ISSN 1644-0692
www.acta.media.pl

ACTA SCIENTIARUM POLONORUM *Acta Sci. Pol. Hortorum Cultus, 14(3) 2015, 81-92*

FROM POLLINATION TO DH LINES – VERIFICATION AND OPTIMIZATION OF PROTOCOL FOR PRODUCTION OF DOUBLED HAPLOIDS IN CUCUMBER

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Abstract. A complete set of procedures comprising of the induction of haploid embryo development by pollination with irradiated pollen, embryo isolation and *in vitro* culture, haploid plant development, chromosome doubling by regeneration from leaf explants, acclimatization, self-pollination of DH plants and assessment of DH lines was performed in cucumber. Haploid and doubled haploid plants were obtained from all eight genotypes used in the study. The donor plant genotypes were evaluated by comparing the number of haploid embryos and DH plants obtained from each genotype. The influence of the donor plant genotype and its age on the regeneration and the yield of DH plants were evaluated. In order to optimize the method of chromosome doubling, the composition of medium was modified and the explants were divided into four groups with regard to the leaf sector used for regeneration. Acclimatization of DH plants to the greenhouse conditions was improved. Uniform, useful in breeding, DH lines were obtained.

Key words: acclimatization, *Cucumis sativus* L., haploid induction, pollen irradiation, embryo rescue, flow cytometry

INTRODUCTION

Double haploids (DH), if obtained in a sufficient quantity, can provide useful material for cucumber F_1 hybrid breeding. Up to now a few methods of haploid cucumber induction have been described, however, the information on the number of DH lines received and their application in the breeding programmes is rather scarce [Gałązka and Niemirowicz-Szczytt, 2013].

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Pollination with irradiated pollen is a well-documented method of cucumber haploid induction that resulted in induction of haploid plants from diverse plant material such as breeding lines, hybrids and open pollinated cultivars [Niemirowicz-Szczytt et al. 2000, Sztangret-Wiśniewska et al. 2006]. Haploid plants are genetically stable, however, it is necessary to double the number of chromosomes before further use in breeding. In this two-stage method of obtaining DH plants the genotype of the donor plant is a background element, whereas the chromosome doubling becomes the most important factor. Therefore, the aim of this work was to optimize the process of chromosome doubling as described by Przyborowski and Niemirowicz-Szczytt [1994], Nikolova and Niemirowicz-Szczytt [1996] and Faris et al. [2000] and to evaluate the effectiveness of this procedure in obtaining DH lines of cucumber. The procedure consisted of the following steps: (1) induction of haploid embryo development, (2) isolation of embryos from seeds, (3) *in vitro* culture of embryos and regeneration of haploid plants, (4) doubling the number of chromosomes, (5) assessment of the ploidy of regenerants, (6) acclimatization and generative multiplication of DH regenerants, (7) sowing DH seeds in a plastic-covered tunnel, evaluation of uniformity and characterization of DH lines.

MATERIALS AND METHODS

Haploid plant development. The initial material comprised of four cucumber F_1 cultivars: Marcel, Junak, Sonate, Śremski and hybrids resulting from their crosses: Marcel × Śremski, Junak × Marcel, Sonate × Śremski, Śremski × Junak (double hybrids). Eighty plants of each type were cultivated in a greenhouse. In order to induce the development of haploid embryos donor plants were pollinated with pollen irradiated by gamma rays from a cobalt source (RChM Gamma 20 Radiation Chamber) at a dose of 300 Gy [Przyborowski and Niemirowicz-Szczytt 1994]. Four pollinations per plant were applied. After 5–6 weeks embryos were excised and placed on E20A medium [Sauton and Dumas de Vaulx 1987]. Subsequently, the resulting plants were transferred to ½ MS medium [Murashige and Skoog 1962]. As a standard procedure the *in vitro* culture was carried out in a phytotron in the conditions of light, at 25°C and 16-hour photoperiod.

