21	85.6	4.8	9.6	0.0
99	13	69.3	23.0	7.7	0.0
Two times					
16	16	72.2	9.1	18.2	0.0
99	12	66.8	16.6	16.6	0.0
759A	27	96.3	3.7	0.0	0.0
759B	16	87.8	0.0	12.5	0.0
Many times					
759A	24	62.5	16.6	20.9	0.0
759B	14	50.0	28.5	7.1	14.3
Explant immersion					
16	16	93.7	6.3	0.0	0.0
Explant on medium with colchicine					
16	24	75.0	20.8	4.2	0.0

Fig. 1. A comparison of haploid (right), doubled haploid and tetraploid (left) leaves.

of haploids were narrow, with a characteristically elongated central part and the leaves of tetraploids were broad and densely dentated (Fig. 1).

The isolation of chimeras required the observation of older plants in the greenhouse and also checking the number of chromosomes in flower buds taken from various nodes on the

plant. An additional indicator was the shape of flowers developing in successive nodes. Haploid flowers were quite easily distinguished from diploid, chimeric or tetraploid ones (Fig. 2). The chromosome number was analysed in all plants presented in Table 1.

Fig. 2. Morphology of male flowers from haploid (down), tetraploid (upper row, right), doubled haploid (upper row, left) and chimeric (left side) plants.

TABLE 2. Associations of chromosomes in PMCs of cucumber chimeras.

Plant symbol	No of PMCs	% of PMCs ($x=7$) with				No of PMCs	% of PMCs ($2x=14$) with				No of PMCs	% of PMCs ($4x=28$) with			
		II*	I	III	IV		II	I	III	IV		II	I	III	IV
32	32	6.3	93.7	—	—	6	100.0	—	—	—	—	—	—	—	—
33	119	31.1	63.8	5.1	—	30	76.6	6.6	6.6	10.0	1	—	—	—	100.0
44	71	8.5	91.5	—	—	17	76.5	17.6	—	5.9	—	—	—	—	—
46	50	10.0	82.0	8.0	—	4	75.0	25.0	—	—	1	—	—	—	100.0
89	89	4.5	95.5	—	—	23	82.6	13.0	—	4.3	2	—	—	—	100.0

* chromosome configuration (II – bivalent, I – univalent, III – trivalent, IV – tetravalent)

Chromosome associations in PMC of chimeric plants

Detailed cytological investigations were performed for five plants whose morphological characters showed that they were chimeras. The results presented in Table 2 indicate that the greatest number of PMC contained one genome $x = 7$. The chromosomes of haploid PMC were in the form of univalents (Fig. 3) and also in the form of bivalents (up to 31%, plant No. 33).

Fig. 3. PMCs of chimeric cucumber plant with haploid ($x = 7$) set of chromosomes.

Chimeric plants had a small number of diploid PMC ($2x = 14$). Bivalents predominated in these cells (75-100%). Other chromosome configurations in diploid PMCs were univalents, trivalents and tetravalents (Fig. 4 and 5). No chromosomes were found outside the caryokinetic spindle. The few tetraploid PMC ($4x = 28$) contained tetravalents (Fig. 6).

Fig. 4. Meiotic chromosomes of diploid cell obtained after colchicine treatment may be separated into 14 univalents.

Fig. 5. Unbalanced division of diploid PMCs ($2 I + 2 II + 2IV$).

Fig. 6. PMC of tetraploid plant ($6IV + 2 II$).

TABLE 3. Frequency of sporads in haploid, doubled haploid and chimeric anthers.

Ploidy level Plant symbol	No of sporads	Percent					
		Diads	Diads with nuclei	Triads	Tetrads	Poliads	Monads
Diploid 1	120	—	—	—	100.0	—	—
Haploid	1	69	10.1	8.7	—	27.5	53.7
	2	113	22.9	19.5	—	21.2	36.3
	3	181	14.4	13.3	2.2	19.3	50.8
	4	214	11.7	14.0	—	16.3	57.9
	5	220	12.3	13.1	3.1	17.7	53.7
	6	106	4.7	11.3	2.8	19.8	61.3
	7	108	6.5	3.7	3.7	17.6	68.5
	8	82	24.4	24.4	2.4	15.8	32.9
Doubled haploid	156	1.3	—	—	89.7	—	—
Chimera	32	86	10.5	8.1	2.3	55.8	18.6
	33	103	15.6	12.6	1.9	63.1	5.8
	44	82	24.4	9.8	2.4	54.9	1.2
	46	136	23.5	20.5	1.4	33.8	8.9
	89	108	24.0	12.9	1.9	42.6	13.9

