Research Article

Polyploidization Facilitates Biotechnological In Vitro Techniques in the Genus Cucumis

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Received 28 June 2010; Revised 27 September 2010; Accepted 19 November 2010

Academic Editor: Neal Stewart

Prezygotic interspecific crossability barrier in the genus Cucumis is related to the ploidy level of the species (cucumber (C. sativus), $x=7$; muskmelon (C. melo) and wild Cucumis species, $x=12$). Polyploidization of maternal plants helps hybridization among other Cucumis species by overcoming prezygotic genetic barriers. The main objective of this paper is to compare the results of several methods supporting interspecific crosses in cucumber without and with polyploidization (comparison between diploid (2x) and mixoploid (2x/4x) cucumber maternal plants). Mixoploid plants were obtained after in vivo and in vitro polyploidization by colchicine and oryzalin. Ploidy level was estimated by flow cytometry. Embryo rescue, in vitro pollination, and isolation of mesophyll protoplasts were tested and compared. Positive effect of polyploidization was observed during all experiments presented by higher regeneration capacity of cultivated mixoploid cucumber embryos, ovules, and protoplasts. Nevertheless, the hybrid character of putative hybrid accessions obtained after cross in vivo and in vitro pollination was not confirmed.

1. Introduction

Polyploidization (chromosome doubling) can be either natural (spontaneous) or induced. Spontaneous chromosome doubling is due to endomitosis and endoreduplication [1]. Induced polyploidization is routinely used by colchicine treatment; but fewer toxic agents (oryzalin, trifluralin, nitrous oxide, etc.) are now being used [2]. Nevertheless, the colchicine manipulation was still used for chromosome doubling. High concentration and duration of this mitotic toxin was used during the experiments with petiole explants of Echinacea purpurea L. [3]. Polyploidization has played a major role in ornamental plant breeding. Wu et al. [4] focused on the polyploidization experiments within the Oriental lilies (diploid oriental cultivars of Lilium—“Con. Amore” and “Acapulco”) (to obtain diploid eggs). Their results implied that the polyploidization is a powerful method to create novel variations in the Oriental lilies. Induced polyploidization may facilitate to produce hybrids among species containing different basic chromosome numbers [5–7]. Some few preliminary results relating to polyploidization in interspecific hybridization in the genus Cucumis have been reported [8]. This step may overcome the prezygotic barrier, caused by different chromosome numbers, and we can produce interspecific hybrids. Hybridization within the genus Cucumis has been used in breeding programs. By using several biotechnological methods (embryo rescue, in vitro pollination, protoplasts isolation, and fusion), hybrids between C. sativus with C. melo and other Cucumis genotypes were produced in sporadic cases. Chen and Staub [9] restored fertility by chromosome doubling with colchicine in F1 plants from hybridization between C. sativus and C. hystricis.

Within the genus Cucumis, the basic chromosome number is variable caused by two distant origins. Cucumber (C. sativus) with $x=7$ is considered to be of Asiatic origin and muskmelon (C. melo) and wild Cucumis species are of African origin ($x=12$). Transfer of economic important genes such as resistances to various pathogens, found in the wild Cucumis species, is a successful interspecific hybridization methodology that needs to be developed [10].

Embryo rescue saves the immature embryos after interspecific crossing. Hybrids were obtained between C. sativus and other Cucumis genotypes [11, 12]. Furthermore, hybrids
in other crops species have been produced through embryo rescue method (e.g., Brassicaceae [13, 14], Lilliaceae [15], and Lens [16]). The composition of media and the cultivation conditions play an important role in these crosses.

Embryos are rescued in crosses where postfertilization is a problem, and embryo aborts after 18 to 21 days after fertilization. However, an alternative is to pollinate and fertilize eggs in vitro in distantly related species. Isolated ovules and pollen grains are cultivated together in the special media. Interspecific and intergeneric hybrids have been obtained in several cases [17–19]. However, in the Cucumis species, hybrid plants have not been reported. The highest level of regeneration, achieved, was the callus formation [20, 21], and true hybridity was not established [22].

In addition to classic sexual hybridization, distantly related species can be hybridized by somatic hybridization. In Cucumis species, isolation and fusion of protoplasts from C. sativus, C. melo, and wild Cucumis species have been reported [23, 24]. Plants through protoplast fusion have been reported in the genus Brassica [25–27] and genus Solanum [28, 29].