Chromosome doubling in haploids. In order to double the number of chromosomes, 2–3 mm^2 sections (explants) of 1 cm long leaves were cut out and placed on the 1.7 medium and then on the 1.6 medium as described by Burza and Malepszy [1995], and modified by Faris [1999]. Shoots regenerating from the explants were rooted on ½ MS medium. Optimization experiments were carried out on haploids of different origin: Marcel, Sonate, Śremski (three haploids of each) and Junak (two haploids). In order to increase the number of DH plants, the content of 2,4-D in 1.7 medium was increased from 1 to 1.5, 2 and 2.5 $mg \cdot dm^{-3}$. Furthermore, to identify the area of the leaf from which most of the plants regenerated, the explants were divided into four groups with respect to the leaf sector from which they were cut: 1 – the distal part of the lamina, 2 – the proximal part of the lamina, 3 – the distal part of the main vein, 4 – proximal part of the main vein (fig. 1B).

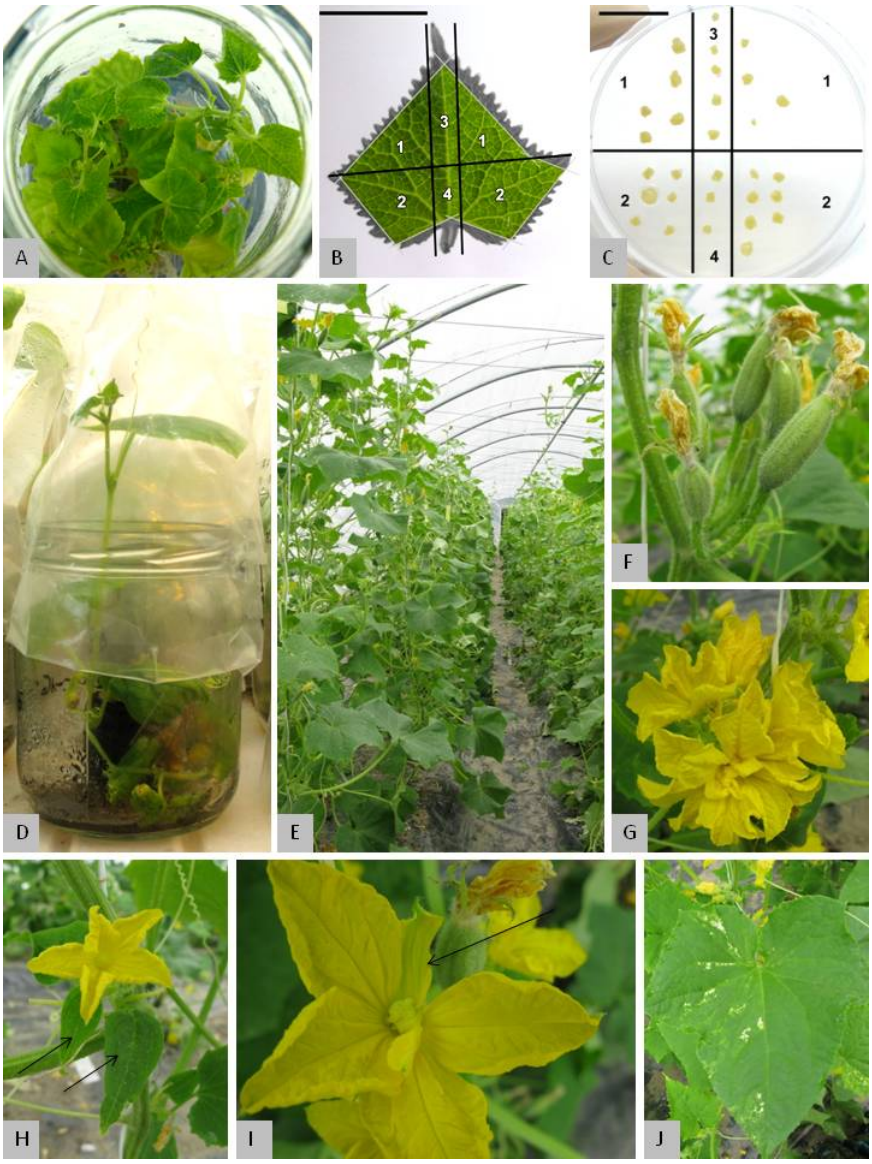

Fig. 1. Selected steps of obtaining DH lines: A – haploid plants *in vitro*; B – chromosome doubling by regeneration from leaf explants – leaf sectors: 1 – the distal part of the lamina, 2 – the proximal part of the lamina, 3 – the distal part of the main vein, 4 – proximal part of the main vein (bar 5 mm), C – example of reaction of explants – callus production in all sectors (bar 20 mm); D – acclimatization – regenerants after transfer to the greenhouse growing in swelling pots; E – DH lines in tunnel; F–J – morphological changes of flowers and leaves: F – many female flowers in one node, G – many male flowers in one node, H – diminutive leaves growing from the corners of the typical leaf (arrows), I – petals deformation (arrow) J – sectorial leaf discoloration

