Acta Bot. Neerl. 39(2), June 1990, p. 153–161

Somaclonal variation in cucumber (*Cucumis sativus* L.) plants regenerated via embryogenesis

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SUMMARY

Plants were regenerated via embryogenesis from leaf explant-derived callus of cucumber (*Cucumis sativus* L. cv. Hokus). Somaclonal variation was evaluated in the regenerated plants (R_1 generation) and in the selfed progeny (R_2). Considerable variation was found in the young R_1 plants, but it was of a transient nature. Mature R_1 plants showed slight abnormalities which were not directly transmitted to the progeny. Seed production of the R_1s was variable and very poor, as was seed germination. This is probably caused by dysfunctioning of generative tissue and cells and might have a genetic base. Of eight R_2 lines analysed, one was tetraploid and five expressed a low extent of variability, probably of a genetic nature. Most of the R_2 plants were phenotypically normal. In a yield analysis, genetic variation useful for improvement of crop performance has not been detected. It is suggested that in cucumber the generative phase acts as a sieve for genetically based somaclonal variation.

Key-words: Cucumis sativus L., cucumber, somatic embryogenesis, somaclonal variation, variants.

INTRODUCTION

The phenomenon of somaclonal variation, i.e. variation displayed among tissue culture-derived plants, has been described for several plant species (Orton 1984; Evans and Sharp 1986; Semal 1986). This variation is caused by changes in the DNA (genetic variation), by alteration of the gene expression (epigenetic variation), or by temporary after-effects of the tissue culture conditions (cf. Meins 1983). Various authors have restricted the use of the term somaclonal variation to those changes that have a genetic base (De Klerk 1990). The genetically based variation has been considered as a possible new source of variation for plant breeding. On the other hand, it would be a severe stumbling block in applications of biotechnological methods for improving plants (Scowcroft *et al.* 1987). In cucumber, *Cucumis sativus* L., procedures of plant regeneration from somatic tissues, suspension cultures as well as from protoplasts have been published (see review: Malepszy 1988; Chee and Tricoli 1988; Colijn-Hooymans *et al.* 1988; Kim *et al.* 1988; Bergervoet *et al.* 1989), but so far only one report has evaluated somaclonal variation in the plants obtained (Malepszy and Nadolska-Orczyk 1989). The emphasis in that study was on morphological characteristics in the selfed progeny of regenerated plants and it was concluded that somaclonal variation occurred only to a low extent (Malepszy 1988).

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In this study, variability in both the regenerated plants and their selfed progeny was evaluated. Attention was given to fertility after self-pollination and frequency of seed germination. The main assessment of the somaclonal variation was extended to the selfed progeny, qualitative as well as quantitative traits were evaluated.

MATERIALS AND METHODS

Plant material

The study was carried out with the highly inbred pickling cucumber (*Cucumis sativus* L.) cv. Hokus (Rijk Zwaan, De Lier). Seeds were sterilized with 2% sodium hypochlorite for 20 min and aseptically germinated in 250 ml honey jars on MS medium (Murashige and Skoog 1962) with 2% (w/v) sucrose. After 8 days, shoot cuttings including the cotyledons were taken and subcultured, two per jar, on the same medium for the production of leaves. Culture conditions were 16 h light (Philips TL 34, 2500 lux) and 25 ± 1°C.

Regeneration procedure

After 3 weeks of seedling growth, the nearly fully expanded second leaf was excised and 5 mm discs were prepared. They were incubated on MS medium supplemented with 3% (w/v) sucrose, 1 g/l trypton (L42, Oxoid), 4 μM 6-benzyladenine (BA) and 4 μM 2,4-dichlorophenoxyacetic acid (2,4-D). The pH was adjusted to 5·6 prior to addition of 0·6% (w/v) bacteriological agar (Oxoid). Autoclaving was carried out at 120°C for 15 min and 25 ml aliquots of medium were dispensed into 9 cm plastic Petri dishes. Five leaf discs were incubated per dish. The cultures were kept in complete darkness at 27 ± 1°C and transferred to fresh medium every 3 weeks.