Characteristics of sporads in anthers of haploids, doubled haploids and chimeras

Table 3 presents the type of sporads occurring in individuals designated as haploids, doubled haploids and chimeras. The high percentage of regular tetrads in the doubled haploid was striking. Chimeras had somewhat fewer tetrads and very few tetrads were found in haploids. Only regular tetrads ensure the formation of viable pollen. All other types of sporads and their high participation indicate the presence of disturbances in meiosis. A fairly equal distribution of sporads in particular classes may indicate a lack of balance in meiotic division in colchicine treated plants.

Fertility of colchicine treated plants

In Table 4 plants are presented in respect to their fertility in 1994 and 1995 (year 1 and 2 of the investigations, respectively). All plants were repeatedly pollinated both by bees and by hand. Table 4 shows that 62.5-70.7% plants did not produce seeds. On the contrary high fertility was only obtained for

10.5-15.6% plants which set 2-3 fruit filled with seeds. These seeds germinated in 95-100%. In class of low fertility 4.4-15.6% plants were found. These plants (class 1 and 2) had a decreased fertility as they set a low number of seeds, often poorly formed and with low germinating ability.

In order to relate the problem of ploidy with plant fertility the data of 1995 are presented in Table 5, for all plants chromosome numbers were checked. Haploid plants were totally infertile. The highest fertility was obtained for doubled haploids, even though one plant in five did not set seeds. On the other hand, chimeras either did not set seeds (40%) or had few poorly formed seeds (class 1 and class 2). Among the analysed plants only 3 were tetraploid. Each of them was different in respect to fertility.

On the basis of the results presented in Table 5 concerning the second year of investigations it may be assumed that most of infertile plants (class 0) during the first year of investigations (Table 4) were haploids and chimeras, plants of class 3 were doubled haploids and plants in class 1 and 2 chimeras.

TABLE 4. Percentage of colchipooids in fertility classes.

Year	No of plants	% of plants in fertility classes			
		Infertile (0)	Low fertility (1)	Medium fertility (2)	Fertile (3)
1	114	70.7	14.4	4.4	10.5
2	32	62.5	15.6	6.3	15.6

TABLE 5. Percentage of different ploidy colchipooids in fertility classes (second year of experiment).

Ploidy level	No of plants	% of plants in ploidy classes			
		Infertile (0)	Low fertility (1)	Medium fertility (2)	Fertile (3)
Haploid	14	100	0	0	0
Diploid	5	20	0	0	80
Chimera	10	40	50	10	0
Tetraploid	3	33	33	0	33
Total	32				

The progeny of colchicine-treated plants (C2 generation)

In 1995 the germination ability of 12 doubled haploids and chimeras of the C1 generation was checked. The seeds of these plants germinated in the range of 95-100%.

Among 180 seedlings (15 for each previously chosen individual) 75 were selected and placed in a greenhouse. The plants were evaluated in respect to shape, stainability of pollen grains and fertility (ability to set seeds).

The progeny of doubled haploids and chimeras were diploid in 98.6%. Morphological characters of progeny obtained after self-pollination of a doubled haploids and chimeras were uniform (e.g. colour of spines on plants, fruit shape, sex). All plants yielded well filled fruits.

The progeny of plants treated with colchicine in 1995 will be tested in 1996. The group of chimeras, whose progeny may show differentiated ploidy, will be of particular importance.

DISCUSSION

Stable and infertile cucumber haploids may in general be propagated only vegetatively under in vitro conditions. The period of storing such plants (renewed by successive passages) may be up to 2-3 years (our unpublished results) but after some time these plants die. For this reason and also in order to obtain homogeneous/homozygous plants it is absolutely necessary to double the chromosome number. In order to allow the system of doubled haploids to be used e.g. in plant breeding the following conditions must be fulfilled (Snape et al. 1986):

- 1) the DH lines should be produced efficiently from all genotypes
- 2) the DH lines should represent a random sample of the parental gametes
- 3) the DH lines should be genetically normal and stable

In the case of this work the third point is of importance. The doubling method should be effective and should not introduce additional variation.