Plant growth and verification of ploidy. Regenerated plants that displayed characteristics typical of a diploid such as sturdy shoot, broad leaf of a typical shape and indentation, were transferred in their culture jars to the greenhouse. After a week the plants were removed from the culture medium, the roots were washed and soaked in a 0.15% solution of Topsin® to prevent fungal infection. Then they were planted in pots with peat substrate and kept shaded. To increase the survival rate of plants, an intermediate step was introduced in the acclimatization process, namely using 42-mm in diameter swelling sterile peat plugs. After acclimatization in the greenhouse, the ploidy of the regenerated plants was verified with FACSVantage flow cytometer (Becton-Dickinson, San Jose, CA, USA) by staining leaf nuclei with 5μg·cm^{-3} DAPI solution in citric/phosphate buffer (0.1M $C_6H_8O_7 \cdot H_2O$ / 0.2M Na_2HPO_4).

DH line production and characterization. The plants determined to be diploid and thus presumably doubled haploids were self-pollinated and the resulting seeds were sown the following year. Twelve progeny plants representing each DH plant were planted in a plastic foil tunnel (fig. 1E). Half of the plants were described in terms of the displayed sex (the number and type of flowers at the nodes of the main shoot) and morphology (the length of the main shoot, internode and petiole, the width of the leaf blade, the colour of fruit spines, the size of ripe fruits), whereas the remaining plants were self-pollinated again. All plants were rated with regard to leaf infection by downy mildew.

Statistical analysis. For statistical analysis SPSS® software from IBM was used. Depending on the type of experiment, one-, two- or three-factorial analysis of variance as well as Duncan's test and Pearson's correlation were used.

RESULTS

Haploid plant development. F_1 hybrids yielded from 0.19 to 0.4 embryos per fruit (e/f), whereas the double hybrids yielded from 0.46 to 0.59 e/f. Single hybrids gave a total of 272 embryos and 93 haploid plants (fig. 1A). Most of haploid embryos and plants were obtained from Marcel (109 and 36, respectively) and Śremski (65 and 28, respectively). With regard to double hybrids, the number of fruits obtained was lower as compared to F_1 hybrids, however, the number of embryos and resulting plants was much higher (472 and 166 respectively). In addition, double hybrids were more uniform in terms of the production of haploid embryos and plants (tab. 1).

Chromosome doubling in haploids – the standard method. Almost all the haploids (246 out of 259) were subjected to doubling by regeneration, but only 87 of them produced regenerated plants and 33 were successfully doubled and selfed to give DH lines (DHLs). The highest number of DHLs was obtained from Marcel (9) (tab. 1). DH regenerants of each type of hybrid were successfully self-pollinated. In addition, the date of initiation of regeneration seemed to play a role because most of the plants were obtained when regeneration was initiated in July and August (tab. 2). It was also apparent that the age of the haploid plant was inversely correlated with the overall capacity to regenerate plants and the number of produced regenerants.