Pale greyish callus was produced which gave rise to the development of protuberances of a bright yellow embryogenic callus after the second and third subculture. After reaching a diameter of about 1 cm, the yellow calli with embryoids were transferred for shoot development to MS medium containing 1% (w/v) sucrose, 0·6% (w/v) agar, 0·5 μM kinetin and 0·1 μM indole-3-acetic acid (IAA), and placed under 16 h light (Philips TL 34, 2500 lux) at 25 ± 1°C. Shootlets were transferred for rooting to the same medium without hormones. After about 2 weeks on this medium, plantlets were ready for acclimatization in small rockwool plugs in the greenhouse.

Evaluation of the regenerated plants (R_1)

Plants were grown to maturity in the greenhouse on 12-cm thick rockwool slabs in bags of plastic film. Water and nutrients were given by means of trickle irrigation, each plant having a dripper. Growth characteristics and typical abnormalities of the plants were evaluated. The ploidy level was determined by measuring the amount of DNA in isolated nuclei using a flow cytometer (Galbraith *et al.* 1983). At least two selfings were made per plant to produce R_2 lines. Seed production was evaluated for each R_1 plant and average seed weight determined by weighing 100 seeds.

Analysis of selfed progenies (R_2)

Seeds were selected from 10 R_1 plants, including five normal, four variant, and one tetraploid plant. 'Hokus' seeds, obtained directly from the seed company, served as control. Forty seeds from each were sown, except for the tetraploid with only 29 seeds. Seed germination was evaluated. Seedlings were potted, not more than 30 per line, and

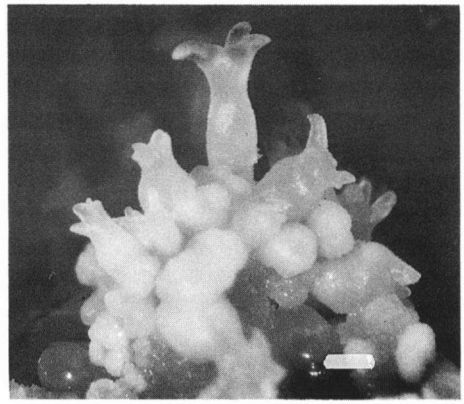

Fig. 1. A yellow callus of *Cucumis sativus* cv. 'Hokus', with healthy embryoids on the top. White bar represents 1 mm.

those dying early and with less vigorous growth were removed. Thereafter, healthy plants were grown under standard rockwool conditions in the greenhouse for evaluation of qualitative variants and for yield analysis. The numbers of plants tested in the yield experiment are shown in Table 3. The order of the plants in the greenhouse was completely randomized. The yield experiment was carried out during the period August–October. Temperature in the greenhouse was set at 23°C day/19°C night, and the water temperature in the rockwool slabs was 25°C. After initiation of flowering, colonies of bees were placed in the greenhouse.

Three quantitative traits were evaluated: (1) plant height, (2) number of leaves, both 8 weeks after sowing, and (3) total number of fruits produced during a period of 5 weeks (fruit harvesting twice a week), starting from first fruiting. The data collected for each trait were submitted to analysis of variance. The variances within the R_2 populations and those in the control population were compared by considering variance ratios.

RESULTS

Plant regeneration. After three subcultures, 43 out of 100 incubated leaf discs had formed yellow embryogenic callus. About 800 embryoids could be discerned on the callus surfaces. On shoot formation medium, however, a large part of the embryoids showed difficulties in forming plants. Only a few embryoids, located on the top of the callus (Fig. 1), elongated and developed into normal shoots, afterwards these formed roots on rooting medium. Eventually, 44 plants were transferred to the greenhouse.

Evaluation of the regenerated plants. In the greenhouse, the R_1 plants initially grew poorly, having short internodes and many leaves with an altered morphology. They also flowered early from the basal nodes. Five of the plants died at this stage. After 2–3 weeks, however, all remaining plants started to elongate. They were phenotypically quite similar to control plants, but some of them could still be distinguished by compact growth and dark green leaves. Five plants formed large, dark green leaves with deeply serrated leaf margins and large flowers. Determination of the nuclear DNA content showed that these plants were tetraploids, while all other plants were diploids.

Table 1. Number of seeds per selfed fruit in R_1 plants of *Cucumis sativus* cv. 'Hokus'. Normal 'Hokus' plants produce 201–300 seeds per fruit

	Classes of seed number			
	0	0–100	101–200	201–300
Number of R_1s	5	14	16	4

Table 2. Numbers of variant plants, at successive stages of plant development, in 10 R_2 lines of *Cucumis sativus* cv. 'Hokus', in comparison with a control (Con.)