Three methods of treating shoot explants with colchicine were used in this work i.e. applying colchicine on the apical shoot meristem, soaking the shoot explant and placing on medium and the placement of shoot explants on medium containing colchicine. In the case of the first method colchicine was applied once, twice and many times. It seems that the method proved effective with double as well as multiple applications of colchicine on shoot meristems (Table 1). In both cases the greatest number of doubled haploids and chimeras was produced. Fertile doubled haploids and chimeras can be used for further generative reproduction.

Among 30 initial haploids in combination with multiple treatment of meristems 1-5 DH were obtained which constituted 7-21% of plants changed after colchicine treatment and a few (17-29%) chimeras and tetraploids (0-14%).

Were the obtained DH stable and normal? In the case of this experiment we have shown that the meiosis of colchicine-treated plants was disturbed. Chimeras especially show a lack of balance which was indicated by the fact that even in diploid PMC the number of bivalents varied from 75-100%. The bivalents are of the end-to-end or side by side type. In addition to diploids, haploid plants and tetraploids were also present.

The presence of different forms of sporads suggests that there was a lack of balance in the meiosis of chimeric plants.

The highest number of tetrads was found in DH (89.7%), fewer in chimeras (33.8-63.1%) and the lowest number in haploids (17.6-27.5%).

This state was reflected in plant fertility. Doubled haploids were in general fertile (Table 5), chimeras had low fertility or were infertile, and haploids were totally infertile. Fertile DH and some chimeras ($2n = 7-14$) may be used for further generative reproduction which as the obtained results show gave uniform and fertile progeny.

As a result of colchicine treatment single fertile or partly fertile tetraploids were also obtained. Tetraploids could be of interest for further study on cucumber polyploids.

It is worth considering whether a different diploidization method could be applied. Our preliminary investigations show that the chromosome number may be doubled due to callus induction and regeneration of plants from haploid leaf explants (Niemirowicz-Szczytt et al. 1994).

Both methods, however, have limitations. The colchicine method requires larger amounts of plant material (a minimum of 10 shoots from 1 initial haploid). The fact that chimeras and tetraploids, which may yield progeny with different ploidy, are formed in addition to DH must be taken into consideration. One of the advantages is that the colchicine-treated plants may be prepared for transferring to the greenhouse in a relatively short time (two months).

The method of callus induction from haploid leaf explants requires little plant material but depends on the genotype and is time-consuming (several months to over a year). The advantage of the regeneration method via the callus phase is also the possibility of obtaining a large number of fertile diploid plants from one explant.

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DIPLOIDYZACJA HAPLOIDÓW OGÓRKA (*CUCUMIS SATIVUS* L.) PRZY UŻYCIU KOLCHICYNY

STRESZCZENIE

Rośliny haploidalne ogórka są całkowicie niepłodne i nie ulegają spontanicznej diploidyzacji. Wykorzystanie haploidów zależy od możliwości podwajania liczby chromosomów i uzyskiwania stabilnych podwojonych haploidów. W celu otrzymania DH cztery różne genotypowo haploidy, rozmnożone wegetatywnie, traktowano kolchicyną. Zastosowano: 1) traktowanie merystemu pędu, 2) moczenie eksplantatów pędu, 3) wykładanie eksplantatów pędu na pożywkę z kolchicyną. Rośliny pokolenia C1 oceniano pod względem cech morfologicznych, cytologicznych i płodności.

Najlepszy wynik 20.9% DH uzyskano po wielokrotnym traktowaniu merystemu kolchicyną. Wyróżniono także dużą grupę chimer (28.5%) a ponadto haploidy i tetraploidy. Rośliny DH były płodne i dały wyrównane potomstwo. Chimery miały obniżoną płodność i cechował je brak stabilności w podziale mejotycznym.

SŁOWA KLUCZOWE: haploidy, ogórek, traktowanie kolchicyną, podwojone haploidy, chimery.