Table 1. The yield of embryos (e), haploid plants (p), and DH plants in cucumber

Hybrids	Name	Fruits obtained (f)	Embryos rescued (e)		Haploid plants						
					received			regenerating shoots			doubled and selfed (DH lines)
			e	e/f[1]	p	p/f[1]	p/e (%)	r	%*	r/e	
Single	M	274	109	0.40 [adef]	36	0.13 [abcdegh]	33.03	17	47.2	0.16	9
	J	256	49	0.19 [bc]	13	0.05 [abce]	26.53	9	69.2	0.18	3
	So	231	49	0.21 [bcd]	16	0.07 [abce]	32.65	6	37.5	0.12	6
	Śr	190	65	0.34 [acde]	28	0.15 [adgh]	43.08	15	53.6	0.23	3
	sum	951	272	0.29	93	0.4	34.19	47	50.5	0.17	21
Double	M × Sr	251	122	0.49 [adefh]	35	0.14 [abceg]	28.69	9	25.7	0.07	2
	J × M	243	112	0.46 [aefh]	39	0.16 [abcdegh]	34.82	6	15.4	0.05	4
	So × Śr	194	115	0.59 [gh]	38	0.20 [adegh]	33.04	13	34.2	0.11	3
	Śr × J	236	123	0.52 [efgh]	54	0.23 [adgh]	43.90	12	22.2	0.10	3
	sum	924	472	0.51	166	0.73	35.17	40	24.1	0.08	12
Total		1875	744	0.39	259	0.14	34.81	87	33.5	0.12	33

M – Marcel, J – Junak, So – Sonate, Sr – Śremski; [1] – values marked with the same letter do not differ statistically (Duncan's test); * – in respect of haploid plants obtained

Table 2. The results of Duncan's test showing the differences in the percentage of leafs regenerating shoots depending by month (months marked with the same letter do not differ statistically)

Month in which the regeneration started		Leaves cultured	Leaves regenerating shoots		% of all leaves regenerating shoots
			No.	%	
January	[abcdgh]	24	0	0	0
February	[abcdfgh]	23	2	0.09	0.05
March	[abcdgh]	48	5	0.1	0.12
April	[abcdfghi]	8	1	0.13	0.02
July	[e]	13	9	0.69	0.21
August	[e]	12	7	0.58	0.17
September	[bdfghi]	21	6	0.29	0.14
October	[abcdfghi]	3	0	0	0
November	[abcdfgh]	34	4	0.12	0.1
December	[fgi]	27	8	0.3	0.19
Total		213	42		

Chromosome doubling in haploids – method optimization. Table 3 shows the results grouped according to the content of 2,4-D in the regeneration medium. The control medium supported production of callus and buds on majority of explants. Most shoots (9), including DH shoots (6) were obtained with the application of 1.5 mg·dm^{-3} 2,4-D.

There was no statistically significant effect of 2,4-D concentration in the medium on production of callus, bud and shoot formation. However, there was a statistically significant interaction between 2,4-D concentration and genotype with respect to the regeneration of callus and bud formation. A total of 10 out of 23 explants that regenerated shoots produced DH shoots. The remaining explants produced mainly haploids.

Table 3. Number and percentage of explants producing callus, buds and shoots at 2,4-D concentration in 1.7 medium (values marked with the same letter do not differ statistically)

2,4-D (mg × dm^{-3})	No. of explants cultured	Explants regenerating					
		callus		buds		shoots (DH)	
		No.	%	No.	%	No.	%
1 (control)	2014	1552	77.06 [a]	48	2.38 [a]	3 (2)	0.15 [a]
1.5	1781	1292	72.54 [a]	26	1.46 [a]	9 (6)	0.51 [a]
2	1854	1306	70.44 [a]	34	1.83 [a]	7 (2)	0.38 [a]
2.5	1420	1009	71.06 [a]	11	0.77 [a]	4 (0)	0.28 [a]
Total	7069	5159	72.98	119	1.68	23 (10)	0.33

The results of regeneration from different leaf sectors indicated that callus, buds and shoots regenerated from all sectors (fig. 1C). Most of the buds were obtained from sector 4 (proximal part the main vein). However, the percentage of explants producing shoots was comparable among all sectors (tab. 4). Moreover, there were no genotypic differences – haploids of different genetic origin did not differ significantly in bud and shoot formation.