This paper reports on utilization of polyploidization in in vitro biotechnological methods which could be used for cucumber breeding. The main aim of this work is to describe and compare the results of these in vitro technique applications in diploid (2x) and mixoploid (2x4x) cucumber plants (used as maternal plants in hybridization). The experiments of in vivo pollination (interspecific), followed by embryo rescue and in vitro pollination (intraspacific and interspecific), and protoplasts isolation in the genus Cucumis were analyzed and evaluated.

2. Materials and Methods

2.1. Plant Source. Various accessions of Cucumis species were used for different in vitro techniques (Table 1). Plant materials originated from the vegetable germplasm collection of the Research Institute of Crop Production (Prague), Department of Gene Bank, at Olomouc, Czech Republic (Web site: (http://www.vurv.cz/), part databases, EVIGEZ) and from the USDA-ARS North Central Regional Plant Introduction Station, Iowa State University, Ames, Iowa, USA. Plants were cultivated in a glasshouse (25°C/15°C day/night) in the Department of Botany, Palacký University in Olomouc, Olomouc, Czech Republic.

2.2. Polyploidization In Vivo. Cucumber seedlings were treated with colchicine (C22H25NO6) (wetted cotton wool on the growth apex with 5 mM and 50 mM colchicine solutions for 2 h; rootlets were submersed in 0.5% colchicine for 24 h; [8]). These influenced plants were used in in vivo cross-pollination and embryo rescue. Oryzalin (C12H18N4O6S) was applied by wetted cotton wool on the growth apex with 30, 60, 90, and 150 μM for 2 h and by submersion of rootlets with 15 and 30 μM for 24 h. These obtained plants were used in in vitro pollination experiments. The ploidy was determined by flow cytometry [8].

2.3. Polyploidization In Vitro. Cucumber embryos were isolated and treated by oryzalin in cultivation medium in Petri dishes (1/2 MS medium). Two concentrations (25 and 45 μM oryzalin) and three different times (8, 24, and 48 h) were examined. The seedlings produced from these treatments were cultivated on OK medium (Table 2) and they were used for protoplasts isolation experiments. The ploidy was determined by flow cytometry.

2.4. Isolation, Staining of Nuclei, and Estimation of Ploidy Level. Flow cytometry was used to estimate ploidy level of in vivo or in vitro cultivated plants after colchicine and oryzalin (polyploidization) treatments. The procedure has been described by Skálová et al. [8]. Relative fluorescence of the nuclei was measured using a PAS flow cytometer (Partec GmbH, Münster, Germany) equipped with a laser.

### Table 1: Cucumis species, abbreviations, and accession numbers used in this study.

| Cucumis species | Abbreviation | Accession number |
|----------------|--------------|-----------------|
| C. sativus (SM-6514/line)* | CS | CZ 09H3900768 |
| C. sativus (Stela F1)** | CSS | CZ 09H3900744 |
| C. sativus (Marketer 430)** | CSM | CZ 09H3900121 |
| C. melo (line MR-1)1 | CM1 | PI 124111 |
| C. melo var. Charentais1 | CM2 | PI 261778 |
| C. melo PMR 452 | CMx | CZ 09H400597 |
| C. melo WMR 292 | CMx | CZ 09H400598 |
| C. melo PMR 52 | CMx | CZ 09H400599 |
| C. anguria var. longipes 1 | CA | PI 249896 |
| C. zeyheri1 | CZ | PI 364473 |
| C. metuliferus1 | CME | PI 292190 |

Explanatory Notes. *Cucumber genotype used for in vivo polyploidization and for following in vivo pollination; ** cucumber genotype used for in vivo polyploidization and for following in vitro pollination; *** cucumber genotype used for in vitro pollination and for following protoplasts isolation; 1 Cucumis genotypes used for in vivo pollination with mixoploid cucumber; 2Cucumis genotypes used for in vitro pollination with mixoploid cucumber.
2.5. Media for Embryo Rescue Culture after In Vivo Pollination. Four types of media were used for embryo rescue of potential hybrid embryos derived from interspecific hybridization of diploid (2x) and mixoploid (2x/4x) cucumber maternal plants with other Cucumis species. Fourteen days after in vivo pollination embryos were cultured on various media (medium OK, ON, CW, and GA; [30]; Table 2). All experiments were repeated (ten embryos were cultured per dish repetitively). The details documented in this experiment design were summarized in Table 3. The variability in the system was reflected using standard deviation.

