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BRIEF COMMUNICATION

Interspecific hybridization of *Cucumis anguria* and *C. zeyheri* *via* embryo-rescue

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Abstract

Embryo-rescue was used to facilitate interspecific hybridization of *Cucumis anguria* L. and *C. zeyheri* Sond. Embryos were excised from developing fruits at one week intervals for six weeks after hand pollination. Medium containing coconut water was the most suitable for initial germination, and a medium with ascorbic acid was the best for embryo development and plant recovery. Viable plants were obtained from embryos and these plants showed morphological characteristics different from both parents. The analysis of the leucine aminopeptidase (LAP) locus revealed three hybrid types, H1.1, H1.2 and H2.

Additional key words: crossability, isozyme analyses, leucine aminopeptidase, phosphoglucomutase.

Interspecific crossing is potentially important for cucumber improvement, since wild species possess valuable characteristics unavailable in the cucumber gene pool (Lebeda *et al*. 2007). Obstacles to interspecific crossing might be overcome by embryo-rescue procedures and the composition of media has the major influence (Chen and Adelberg 2000, Lebeda *et al*. 2007). The development of a successful crossing protocol for *Cucumis anguria* L. × *C. zeyheri* Sond. hybridization could lead to the incorporation of resistance to diseases and pests. Both *C. anguria* and *C. zeyheri* wild species belong to the subgenus *Melo* (n=12) and they are resistant to *Cucumber green mottle mosaic virus* (CGMMV) (Den Nijs and Custers 1990). In contrast, *C. sativus,* belongs to the subgenus *Cucumis* (n=7) (Kirkbride and Dallwitz 1995). Interspecific crossing among wild *Cucumis* spp. has also been performed to determine their compatibilities. *C. anguria* × *C. zeyheri* hybridization was successful (Visser and Den Nijs 1983). The aim of our current research was the improvement and evaluation of embryo-rescue techniques for the production of fertile *C. anguria* × *C. zeyheri* hybrids, the morphological and biochemical comparison of these hybrids, and utilization of these results in future attempts at hybridization in the genus *Cucumis*.

Seeds of three wild cucumber accessions, *Cucumis anguria* L. var. *longaculeatus* PI 249896 (designated CA1), PI 364475 (designated CA2), and *Cucumis zeyheri* Sond. PI 364473 (designated CZ) were obtained from the USDA-ARS North Central Regional Plant Introduction Station, Iowa State University, Ames, Iowa, USA. The plants were cultivated in the glasshouse (day/night temperature 25/15 °C, air humidity 50 %, natural irradiance) at the Department of Botany, Palacký University in Olomouc, Czech Republic. *C. zeyheri* served as the paternal parent and two accessions of *C. anguria* were used as the maternal parents for crossing. The fruits were harvested at one-week intervals, starting 7 d after hand pollination (AHP) and ending after six weeks. Fruit surfaces were sterilized with 70 % ethanol (2 min), and seeds and embryos were excised by using a stereoscopic binocular microscope (*Carl-Zeiss*,

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Abbreviations: AHP - days after hand pollination; BA - benzyladenine; CW - coconut water; GA - gibberellic acid; IAA - indoleacetic acid; IBA - indolebutyric acid; KIN - kinetin; MS medium - Murashige and Skoog medium.

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Jena, Germany) under sterile conditions. Forty embryos were dissected from each harvested fruit at each weekly interval. They were cultured in darkness on various media in glass tubes held at 22 - 24 °C for six weeks. The basic medium contained Murashige and Skoog (1962; MS) salts supplemented with benzyladenin (BA; 0.01 mg dm^{-3}), indolebutyric acid (IBA; 0.01 mg dm^{-3}), sucrose (20 g dm^{-3}) and agar (8 g dm^{-3}). Specific additions supporting embryogenesis were added (Table 1) and four media were tested. After germination, seedlings were transferred to Erlenmeyer flasks and grown in a controlled environment (day/night temperature of 22/18 °C, 16-h photoperiod, irradiance of 32 - 36 μmol m^{-2} s^{-1}) for six weeks. Resulting plants were transferred into *Perlite* (*AGRO CS*, Česká Skalice, Czech Republic) for four weeks and then to an organic substrate *Florcom SP* (*BBcom*, Letohrad, Czech Republic), and grown to maturity in the glasshouse. This experiment was not repeated, since improved protocols were identified to allow for concentrated focus on *C. sativus* by wild *Cucumis* spp. hybridization experiments.