Table 4. Number and percentage of explants producing callus, buds and shoots from the leaf sector (values marked with the same letter do not differ statistically)

Sector*	No. of explants cultured	Explants regenerating					
		callus		buds		shoots (DH)	
		No.	%	No.	%	No.	%
1	2521	1526	60.53 [a]	21	0.83 [a]	6(0)	0.24 [a]
2	3301	2612	79.13 [b]	72	2.18 [b]	12(7)	0.36 [a]
3	670	530	79.10 [b]	8	1.19 [b]	2(1)	0.30 [a]
4	577	491	85.10 [b]	18	3.12 [c]	3(2)	0.52 [a]
Total	7069	5159	72,98	119	1,68	23(10)	0.33

* Sectors: 1 – the distal part of the lamina, 2 – the proximal part of the lamina, 3 – the distal part of the main vein, 4 – proximal part of the main vein

Acclimatization of regenerated plants to greenhouse conditions. Despite careful treatment in the initial phase of growth in the greenhouse, almost 60% of the plants did not survive (tab. 5). Therefore, the acclimatization procedure was modified as follows:

the shoots rooted in ½ MS medium were transferred to sterile swelling plugs, watered with 0.15% Topsin®, put back into the jar and left in the phytotron. The jars with plants that continued to grow were unsealed, and after several days were transferred to the greenhouse (fig. 1D). During this period the plants displayed an increase in the colour intensity and leaf thickness. After 1–2 weeks the plants were transplanted from swelling plugs into standard pots filled with peat substrate and handled according to the standard procedure as described previously. After the application of the modified acclimatization method over 80% of plants transferred to the greenhouse survived (tab. 5).

Table 5. The effect of the improved acclimatization method

Origin	Standard acclimatization			Improved acclimatization		
	plants acclimatized	plants that survived		plants acclimatized	plants that survived	
	No.	No.	%	No.	No.	%
Marcel	26	15	57.7	67	58	86.6
Junak	5	2	40	69	61	88.4
Sonate	0	0	0	22	9	40.9
Śremski	19	4	21.1	3	3	100
Total	50	21	42	161	131	81

Table 6. Morphological characteristics of DH lines of different origin

Line	Main shoot			Leaves (mm)			Degrees of infestation*		
		average length (cm)							
	n	plant (SD)	internode (SD)	n	average petiole length (SD)	average lamina width (SD)	average lamina length (SD)	9.08	13.08	16.08
22/M/17	6	245.0 (12.2)	6.2 (0.5)	6	210.8 (9.7)	206.7 (25.4)	140.8 (13.9)	2–3	4	5
28/M/18	6	306.5 (20.1)	8.0 (0.8)	5	234.0 (15.2)	236.0 (4.2)	174.0 (10.8)	1–2	2	2
38/J/5	6	341.3 (66.4)	8.6 (1.2)	3	185.0 (13.2)	200.0 (13.2)	151.7 (10.4)	1	2	2
39/S/23	5	289.2 (22.9)	7.1 (0.5)	5	142.0 (8.4)	182.0 (18.2)	135.0 (10.0)	1	1	2
42/Sr/7	6	339.2 (56.0)	7.6 (0.6)	6	194.2 (14.6)	210.0 (6.3)	160.0 (7.1)	5	8	9
44/P×M/78	5	309.0 (17.0)	7.4 (0.3)	5	144.0 (25.1)	196.0 (13.4)	159.0 (2.2)	4	8	9

M – Marcel, J – Junak, So – Sonate, Sr – Śremski SD – standard deviation; * – 9-degree scale of infestation by downy mildew: 1 – no symptoms, 9 – the highest degree of infestation; n – number of plants characterised

Characteristics of the DH lines. Plants within the lines were visually uniform (fig. 1E). The lines that originated from the same haploid plant had similar morphological characteristics. Among the 24 described lines, 10 were monoecious and 14 were female. Nine lines displayed sex type that was different that of the donor plant. Most of

the lines had 1–2 flowers per node, but there were also some plants with 3–5 female flowers (and fruits) per node (fig. 1F) or 7–11 male flowers (fig. 1G). All lines displayed a typical green leaf colour and white fruit spines. Some plants were characterised by an altered morphology of individual flowers, diminutive leaves growing from the leaf angles and bright leaf sectors (fig. 1H–J) respectively). Seven lines showed a lower degree of infestation by downy mildew than the donor cultivars. tab. 6 shows an example of the morphological characteristics of several DH lines of different origin.

