Plant Regeneration from Cotyledon Protoplasts of Cucumis melo L. cv. Cantaloup Charentais.

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In a previous work (1) we described suitable methods for obtaining callus from mesophyll protoplasts of axenic plants of three melon cultivars. Recently we have reported the morphogenic response of those kind of calli, both via embryogenesis and organogenesis (2). Whole plants could be regenerated from protoplast-derived calli growing on a definite sequence of media. In this paper we show the achievement of plant regeneration from protoplasts originating from another source, namely cotyledons from seedlings of Cucumis melo L. 'Cantaloup Charentais'.

Methods.- Seeds of melon were surface sterilized by immersion in 12.5% commercial bleach (equivalent to 6.25 g/l of active chlorine) for 20 min. After three rinses with sterile distilled water, they were aseptically sown on agarified MG medium (1) in 20x195 mm test tubes. Cotyledons from 11-13 days old seedlings were used for protoplast isolation, either directly or after preculturing them in C medium (1) for two days. Strips of 1-2 mm width from these explants were placed in 100 ml Erlenmeyer flasks containing 8 ml of enzyme solution at the rate of 0.15 g of tissue/ml of enzyme, following the procedure previously described (1). After removing the enzyme solution, protoplasts were purified by flotation over 20% sucrose, picking up the ring which appeared in the interphase formed between the mannitol solution containing the protoplasts and the pad of sucrose. The protoplast yield was estimated by four cell counts in a Howard mould-counting chamber. Protoplasts, at a initial density of 1.5-2 $\times 10^5$ cells/ml, were cultured on 50 mm Petri dishes containing 4 ml of ZEPC 0.6M-mannitol liquid medium (1), at 27°C under darkness, for 20 days. Cells and small cell aggregates originating from protoplasts were transferred to the same liquid medium but at 0.3M-mannitol, at the rate of 2-4 $\times 10^4$ units/ml. After 20 days more in culture, the cells and small cell clusters were subcultured in a modified CEN medium (2) consisting of Murashige and Skoog (4) salt solutions, 1 mg/l thiamine-HCl, 100 mg/l myo-inositol, 1 g/l casaminoacids, 30 g/l sucrose, 0.1 mg/l indole-3-acetic acid and 10 mg/l kinetin, at the rate of 0.5-1.0 $\times 10^4$ units/ml, in 100 ml flasks containing 20 ml of liquid medium and incubated at 25.0±0.5°C and constant illumination (400 lux) on an orbital shaker at 95 r.p.m. In order to obtain organogenesis, the resulting microcalli were successively transferred every 15 days to the sequence of solid media IK 0160 (3), IK 0060 (3) and a modified NB 00101_s (2) to content 10 g/l sucrose. All seedlings and callus cultures were incubated in a growth chamber at 27±2°C during the 16 h light period (1,500 lux, cool fluorescent tubes Gro-lux, Sylvania) and at 24±1°C during the dark.

Results.- Table 1 shows the protoplast yields obtained from cotyledons under the two conditions assayed and their behaviour when they were cultivated in ZEPC 0.6M-mannitol liquid medium. Contrarily to what happened with mesophyll protoplasts (1) we do not observe here a decrease of yield due to the preculture of the source of protoplasts; in fact, the number of protoplasts per gram of tissue obtained from precultured as well as from non-precultured cotyledons was similar to the one produced by precultured leaves of axenic plants (1).

Morphologically, cotyledon protoplasts are very close to mesophyll ones, presenting a great quantity of big chloroplasts distributed in the cytoplasm and a notable vacuolization, but being clearly superior in size and containing a higher number of other intracellular organelles (mainly amyloplasts) which makes it necessary their purification by floating them over a pad of sucrose.