R_2 lines		Seedlings		Young plants		Mature plants	
No.	Variant R_1 parent	Total	Cripple	Total	Dying/less vigorous	Total	Qualitative variants
1	—	18	1	17	1	16	1 de.
2	—	19	—	19	1	18	—
3	—	27	—	27	—	27	—
4	—	30	1	29	2	27	1 l.e./2 de./1 wi.
5	—	1	1	—	—	—	—
6	s.i.	39	—	30	—	30	—
7	dw.	22	2	20	—	20	5 d.l.
8	dw.	40	—	30	2	28	1 d.l.
9	d.l.	0	—	—	—	—	—
10	4x	7	—	7	1	6	6 4x
Con.		40	—	30	—	30	—

From each R_2 40 seeds were sown. Not more than 30 seedlings were potted per line. The tetraploid R_2-10 had only 29 seeds.

s.i. = short internodes and dark green leaves; dw. = dwarf; d.l. = distorted leaves; 4x = tetraploid; de. = determinate apex; l.e. = long epicotyl; wi. = wilting.

Seed production of the R_1 plants was low compared with 'Hokus', and showed a large variation, ranging from 0 to 300 seeds per fruit (Table 1). Five plants, of which four were tetraploids, produced parthenocarpic fruits only. One tetraploid produced one fruit with 29 seeds from three pollinated flowers. The 100-seed weight ranged from 2·6 to 3·0 g for seeds from the R_1 plants, whereas the 100-seed weight of the control seeds was 2·2 g.

Analysis of selfed progenies. Seed germination of the R_2 lines was variable, ranging from 0 to 100%, and much lower than in the control (Table 2). Similar germination results were found with 10 other seed samples germinated in Petri dishes on moistened filter paper. Many seeds were brown, although containing normal embryos, whereas others contained mis-shapen embryos (Fig. 2). Several seeds only germinated partly.

At the seedling stage, a small number of seedlings were cripple, showing failure of hypocotyl growth and large white parts on the cotyledons (Table 2). One seedling had pale

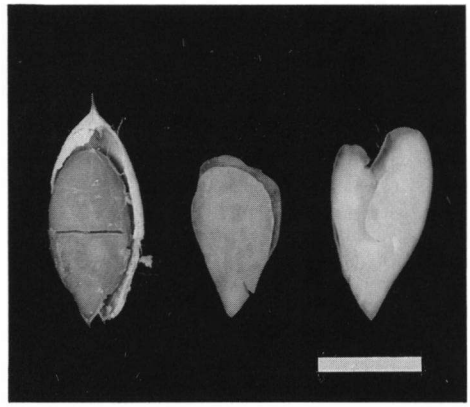

Fig. 2. Abnormal, unviable embryos in the R_2 generation of *Cucumis sativus* cv. 'Hokus'. From left to right: brittle embryo from a brown seed, embryo of reduced size, and embryo with abnormally folded and mis-shapen cotyledons. White bar represents 5 mm.