DISCUSSION

Haploid plant development. It was possible to obtain haploid embryos and plants in all genotypes, therefore the described here method can be considered as universally applicable. Compared to a very high yield ranging from 1.2 to 4.8 e/f obtained by Przyborowski and Niemirowicz-Szczytt [1994] and Çağlar and Abak [1996a, b], the presented here results are not satisfactory for the F_1 hybrids, but comparable for the double hybrids (from 0.46 to 0.59 e/f). Various researchers report obtaining cucumber haploids by the means of pollination with irradiated pollen; this methodology is constantly being improved and tested on other cucurbit plants. Since the methodology is comparable, it can be inferred that the genotype of the donor plant is responsible for differences in the efficiency of haploid embryo production.

Approximately 20% more embryos and twice as many haploid plants were obtained from a similar number of fruits from the double hybrids as from single hybrids. These results could be due to the heterosis effect, which is strongly manifested in the yield of fruits, depending mainly on the number of female flowers and earliness in cucumber.

Chromosome doubling in haploid plants. The percentage of DH plants obtained by regeneration from haploid leaf explants was variable and generally low. From 246 haploids subjected to doubling only 33 DHLs were obtained. Sztangret-Wiśniewska et al. [2006] observed a similar reduction of plant material.

It was assumed that increasing the content of 2,4-D in the medium would lead to an increase in doubled plant regeneration. It is known that 2,4-D may affect genetic stability of tissue *in vitro* [Machakova et al. 2008]. However, the effect of concentration of 2,4-D in the medium on shoot regeneration was not significant. According to Usman et al. [2011], who demonstrated a positive effect of increasing the 2,4-D content in the medium for embryogenesis in cucumber leaf explants culture, it seems appropriate to repeat the experiment using more haploid plants with a higher capacity to regenerate shoots.

Our results suggest that the developmental age of tissues subjected to regeneration can have a significant influence on regeneration and doubling efficiency. This observation is supported by the results obtained by Kwack and Fujieda [1988] and Faris et al. [2000].

During the optimization experiments the best results were obtained with explants prepared from leaf sectors containing many veins, probably due to the presence of cambium in the leaf veins. The elimination of the distal part of the leaf blade would reduce the amount of work and needed materials and should be tested in the future.

A gradual reduction in plant material was observed in subsequent steps of DH line production, in particular during the doubling of chromosomes and the acclimatization process. This phenomenon was also described by Dirks [1996] and Gémes Juhász et al. [2002]. Thus the doubling stage presents a "bottleneck" that requires further optimization. This is especially so because an inefficient method of doubling restricts the use of DH lines and makes the comparison of the DH lines and conventional lines difficult [Yesitir and Sari 2003, Sztangret-Wiśniewska et al. 2006]. Our results indicate that each cultivar requires a different, specifically optimized protocol of chromosome doubling – both for the regeneration from haploid leaves and the colchicine treatment. For example, Lim and Earle [2009] increased the number of doubled melon plants by immersing shoot explants in a colchicine solution, and then placing the explants on a medium containing a combination of growth regulators that supported the growth by inhibiting the effect of colchicine. Other experiments by Gałązka [unpublished] suggest that enriching the 1.7 medium with colchicine may increase the chance of obtaining doubled haploids from leaf explants.

Acclimatization of regenerated plants to the greenhouse conditions. In order to alleviate the stress of acclimatization, the growth conditions of plants were modified beginning with the culture in the phytotron. Previous protocols called for the plants to be transferred directly from the phytotron to the greenhouse, a process resulting in a drastic change of lighting and temperature. Once in the greenhouse the medium was replaced by a potting substrate and then humidity was gradually lowered. The resulting survival of plants was about 40%. The optimized protocol called for the acclimatization to be initiated in the phytotron by placing the plants and the culture medium in swelling plugs and by lowering the humidity. Subsequently the plants were transferred to the greenhouse, where more than 80% of plants continued to grow. A higher percentage of plants surviving acclimatization was noted among plants derived from the double hybrids than from the F_1 hybrids, which was likely due to the heterosis effect.

The root system of cucurbits is susceptible to damage. Furthermore, according to Fila et al. [1998] the root system of plants from *in vitro* culture is less conductive. Therefore, it seems that the number of plants surviving acclimatization was higher after using sterile swelling plugs because of more optimal conditions for root development.