2.6. Media for In Vitro Pollination. Three types of cultivation media were used for in vitro fertilization. Diploid (2x) and mixoploid (2x/4x) cucumber ovules were pollinated by cucumber and muskmelon pollen grains (medium YS, CP, and N; [21, 31]; Table 2). Diploid ovules after successful in vitro fertilization (inspected using microscope Olympus CK40) were transferred on media for embryo rescue described above. The mixoploid ovules after fertilization were cultivated on medium for generating embryo-derived calluses (medium MSN; [31]; Table 2). All experiments were repeated (ten ovules were cultured per dish repetitively). The details documented in this experiment design were summarized in Table 4. The variability in the system was reflected using standard deviation.

2.7. Protoplast Isolation. Mixoploid (2x/4x) cucumber plants (obtained after in vitro polyploidization) were used for mesophyll protoplast isolation. They were isolated according to Navrátílová et al. [34]. Due to high level of contamination in mixoploid isolated protoplasts, the antibiotics were added to enzymatic solution (400 mg/l ampicillin, 100 mg/l chloramphenicol). The regeneration efficiency (the first cell division, microcallus, and callus formation), the viability, and the density of protoplasts were compared. Viability of protoplasts was established using an Olympus fluorescent microscope BX 60, fluorescein diacetate (FDA) stain [35], and a BW filter. Density of protoplasts was determinate by haemocytometer. The variability in the system was reflected using standard deviation.

2.8. Detection of the Putative Hybrids. The DNA of the putative hybrid plants originated from embryo rescue approach (after in vivo cross-pollination) and ovules after in vitro cross-pollination were isolated by standard CTAB procedure. The PCRs with specific primers for ITS regions (internal transcribed spacers) were performed by using FastStart PCR Master Kit (Roche). The PCR products were checked by agarose electrophoresis, purified using GeneElute PCR Clean up Kit (Sigma), cloned into the pGEMT vector (Promega), and introduced into E. coli. Selected bacterial colonies were cultured and used for plasmid DNAs isolation. Isolated plasmid DNA was sequenced and sequences were compared with both muskmelon and cucumber ITS sequences originated from EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/). Also BLAST 2.0 program was used to find sequence similarity and homology.

### Table 2: Cultivation media used in this study.

| Medium | Composition of media | References |
|--------|----------------------|------------|
| OK*    | MS medium + 20 mg/l ascorbic acid, 0.01 mg/l IBA, 0.01 mg/l BA, 20 g/l sucrose, 8 g/l agar | [30] |
| ON*    | MS medium + 1 g/l casein hydrolysate, 0.01 mg/l IBA, 0.01 mg/l BA, 20 g/l sucrose, 6 g/l agar | [30] |
| CW*    | MS medium + 5% coconut water, 200 mg/l α-glutamine, 0.01 mg/l IBA, 0.01 mg/l BA, 60 g/l sucrose, 6 g/l agar | [30] |
| GA*    | MS medium + 0.3 mg/l GA3, 0.01 mg/l IBA, 0.01 mg/l BA, 20 g/l sucrose, 8 g/l agar | [30] |
| YS**   | 600 mg/l Ca(NO3)2 x H2O, 100 mg/l H3BO3, 80 g/l sucrose, 8 g/l agar | [21, 31] |
| CP**   | MS medium + 9.5 mg/l glycine, 500 mg/l casein hydrolysate, 40 g/l sucrose, 4 mg/l IAA, 0.5 mg/l KIN, 5 mg/l GA3, 40 g/l sucrose, 8 g/l agar | [21, 31] |
| N**    | N medium + 500 mg/l casein hydrolysate, 50 g/l sucrose, 8 g/l agar | [21, 31] |
| MSN+++ | MS medium + 30 g/l sucrose, 0.5 mg/l NAA, 1 mg/l KIN, 8 g/l agar | [31] |

* Explanatory Notes. OK, ON, CW, GA, YS, CP, N, and MSN: the abbreviations for media used during the experiments; MS medium [32]; N medium [33]; * media used for embryo rescue after in vivo pollination; ** media used for in vitro pollination; *** medium used for embryogenesis after in vitro pollination; IBA: indole-3-butyric acid; BA: benzyladenine; GA3: gibberellic acid; KIN: kinetin; NAA: α-naphthalene-acetic acid.