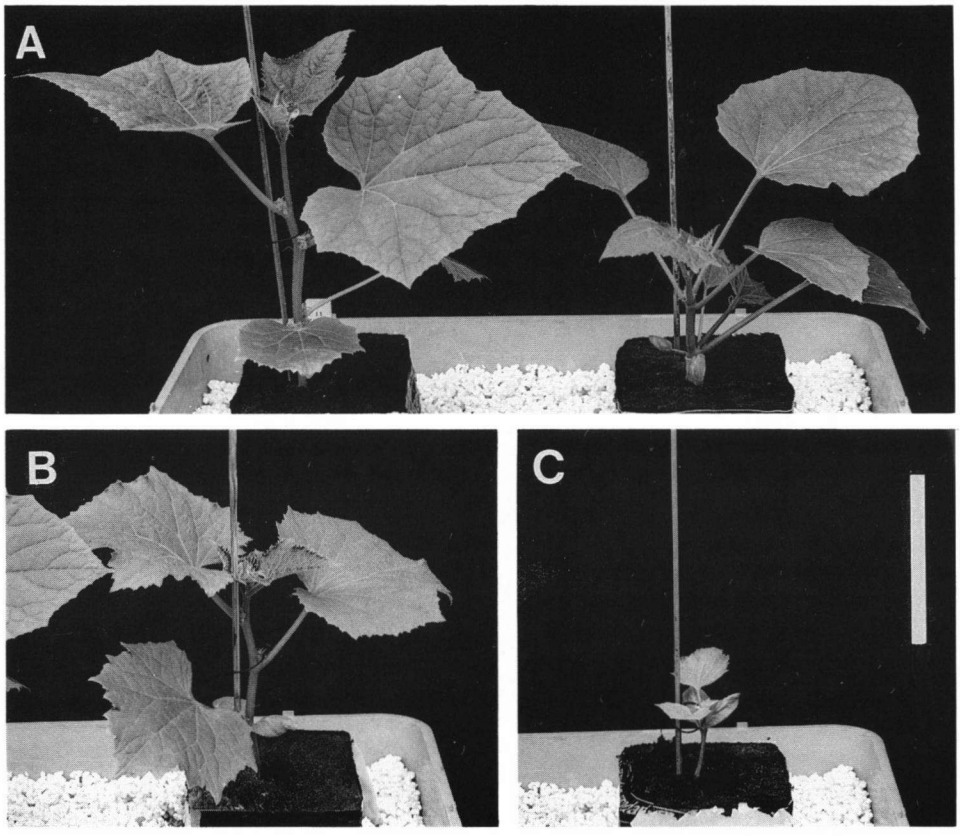

Fig. 3. Variants in the R_2 generation of *Cucumis sativus* cv. 'Hokus'. (a) Control plant (left) beside a plant with determinate growth of the apex and side-shoot formation from the base; (b) tetraploid plant with dark green leaves and deeply serrated leaf margins; (c) cripple seedling with pale green cotyledons. White bar represents 10 cm.

Table 3. Means, extreme values, and variances of quantitative traits of R_2 lines of *Cucumis sativus* cv. 'Hokus', in comparison with a control (Con.)

	Plant height (cm)				No. leaves				No. fruits			
R_2 (*n*)	$\bar{x}$	Min.	Max.	Variance	$\bar{x}$	Min.	Max.	Variance	$\bar{x}$	Min.	Max.	Variance
1 (16)	184*	155	208	272	22·8*	20	25	1·6	19·5	14	27	14·1
2 (18)	193*	159	205	148	23·4*	22	25	1·1	20·2	11	29	17·6
3 (27)	184*	155	207	159	23·0*	21	25	1·2	19·6	12	26	13·6
4 (27)	174	120	208	312	21·7	16	25	3·0*	18·5*	3	27	26·1
6 (30)	188*	156	212	195	22·7*	21	24	0·9	21·5	7	28	29·2
7 (20)	171	116	203	545*	21·3	18	24	2·3	14·0*	4	25	30·3
8 (28)	161	108	200	367	20·5	16	23	1·8	16·9*	5	29	28·1
10 (6)	129*	115	139	100	20·5	20	21	0·3	23·7*	19	32	21·5
Con. (30)	168	131	196	208	20·8	19	23	1·2	21·1	11	30	26·9

*Significantly different from the control ($P < 0{\cdot}05$).

green cotyledons and formed very small leaves (Fig. 3c). At the young plant stage, a few plants suddenly died or had clearly reduced vigour. These plants were removed. In the R_2 generation, the young plants never showed the typical difficulties in initial growth as described for the R_1 generation.

From the plants which reached the mature plant stage, only a few showed qualitative variations (Table 2). In R_2-1, one plant was found with determinate growth at the first node (Fig. 3a). R_2-4 included one plant with a long epicotyl (6 cm instead of the normal 1 cm), two plants with determinate growth, which was expressed in one or two nodes along the stem, and one plant which suffered from wilting in full sunlight. In R_2-7, five plants formed distorted leaves with reduced leaf blades. The expression of this malformation equals a 1:3 segregation, but the same variant in R_2-8 occurred less frequently. In R_2-10, all plants had dark green leaves with deeply serrated leaf margins, typical of the tetraploidy (Fig. 3b). On average, the percentage of qualitative variants was approximately 10%. On the whole, most of the R_2 plants did not show conspicuous phenotypic variation.