The behaviour of the protoplasts in the ZEPC liquid medium was absolutely dependent on the preculture of cotyledons. So, precultured cotyledons produced viable protoplasts with 75-80% of cell wall regeneration within 2-3 days of culture, and sustained divisions in the 45% of the regenerated cells after 20 days in culture. On the contrary, the non-precultured cotyledons gave rise to protoplasts which experimented cell wall regeneration at very low rates and only exceptionally initiated first divisions; sustained divisions were never observed. Previously (1) we already pointed out the advantageous effect of preculturing the source of protoplasts in the case of varieties with low response and/or when mother plants were grown under suboptimal conditions; the present results using cotyledons as source of protoplasts are a new evidence in this direction.

The percentage of cells undergoing sustained divisions in the ZEPC 0.6M as well as their further growth after being subcultured in the ZEPC 0.3M liquid medium did not differ from the data reported for mesophyll protoplasts (1), reaching in both cases a high level of mitotic activity.

When cells and small cell clusters grown on ZEPC 0.3M were transferred to the CEN agitated liquid medium, whitish or light-green microcalli were formed after 20 days in culture. These could be collected by being filtered through a 100 µm nylon mesh. Batches of around 250 mg (fresh weight) of microcalli were placed on IK 0160 solid medium and grown for 15 days, giving rise to big whitish and friable calli carrying several green zones of apparently organized growth and some peripheral true buds. The transfer of these organized tissues to auxin-free IK 0060 solid medium encouraged their growth, though the amount of unorganized friable callus formed was still high. The quantity of unorganized tissue decreased gradually after two passages of the green zones through NB 00101_S medium, where compact and green calli were developing greatly, giving rise to the formation of a great number of buds and shoot-buds. Clearly distinguishable shoots could be observed in some calli after their third passage through the NB 00101_S medium. The number of calli presenting developed shoots and the number of shoots per callus depended on two factors: the exclusive transfer of organized zones of calli in each subculture and the short interval elapsed among them (10-15 days every time). Table 2 summarizes the results obtained after each step of this procedure.

In order to obtain whole plants, the clearly developed shoots were individually transferred to a modified NB 00101_S medium (without 6-benzylaminopurine or casaminoacids). Most of the shoots displayed clear abnormalities due, perhaps, to changes in their chromosome number and were unable to root. However, some of them became whole plants (usually rosette-shaped plants and occasionally normal plants) which could be propagated in vitro by subculturing their apices and axillary buds in the abovementioned medium.

In short, we can now avail ourselves of two different protoplast systems which, under the proper cultural conditions, allow a moderate rate of plant regeneration, via organogenesis, in melon.

Nowadays, in our laboratories, we are using the protoplast fusion technique in order to obtain interspecific somatic hybrids between Cucumis melo L. and several wild species of genera Cucumis and Cucurbita, in which resistances to plagues and diseases have been described. The obtention of such a kind of hybrid plants should represent the first step in our final goal, i.e. the transfer of resistance genes from the wild species to the cultivated one. So the attainement of plant regeneration from protoplasts of melon is an important step towards the stated end.

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Table 1. Isolation and culture of protoplasts from non-precultured (NP) cotyledons and from precultured (P) ones in C medium for 20 days.

	Isolation	Culture
	Protoplast yield ($\times 10^6$)/g of tissue	% of cells undergoing divisions
NP	0.82 ± 0.53	0.0
P	1.37 ± 0.77	45.30 ± 9.57

Table 2. Morphogenic response of protoplast-derived calli after subculturing for 15 days in each of the succesive solid culture media.

Sequence of media	No. of calli	% of calli with buds[z]			% of calli with shoot-buds	% of calli with shoots
		++	+	-		
1)IK 0160	177	77.4	22.6	0	1.7	0
2)IK 0060	138	97.8	2.2	0	31.2	0
3)NB 00101$_S$	93	77.4	22.6	0	35.5	0
4)NB 00101$_S$	59	94.9	0	5.1	59.4	0
5)NB 00101$_S$	41	100.0	0	0	100.0	9.7
6)NB 00101$_S$	56	100.0	0	0	100.0	10.7
7)NB 00101$_S$	64	100.0	0	0	100.0	20.0

[z] ++ : calli with numerous buds peripherally distributed.
+ : calli with a few buds appeared in discrete zones.
- : calli without buds.