### Table 3: Summary of cross in vivo pollination C. sativus (2x; 2x/4x) × Cucumis spp.

| Ploidy of plants/abbreviation | No. of IH pollination | No. of obtained fruits | No. of isolated protoplasts | No. of isolated embryos | No. of regeneration |
|-------------------------------|-----------------------|------------------------|-----------------------------|-------------------------|---------------------|
| 2x (CS)                       | 80                    | 36                     | 980                         | 420                     | 12                  |
| 2x/4x (CSC)                   | 111                   | 72                     | 2376                        | 704                     | 33                  |

* Explanatory Notes. IH: interspecific hybridization.
3. Results and Discussion

Induced polyploidization through colchicine and oryzalin treatments in cucumber was effective. There were, always, obtained mixoploid plants (2x/4x) in diploid cucumber maternal plants after colchicine pretreatment (5 mM and 50 mM, wetted cotton wool on the growth apex and submersion of rootlets). These plants were used for in vivo hybridization experiments. The effect of oryzalin was evaluated to induce mixoploidy at a lower concentration (15, 30, 60, 90, and 150 μM, wetted cotton wool on the growth apex and submersion of rootlets; 25 and 45 μM oryzalin in 1/2 MS medium). Oryzalin is not as toxic as colchicine; in addition, in contrast with oryzalin, colchicine is carcinogenic. The use of less dangerous polyploidization reagents was suggested in other studies [2, 36]. The mixoploid plants, arisen after oryzalin treatment, were used for in vitro pollination and for protoplasts isolation. Methods of in vivo cross-pollination and embryo rescue with mixoploid cucumber maternal plants were tested for the first time. Firstly, there were processed 80 pollination treatments with diploid (2x) cucumber maternal plants, and further 111 treatments with mixoploid (2x/4x) cucumber maternal plants (as sources of pollen selected Cucumis spp. were used, details were specified in Table 1). In diploid plants, thirty six pollinations were successful (45%; in majority cases again C. melo was the most successful pollinating partner) and 980 seeds and 420 embryos were isolated. In case of mixoploid plants, seventy two pollinations were successful (65%; in majority cases again C. melo was the most successful pollinating partner) and 2,376 seeds and 704 embryos were obtained. Figure 1 shows the difference between successful hybridization in Cucumis species using diploid and induced mixoploid lines. Cucumis zeyheri, C. metuliferus, and especially C. melo (var. Charentais) were suitable parents, identified, during this study. These species also showed satisfactory results during embryo rescue experiment [30]. Hybridization using mixoploid maternal plants (documented in Figure 3(a)) showed better results compared to diploid as maternal plants because higher number and higher final regeneration level of the hybrid embryos were observed. Only callus formation, as the highest level of regeneration, was achieved during cross-pollination of diploid cucumber and muskmelon (Figure 3(b)). On the other hand, the intact plants were obtained from cross-pollination between mixoploid cucumbers and muskmelon (C. melo var. Charentais) (Figure 3(c)). The putative hybridism of these plants was inspected. All the sequenced samples of cloned ITS regions were determined as C. sativus. The homology of obtained sequences with C. sativus ITS sequence (AJ488213) was between 98 and 99.5%. Only one sequence showed high level of sequence differences, but BLAST analysis revealed a relation of this sequence to C. sativus genome. The differences are probably results of rather a large-scale mutagenesis in mixoploids after colchicine treatment than those recorded in the interspecific hybridization. Embryo rescue rate is related with the composition of media. It proves that specific components have positive effect on young putative hybrid embryos. Addition of coconut water, casein hydrolysate, and gibberellic acid facilitates rescue of hybrid embryos [8, 30, 37, 38]. Based on this evidence, we obtained the best cultivation results on medium GA in both cases (diploid and mixoploid cucumber maternal plants). The half of obtained microcalluses from crosses between 2x × 2x plants were grown on medium GA (four microcalluses C. sativus × C. melo; two microcalluses C. sativus × C. metuliferus). Sixteen regenerants were obtained in mixoploid × 2x (eight plants and six microcalluses C. sativus × C. melo; two microcalluses C. sativus × C. metuliferus).

We also used in vitro pollination and fertilization to generate hybrid. In vitro pollination and following fertilization methods were tested in other genera (e.g., genus Cichorium [17]; family Brassicaceae [19]). In our study, pollen grains and ovules were isolated from diploid (2x) cucumber and cultivated together. The muskmelon pollen grains were also used to pollinate diploid cucumber ovules. For in vitro pollination and fertilization of mixoploid cucumber ovules, we used mixoploid cucumber pollen and muskmelon pollen grains again. The results of successful fertilization after these pollination experiments were summarized in Table 3. Figure 2. shows the differences between the number of successful in vitro pollination in diploid and mixoploid cucumber ovules. The number of obtained regenerated ovules (green ovules, or callus formation on ovule tissue) was higher in mixoploid maternal plants (57% for diploid cucumber pollen grains; 25% for mixoploid pollen grains; 52% for muskmelon pollen grains), than in diploid maternal cucumber plants (38% for diploid cucumber pollen grains; 26% for muskmelon pollen grains). The highest level of regeneration was microcallus and no intact plants were obtained in both cases—in diploid (Figure 3(d)) and mixoploid cucumber ovules (Figure 3(e)). Thus, hybrid character of microcalluses derived from diploid ovules was not confirmed [22]. Sequences of the cloned ITS region from mixoploid fertilized

