

Isolation and Culture of Protoplasts from Cucumis metuliferus and Cucurbita martinezii and a Method for their Fusion with Cucumis melo Protoplasts.

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The incorporation of resistance to diverse plagues and diseases into cultivated varieties of melon is one of the most interesting targets of the genetic improvement of such a crop. Some wild species of the family Cucurbitaceae are very important sources of extraspecific variation as several of those resistances have been described in them. Thus, resistances to Sphaeroteca fugilinea SLECHT and aphids (5), pumpkin mosaic virus and watermelon mosaic virus I (13) and Meloidogyne spp. (4) have been reported in Cucumis metuliferus, while resistances to cucumber mosaic virus and Erysiphe cichoracearum sp. cucurbitae (3) have been referred in Cucurbita martinezii. In principle, protoplast fusion should help to overcome the incompatibility barriers existing for the sexual cross between melon and those wild species. In this paper we describe the isolation and culture of protoplasts from both wild species and a method to induce the fusion of these protoplasts with mesophyll protoplasts from axenic plants of Cucumis melo 'Cantaloup Charentais'.

Methods.- Seeds of C. melo 'Cantaloup Charentais', Cucumis metuliferus NAUD (gently supplied by Dr. Jacobs, University of Stellenbosch, South Africa) and Cucurbita martinezii BAILEY (gently supplied by Dr. Dumas de Vaultx, INRA, Montfavet, France) were surface sterilized and germinated on MG medium as previously described (9,10). The source of protoplasts from each plant species were as follows: i) C. melo, leaf segments precultured two days in C medium from axenic plants grown on MEL medium (9); ii) C. metuliferus, two days precultured cotyledons in C medium as well as non-precultured ones and calli from a cell line kept by subculturing every 15 days in CCM medium (C medium + 10% coconut milk) at 27±0.5°C; iii) C. martinezii, cotyledons as above and small roots (1-2 cm in length) precultured three days in C medium, obtained from axenically propagated plants by rooting the shoot apices on MB3 medium (10).

Enzyme solutions and protoplast isolation methods for each kind of source were: i) for melon mesophyll protoplasts, those previously reported (10) but at the rate of 0.15 g of tissue per ml of enzyme solution; ii) for cotyledons from both wild species, those described for C. melo cotyledons in this issue (14); iii) for calli from C. metuliferus cell line, the same method but using 2 g of calli/8 ml of RSM enzyme solution consisting of 2% cellulase Onozuka-RS + 1% Macerozyme + 0.6M-mannitol on Murashige and Skoog (12) salt solution; and iv) for roots from C. martinezii, the same method but using 1g of tissue/8 ml of the enzyme solution utilized for melon mesophyll protoplasts. Protoplasts were cultured, in all the cases, on ZEPC 0.6M-mannitol according with the procedure and conditions described earlier (9) for melon mesophyll protoplasts.

Protoplast fusion procedure: it consists in a modification of the Kao et al. (7) ones. Protoplasts from both parentals were purified by flotation over 20% sucrose and resuspended in the M solution (100 mM CaCl₂.2H₂O + 300 mM glucose, at pH 6.3) at the rate of 3-4 × 10⁵ protoplasts/ml. Both suspensions were mixed at equal parts (v:v) and a volume of 0.5 ml of the mixture was settled out on a 55 mm plastic Petri dish for 10 min. Then, 0.5 ml of MPEG solution

(M solution + 60% PEG-6,000 at pH 6.3) were added to the mixed protoplasts and the mixture was kept static for another 10 min period. The resulting milliliter of aggregated protoplast suspension was eluted with 5 ml of KMml0 solution, a modification of that from Keller and Melchers (8) consisting of 100 mM CaCl₂ 2H₂O + 300 mM mannitol, buffered at pH 10 with glycine 50mM-NaOH. After 20 min the liquid medium was removed and 5 ml of ZEPC 0.6M-mannitol were added to the protoplasts which had remained attached to the bottom of the dish. This step was repeated once in order to eliminate the fusogenic solution and, finally, another 4 ml of ZEPC medium were added to culture the protoplasts and the fusion products.