Comparative analysis of parental and hybrid plants was conducted on morphological traits following Křístková *et al*. (2003) during their growth and development in the glasshouse, and compared to published descriptions (Kirkbride and Dallwitz 1995). These traits were qualitatively evaluated in our experiment and included examination of shape of the leaf laminae, puberulent or glabrous anther thecae, sulcate or terete pedicel of female flowers in outline and finally, sulcate or terete pedicel of fruits in outline. The hybrid character of the obtained plants was confirmed by isozyme analysis. Fresh young leaf tissue (0.1 g) was mechanically ground with *Dowex-C1(1-X8)* containing quartz sand and homogenized on ice in 0.3 cm^3 extraction buffer (Vallejos 1983). The crude extracts were centrifuged at 16 000 *g* for 10 min (-4 °C), and clear supernatants were stored in Eppendorf tubes at -80 °C. Samples were thawed and loaded on polyacrylamide gel (8.2 % separation ge1, 4 % concentration gel) for the electrophoresis which was performed at 35 mA, 390 V for 2 h (adjustable height dual gel electrophoresis unit, *Sigma-Aldrich*, Prague, Czech Republic). Four enzymes, glutamate-oxalo-acetate transaminase (GOT), leucine amino-peptidase (LAP), 6-phosphogluconate dehydrogenase (6PGDH), and phosphoglucomutase (PGM) were used for analysis following the methods of Vallejos (1983). Two maternal plants (CA1 and CA2), one paternal plant (CZ) and plants obtained after cross pollination of these, grown on four culture media (Table 1), were tested. Identification of hybrid types was made according to observed banding patterns, assuming the isozyme subcellular locations and quaternary structures reported for other plants (Wendel and Weeden 1989).

Interspecific hybrids between *Cucumis anguria* × *C. zeyheri* were obtained in this study. Previous studies described hybrids among other *Cucumis* spp. The formation of embryos was reported in crosses of *C. metuliferus* × *C. melo*, and putative unconfirmed hybrid embryos were obtained from crosses between *C. metuliferus* and *C. sativus* and between *C. zeyheri* and *C. metuliferus* (Den Nijs and Custers 1990). Crosses between *C. africanus* and other wild *Cucumis* species also produced fruits with seeds (Den Nijs and Visser 1985).

Differences in germination and regeneration of embryos and seeds from *C. anguria* × *C. zeyheri* and plant formation on four media were observed (Table 1). Fruits harvested one week AHP were immature, their seeds were cultured intact, and mostly callus formation was observed. Plants were obtained from all other fruit ages (2 - 6 weeks AHP). The greater initiation of immature embryos germination was recorded on media with coconut water (CW) and gibberellic acid (GA) (Fig. 1*A*). Regeneration of older embryos and plant formation was optimized on media with ascorbic acid (OK) and casein hydrolysate (ON) (Fig. 1*B*,*C*). Over the course of embryo-rescue culturing, there are substantial differences in the response and regeneration capacity of hybrid embryos to various additions to the culture media, as has been noted by earlier studies. For example, MS medium supplemented with casein hydrolysate, nicotinic acid, IAA and KIN was used to obtain viable hybrid plants from *C. melo* × *C. metuliferus* (Beharav and Cohen 1995). Ondřej *et al.* (2000) and Skálová *et al.* (2005) observed positive influences of GA and CW on regeneration of wild *Cucumis* spp. immature embryos. Vasudevan *et al.* 2007 reported positive effects of BA and α-naphthaleneacetic acid (NAA) on the cucumber regeneration from embryonal axis.

Table 1. Effect of composition of tested media OK (MS + 20 mg dm^{-3} ascorbic acid) ON (MS + 100 mg dm^{-3} casein hydrolysate), GA (MS + 0.3 mg dm^{-3} gibberellic acid) and CW (MS + 5 % coconut water) and time after hand pollination (1 - 6 weeks) on the percentage of successful embryo regeneration and plant formation.

Medium	Embryo regeneration [%]						Plant formation [%]					
	1	2	3	4	5	6	1	2	3	4	5	6
OK	40	60	70	58	90	75	0	20	65	58	70	75
ON	0	80	50	63	100	75	0	30	40	42	80	75
GA	40	40	80	84	80	100	0	40	45	47	30	50
CW	40	80	85	47	50	50	0	80	60	32	30	50