| Ploidy of plants/abbreviation | Σ♂ | No. of ♀ and Σ♂ | Σ♀ | No. of successful fertilization | No. of regenerated ovules |
|-------------------------------|----|----------------|----|----------------|--------------------------|
| 2x (CSS)                      | CSS| 1610           | 570| 220                      |                          |
|                               | CMx| 610            | 370| 96                       |                          |
|                               | CSM| 310            | 155| 89                       |                          |
| 2x/4x (CSSO)                  | CSSO| 280           | 230| 58                       |                          |
|                               | CMx | 780           | 250| 130                      |                          |

Table 4: Summary of in vitro pollination of C. sativus (2x; 2x/4x) × Cucumis spp.
obtained after self-pollination ovules of Cucumis spp. with low percentage of seedlings (2.2-2.3%). Popielarska [39] reported only four successful experiments. The necrosis appeared mostly after the longer cultivation. Microcalluses with size maximally 2 mm were cultivated for twelve weeks without change.

The necrosis was observed in in vitro culture of regenerated ovules after transferring them on these media. The microcalluses with size maximally 2 mm were cultivated for twelve weeks without change. The necrosis appeared mostly after the longer cultivation. Microcalluses with size maximally 2 mm were cultivated for twelve weeks without change.

The protoplasts were obtained from mesophyll tissues of mixoploid cucumber plants produced after in vitro polyploidization. The flow cytometry results showed that the ploidy of protoplasts was 2x/4x/8x. Thirty five isolations were performed for mixoploid cucumber accessions; 37% isolation and following cultivations of 2x/4x/8x protoplasts were successful (protoplasts showed the regeneration efficiency, the first cell division (Figure 3(f)), and the microcallus and callus formation (Figure 3(g)). Gajdová et al. [23] recorded an average of 25% regeneration efficiency of diploid cucumber protoplasts. Therefore, the mixoploid cucumber mesophyll protoplasts reached a higher regeneration capacity than diploid protoplasts. The determined average of mixoploid protoplast viability was 75% (±14%) (Figure 3(h)) and the average density per 1 g of mesophyll tissue was 3.7 × 10^6 (±1.9 × 10^6). Navrátilova et al. [40] found 86.09% cucumber protoplast viability and the average density 4.36 × 10^6 per 1 g of mesophyll diploid cucumber tissue. Nevertheless, Gajdová et al. [41] also summarized the average yields of mesophyll protoplasts for different diploid cucumber genotypes (the averages from 1.98 × 10^6 to 11.85 × 10^6). The results obtained from experiments with mixoploid protoplasts will be helpful in protoplasts interspecific fusion (somatic hybridization).

4. Conclusions

The polyploidization pretreatments employed for generating interspecific hybridization used in this study are reported for the first time in the genus Cucumis. Mixoploid (2x/4x) plants were obtained after colchicine and oryzalin treatment. Several in vitro techniques have been utilized for facilitating interspecific hybridization (embryo rescue, in vitro pollination, and protoplasts isolation) and the results concerning the usage of diploid and mixoploid cucumber maternal plants were analyzed and compared. The positive influence of this procedure was proved especially for in vitro cross-pollination between cucumber and other Cucumis species.
The intact plants were obtained after crossing mixoploid cucumber plants with muskmelon. The mixoploid character of cucumber ovules during *in vitro* pollination and fertilization was demonstrated. However, only calluses were obtained in both cases (in diploid and mixoploid ovules). Mixoploid protoplasts were isolated from mesophyll of plants treated by oryzalin. The average viability and density and regeneration capacity of protoplasts were evaluated suggesting for projected somatic hybridization. In the end, the polyploidization pretreatments substantially facilitated individual *in vitro* techniques, especially regeneration efficiency in mixoploid embryos and ovules.
Acknowledgments
The authors thank Professor Ram J. Singh (Illinois University, Urbana, USA) for reading and remarks on the first draft of the paper. This research was supported by grant MSM 6198959215 (Ministry of Education, Youth and Sports of the Czech Republic).

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