There are several methods of *in vitro* plant hardening. However, there is no literature reference as to the use, either *in vitro* or *in vivo,* of swelling plugs. These plugs due to their universal applicability could be used for plant acclimatization in other species.

DH lines. The production of seeds from the doubled haploids was challenging, and it significantly reduced the number of DH plants. Only 33 DHs were successfully self-pollinated. In the case of female plants it was possible to stimulate the formation of male flowers by the application of silver nitrate. However, a greater obstacle is a reduced fertility of DH plants as noted by Gémes Juhász et al. [2002] and Sztangret-Wiśniewska et al. [2006].

The published so far papers on haploid and doubled haploid induction in cucumber focus primarily on the efficiency of the methodology (number of H and DH plants) and do not provide information on the number of doubled haploids that reproduce and give DH lines and could be used for breeding [Faris et al. 2000, Diao et al. 2009]. The use of DH lines in the F_1 hybrid production is reported only sporadically. In presented here

research, the characteristics of 24 DH lines were described. The collected data indicates that the DH lines are uniform in terms of morphological traits and are of value for cucumber breeding.

CONCLUSIONS

Pollination with irradiated pollen resulted in production of haploid plants from eight cucumber genotypes. A weakness of this method is the need to double the number of chromosomes in the obtained plants. A satisfactory number of haploid plants was received, however, a gradual reduction of plant material was observed after subsequent steps of chromosome doubling and plant acclimatization in the greenhouse.

The presented here results indicate that progress in the area of DH production is difficult and can be achieved by small steps only. Despite an extensive testing of various parameters, the presented here results can be hardly considered satisfactory, as the tested protocols, did not significantly increase the number of haploids and DH lines. Nevertheless, the reduction in the yield of plant material was diminished by the use of a mild form of plant acclimatization. Ultimately, numerous valuable DH lines with interesting features that may be beneficial in a breeding program were obtained.

ACKNOWLEDGEMENTS

The research was supported by a project grant from Polish Ministry of Agriculture and Rural Development (MRiRW: HOR hn 2008–13/92). The authors wish to thank Mirosława Koronkiewicz, Michał Tracz, Aleksandra Korzeniewska, Teresa Gałecka, Elżbieta Gniazdowska and Joanna Szewczyk for their assistance during research.

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OD ZAPYLENIA DO LINII DH – WERYFIKACJA I OPTYMALIZACJA PROTOKOŁU OTRZYMYWANIA PODWOJONYCH HAPLOIDÓW OGÓRKA

Streszczenie. Przeprowadzono pełną procedurę otrzymywania podwojonych haploidów ogórka, która składała się z następujących etapów: indukcji rozwoju haploidalnych zarodków poprzez zapylenie napromieniowanym pyłkiem, izolacji i kultury *in vitro* zarodków,

rozwoju roślin haploidalnych, podwojenia liczby chromosomów poprzez regenerację z eksplantatów liściowych, aklimatyzację, samozapylenia podwojonych haploidów i charakterystyki linii DH. Haploidy i podwojone haploidy otrzymano w przypadku wszystkich ośmiu genotypów wykorzystanych w doświadczeniach jako rośliny donorowe. Rośliny donorowe porównano pod względem liczby haploidalnych zarodków i roślin DH jaką z nich otrzymano. Oceniono wpływ genotypu i wieku rośliny donorowej na regenerację i liczbę otrzymanych regenerantów DH. W celu optymalizacji metody podwajania liczby chromosomów zmodyfikowano skład pożywki, a eksplantaty podzielono na cztery grupy w zależności od sektora liścia z którego zostały pozyskane. Ulepszono sposób aklimatyzacji roślin DH do warunków szklarniowych. Otrzymano wyrównane linie DH, które mogą być wykorzystane w hodowli.

Słowa kluczowe: aklimatyzacja, *Cucumis sativus* L., indukcja haploidów, napromieniowany pyłek, kultura zarodków, cytometria przepływowa

Accepted for print: 19.03.2015

For citation: Gałązka, J., Słomnicka, R., Góral-Radziszewska, K., Niemirowicz-Szczytt, K. (2015). From pollination to DH lines – verification and optimization of protocol for production of doubled haploids in cucumber Acta Sci. Pol. Hortorum Cultus, 14(3), 81–92.