Results.- Protoplast isolation and culture from the wild species. Protoplast yields were very different depending on the kind of source utilized; thus, cotyledons of both species, regardless of their preculture in C medium, yielded $0.5-3.0 \times 10^6$ protoplasts/gram of tissue, similarly to those obtained with melon using the same source of material (14), while calli from the Cucumis metuliferus cell line and roots from the axenically propagated Cucurbita martinezii plants produced lower yields, around $0.8-2.0 \times 10^5$ protoplasts/gram.

Morphologically, cotyledon-derived protoplasts are similar to the melon cotyledon ones, and not much different from the melon mesophyll protoplasts (close vacuolation degree and chloroplasts distribution, but being clearly superior in size). On the contrary, calli- and root-derived protoplasts are similar in size to melon mesophyll ones but more cytoplasmic and lacking mature chloroplasts.

The response to culture in ZEPC medium of the cotyledon protoplasts from both wild species was, again, absolutely dependent on the preculture of the plant material (9,14), as non-precultured cotyledons produced protoplasts which, although regenerated their cell wall and initiated one or two cell divisions, were unable to grow further, whereas precultured cotyledons gave rise to protoplasts that maintained their mitotic activity in similar way to what melon mesophyll protoplasts did (9) and, after 20 days in culture, they could be transferred to ZEPC 0.3M-mannitol to continue their growth.

Calli and roots produced a too low protoplast yield to allow a systematic study of their behaviour in ZEPC medium. Nevertheless, their morphology, completely different from the melon mesophyll protoplasts, makes them specially useful for fusion experiments, because it facilitates the determination of fusion frequencies and, above all, they are extremely advantageous if the selection method to be used is that of visual identification and mechanical harvest of the heterokaryons.

Protoplast fusion. Several factors, such as the initial protoplast density, the type and concentration of PEG, the duration of treatment with the high Ca⁺⁺/PEG solution, the time elapsed in the high pH solution, etc., were systematically studied in preliminary experiments. As a result of these studies, the most suitable method to fuse mesophyll protoplasts of melon with calli-derived protoplasts of Cucumis metuliferus and with roots-derived protoplasts of Cucurbita martinezii is the one described under 'Methods'.

PEG-6,000 gave better results than PEG-1,500. Out of the 20%, 30% and 40% concentrations studied, the one with 30% originated an acceptable protoplast aggregation degree without affecting their viability. The aggregation period

of 10 min was the most advisable one, as shorter periods produced lower fusion frequencies while longer times resulted in reduced cell viability. The time elapsed in contact with the high pH solution was, also, critical, since periods longer than 20-25 min strongly affected the viability of the protoplasts, although higher fusion frequencies could be achieved. Finally, the initial protoplast density was very important as concentrations higher than $3-4 \times 10^5$ protoplasts/ml led to the formation of excessive amounts of polykaryons and multiple fusion bodies, which are highly unstable, whereas lower initial densities caused too low fusion frequencies. Summing up, by using the adequate conditions and the procedure described above, fusion frequencies ranging from 5% to 12% in Cucumis melo (x) C. metuliferus and from 1% to 6% in C. melo (x) Cucurbita martinezii have been obtained in our laboratory.

We are currently applying this method to several protoplast systems, using the abovementioned sources of protoplasts according to the chosen selection procedure for the fusion products, with the following strategies: i) selective system, by visually identifying and mechanically selecting the heterokaryons and culturing them in microdroplets of conditioned culture media; in this case we are fusing mesophyll protoplasts of C. melo with calli protoplasts of C. metuliferus and with root protoplasts of Cucurbita martinezii; ii) natural semiselective system, based on the fusion of melon mesophyll protoplasts with non-precultured cotyledon protoplasts of both wild species; and iii) induced semiselective system, fusing melon mesophyll protoplasts with physical or chemically inactivated protoplasts from precultured cotyledons of both wild species. The third system has allowed, in some cases (1,2,11) the transfer of limited quantities of genetic information from the donor species (the inactivated protoplasts) to the recipient species (the undamaged protoplasts) and, therefore, could be of great interest from the point of view of plant breeding. Moreover, the application of this semiselective system to the fusion of protoplasts between species belonging to different genera could permit the obtention of asymmetric hybrids which can be of greater interest than the symmetric ones, since, usually, the former do not present as many infertility problems, developmental disarrangements and rooting difficulties as the latter (6).

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