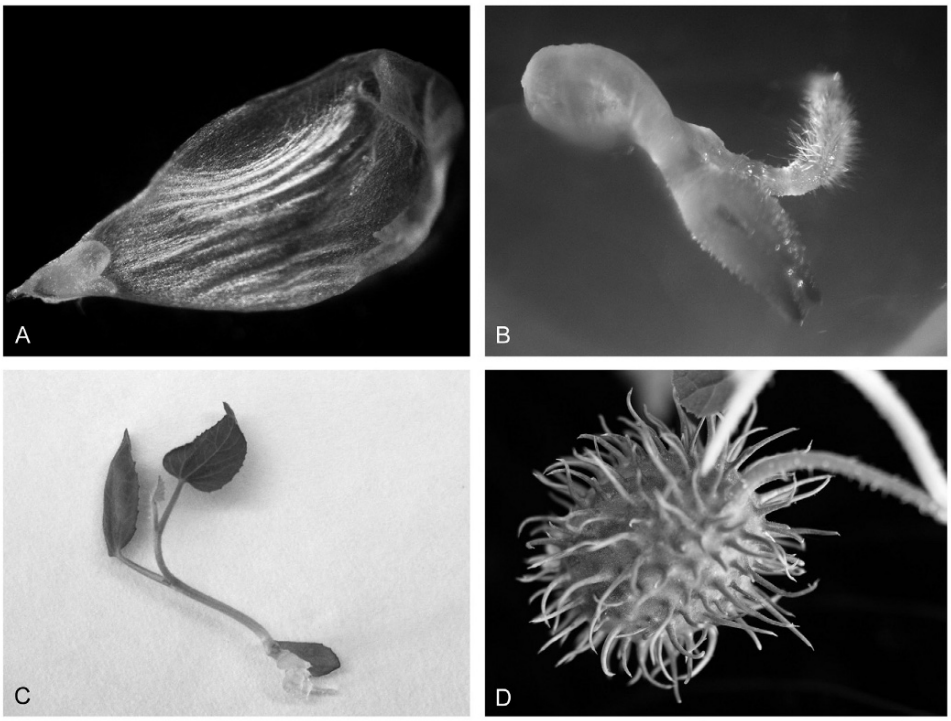

Fig. 1. Hybrids obtained after interspecific hybridization *Cucumis anguria* × *C. zeyheri*. *A* - Hybrid embryo isolated 3 weeks AHP on CW medium. *B* - Direct regeneration of a hybrid embryo isolated 4 weeks AHP on OK medium. *C* - Hybrid plant obtained from an embryo isolated 5 weeks AHP and grown on ON medium. *D* - Fruit from a hybrid plant (H1.2).

Lebeda *et al*. (1999) used isozyme analyses of peroxidase (PER), esterase (EST) and acid phosphatase (AP) for confirming the hybrid origin of embryos from hybridization of *C. sativus* × *C. melo*. We used four enzymes (GOT, PGM, LAP and 6PGDH) for this type of analysis. The analysis of GOT was monomorphic and the analysis of 6PGDH had poor results in the preliminary analyses; therefore they were excluded from final analyses. Two analyses (PGM and LAP) allowed the detection of polymorphisms. Based on analysis of PGM, three parental types (CZ, CA1 and CA2) and two hybrid types (H1 and H2) were defined. H1 hybrid isozyme pattern was observed in all progenies resulting from fruits harvested 2, 3, 5 and 6 weeks AHP (cross CA1 × CZ) and the H2 morphotype was observed in progenies derived from fruits harvested 4 weeks AHP (cross CA2 × CZ). Based on analysis of LAP, six isozyme patterns were defined, the same three parental types as observed in the PGM and three hybrid types. The H1.1 hybrid pattern was observed in all progenies derived from fruits harvested 2 and 3 weeks AHP and the majority of progenies from fruits harvested 5 and 6 weeks AHP. The H1.2 morphotype was observed in progenies derived from fruits harvested 5 and 6 weeks AHP which were cultivated on medium with CW. H2 morphotype was recorded in progenies derived from fruits harvested 4 weeks AHP again (cross CA2 × CZ).

The most informative trait indicating the hybrid character of these plants was the leaf lamina shape at anthesis. Hybrid types H1.1 and H1.2 were intermediate to their parents. Hybrid H2 was morphologically similar to the paternal genotype. The other evaluated traits were the male-flower anther thecae, with hybrids showing the paternal phenotype, and the female-flower pedicels and fruit appearance, which were of maternal phenotype for H1.1 and H1.2 but paternal or intermediate phenotype for H2. Hybrids H1.1, H1.2 and H2 produced seeds (Fig. 1*D*).

To summarize, interspecific hybrids derived from *C. anguria* × *C. zeyheri* that displayed isozyme progeny types H1.1 and H1.2 mainly showed intermediate characters and isozyme progeny type H2 tended to show paternal characters. In contrast with report for hybrid *Cucumis* × *hytivus* (Chen and Staub 1996) and review of such hybridization events (Schwarzbach *et al*. 2001), we did not observe any novel hybrid characters.

To conclude, embryo- and seed-rescue culture resulted in successful in hybridization of *C. anguria* × *C. zeyheri*, as hybrid plants were obtained. New types of media were tested and improved for interspecific hybridization among *Cucumis* spp. Isozyme and morphological analyses were used for confirmation of hybrid origin.

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