In the analysis of the quantitative traits, R_2 lines were found which outperformed the control (Table 3). Average plant height and leaf number of the R_2s 1, 2, 3 and 6 were significantly higher than in the control. These R_2s, however, were not different from the control in number of fruits. R_2s 4, 7 and 8 produced significantly smaller numbers of fruits than the control. The tetraploid R_2-10 differed from the control in plant height as well as in number of fruits. Statistical evaluation of the variances showed only two R_2 variances to be significantly different from that in the control, namely in R_2-7 for height and in R_2-4 for leaf number. However, these significant differences are mainly due to distinctly less vigorous plants.

DISCUSSION

In the pickling cucumber 'Hokus', on the whole, a high degree of somaclonal variation was found upon regeneration via embryogenesis. However, after self-pollination of the regenerated plants, the number of variant type plants in the R_2 was low. Most of the plants in that generation did not show conspicuous phenotypic variation. This is in accordance with earlier reports by Malepszy (1988) and Malepszy and Nadolska-Orczyk (1989).

Much variation was observed after transplanting the *in-vitro* plantlets into rockwool plugs. Poor, compact growth and precocious flowering were the most obvious characteristics, but they disappeared after some time and were never exhibited in the R_2 generation. Similar observations have been made by Ziv and Gadasi (1986) and Colijn-Hooymans *et al.* (1988). The altered appearance of the young R_1 plants has probably been due to hormonal imbalances as a direct result of the tissue culture procedures.

Variability occurred to a moderate extent at the mature stage of the R_1 plants. The variation concerned dwarf growth and deformed leaves, but was not transmitted to the selfed progeny. Such variation might be related to physiological after-effects or temporary changes in gene expression due to the tissue culture history, rather than to mutational changes (Lörz and Brown 1986). An obvious mutational event expressed in the R_1 generation was tetraploidy, which was transmitted to the selfed progeny.

Considerable variation came to expression during generative multiplication. Seed set of the R_1 plants was very poor. This has also been observed in other crops after tissue culture (Engler and Grogan 1984). An important reason might be the presence of gross chromosomal abnormalities caused by tissue culture, leading to abnormal meiosis. Part of the low

seed set was associated with tetraploidy, which generally causes decreased fertility in cucumber (Smith and Lower 1973; den Nijs and Visser 1984). In addition to low fertility of the R_1 plants, seed germination of the R_2 generation was very variable and generally low. Defective seed variants and early variants affecting seedling development are commonly found in other plants after tissue culture (McCoy and Phillips 1982; Gavazzi *et al.* 1987; Lee and Phillips 1987). Whether these variants in cucumber have a genetic basis, or have been brought about by the preceeding maternal problems during sexual multiplication is not clear and should be examined in progeny tests of further generations.

Only a low level of variability was found at the R_2 seedling and plant stages of development. Variation concerned developmental abnormalities, less vigorous growth and early death. Only a few plants showed qualitative variations, which are most probably simply inherited. Most of these variants are similar to known cucumber mutants (cf. Robinson *et al.* 1976; Kubicki 1983; Pierce and Wehner 1989). Whether they are identical to them has to be checked in complementation tests.

Regarding the quantitative traits, it must be concluded that genetic variation, useful for improvement of performance of the crop, has not been detected. This may be due to the high level of variation in the control 'Hokus'. It is generally known in cucumber that growth and yield are subject to considerable environmental influence (Lower and Edwards 1986). Remarkably, some R_2s exceeded the control in plant height and number of leaves. The higher seed weight of these R_2s might have contributed to their better early performance, rather than a homozygous nuclear or cytoplasmic mutation.

Finally, as an interpretation of the results, the hypothesis might be put forward that much of the somaclonal variation that appeared from the cucumber tissue culture is of a genetic nature, but that a large part of it is lost during generative multiplication. The sexual process might act as a sieve for aberrant mutational events. This could be why only a small number of the supposed genetic variants were present. If this is true, then it will be difficult to discover new genetic traits, such as resistances which have not been found within the species so far. For the application of biotechnological methods for improvement of the crop, the initially high extent of somaclonal variation might be troublesome. The supposed strong generative sieve, however, will exclude several aberrations from entering into the progeny after selfing or crossing.

ACKNOWLEDGEMENTS

The authors thank J. H. W. Bergervoet for technical assistance, and Dr C. M. Colijn-Hooymans, Dr J. J. M. Dons and Dr S. P. C. Groot for critically reading the manuscript.

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