Improving Culture Efficiency of *Cucumis metuliferus* Protoplasts

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Although we have made great progress in developing resistance to root – knot nematodes in cucumber, no source has been created or screened to resistance to *M. incognita,* the main species affecting cucumber (9, 10). Resistance to *M. incognita* exists in horned cucumber (*Cucumis metuliferus*), but efforts by many research groups (including our own) to make crosses of *Cucumis* species with cucumber have failed. Somatic hybridization using protoplast fusion is an alternative for utilizing *C. metuliferus* germplasm. Plants have been regenerated from protoplasts isolated from *C. sativus* (2, 4, 7), but not from *C. metuliferus.* To apply protoplast fusion, a reliable technique for isolating and culturing *C. metuliferus* protoplasts is often a prerequisite. Several reports suggest that large improvements in plating efficiency can be made by culturing protoplasts in a medium solidified with agarose (2, 3, 8).

The objective of this study was to compare agarose-disc culture to liquid culture, with and without weak light, for stimulating protoplast division of *C. metuliferus* cotyledon protoplasts. We also evaluated the efficiency of agarose-disc culture using different species (*C. sativus* and *C. metuliferus*), and protoplast sources (cotyledon or true leaf). Plating density, using nurse cultures, and center disc size (10 or 50 µl) were varied as well.

Methods. Seeds of two cultigens of cucumber ('Sumter' and Wisconsin SMR 18) and three plant introduction accessions of *C. metuliferus*, PI 482452, PI 482454 and PI 482461 were used. Sterilized seedlings were prepared as described as previous report with C1 medium (6). The protocols of protoplast isolation and mixture of enzyme solution with C2 medium were also same as previous report (6). Number of protoplasts was estimated using a hemacytometer after isolation, and viability was determined using fluroscein diacetate (11).

Influence of light and culture condition Only *C. metuliferus* PI 482454 were used in this experiment. For agarose culture, 10 ml of C2 medium with 1.2% (w/v) agarose (Sea Plaque FMC BioProducts, Rockland, Maryland) was used. Protoplasts were plated in agarose for a final density of 1×10^5 protoplasts per ml. Five 100 µl drops each of this solution were pipetted into 10 × 60 mm petri plates as described by Dons and Bouwer (3). After gelling, 4.5 ml of liquid C2 media was added. One and two weeks after isolation, the liquid medium was removed and C2 medium containing 0.275 or 0.25 M mannitol, respectively, was added. Agarose-disc cultures were maintained on a gyratory shaker at 30 rpm (3) at 30°C, under a 16 h photoperiod of cool white fluorescent lights (13.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$) (chamber 1), or in the dark at 30°C (chamber 2).

For liquid culture, protoplasts were cultured in 5 ml of C2 medium at a density of 1×10^5 protoplasts per ml in 10 × 60 mm petri plates and were maintained under the same conditions as the agarose-disc cultures, except keeping in a stationary position. Seven days after protoplast release, 1 ml of C2 medium, with only 0.15 M mannitol was added to each plate. Liquid culture plates were swirled one min per day to increase aeration. Estimates of protoplast division (PD), measured as the percentage of protoplasts which had undergone one cell division, and more than one division were made four and eight days after isolation. Cell wall regeneration was determined by observed changes in protoplast shape, and actual counts of cell division. The experiment was a split-block treatment arrangement in a randomized complete block design with three replications. There were three plates per treatment. Chambers were designated as whole plots, flasks were subplots, and culture techniques were sub-subplots.

Influence of genotype and tissue source. Two cultigens of cucumber ('Sumter' and Wisconsin SMR 18) and three accessions of *C. metuliferus*, PI 482452, PI 482454 and PI 482461 were used in this experiment. Protoplasts were isolated from cotyledons from five- to seven-day-old plants and leaves from 12- to 14-day-old plants. Cotyledon protoplasts were isolated from all five cultigens, while leaf protoplasts were isolated only from Wisconsin SMR 18 and PI 482454. In each replication, there was one flask per tissue type, with each flask contributing two plates to each treatment combination. To allow for adequate sampling, the lowest density, 10 µl disc treatments had three plates.

Agarose discs were prepared as described above, except that the final protoplast density of the center discs was reduced and nurse-culture discs were added to each plate. For each plate, four 100-µl nurse culture discs at a density of 1×10^5 protoplasts per ml were pipetted into the plate. A 10- or 50-µl disc of an estimated density of 5×10^2, 1×10^3, or 1×10^4 protoplasts per ml was then pipetted into the center of each plate. These center discs were used for evaluation of PD and plating efficiency (PE). Estimates of PD and cell wall regeneration were made four and eight days after isolation. PD were measured as described above. PE were estimated with the disc (s) by calculating the percentage of protoplasts which produced microcalli of 16 cells or more after 21 days. The experiment was a split-split plot treatment arrangement in a randomized complete block design with three replications. Mean comparisons for variables were made among treatments using Fisher's protected LSD (5% level).

Results. A wide range of concentrations of NaOCl (0.26 to 2.6%) were used to sterilize seeds of *C. metuliferus*, resulting in extremely low germination (0 to 5%). Use of the industrial disinfectant LD resulted in excellent germination rates (approximately 80%), and seed contamination of only 4 to 8% on C1 media (data not presented).

Influence of light and culture conditions. The number of viable protoplasts isolated per g of *C. metuliferus* PI482454 cotyledon tissue was $(1.25 \pm 0.22) \times 10^7$. Protoplast size varied from 10 to 50 µm in diameter. Cell wall regeneration and protoplast division began two to three days after isolation, regardless of treatment. After four days of culture, a significant difference in amounts of divided once (PD4-1) and more than once (PD4-2) protoplasts existed between agarose and liquid culture. No effect due to presence or absence of light was observed for PD4-1 and PD4-2 in either liquid or agarose culture (Table 1). Eight days after isolation, the percentage of divided once (PD8-1) and more than once protoplasts (PD8-2) was measured again, and significant differences between agarose and liquid culture still existed (Table 1). At that time, the presence of light resulted in significant increases for multiple protoplast divisions (PD8-2) only within agarose culture, but not in liquid culture.

The positive effect of using agarose medium on increasing both the number of protoplasts that had divided once and more than once support findings of numerous other researchers (1, 2, 5, 8) concerning the benefits of culturing protoplasts in agarose. The positive influence of weak light on multiple protoplasts division was only seen in agarose culture after eight days. Data from this experiment suggests that weak light is neither necessary or inhibitory for division.

Genotype and tissue source study. Protoplasts from cotyledon tissue varied in size from 10 to 50 µm in diameter. Primary leaf protoplasts for both species were more uniform in size, and varied in diameter from 10 to 20 µm in diameter. Yields and viability of protoplasts varied according to species and tissue type; all yielded acceptable quantities of viable protoplasts (Table 2).

After four days of culture, determination of multiple protoplast division (PD4) indicated a strong trend toward increased division with increased density was seen for both disc size treatments, and a more significant change was seen between 10 µl and 50 µl disc size treatments with culture densities of 1×10^3 and 1×10^4 protoplasts per ml (Table 3). A slower response of protoplasts in 10 µl disc treatments at a density of 1×10^3 protoplasts per ml was observed (Table 3).

Comparing different density treatment, significant effects due to density could also be seen within cotyledon protoplasts of both cucumber cultivars and one accession of *C. metuliferus* [(PI 482454 (Table 4). Increased density consistently caused an increase in multiple protoplast division. The percentage of protoplast division of 1×10^4 protoplasts per ml was higher than those of the other low densities after 4 to 8 day culture. However, disc size appears to have little long-term effect on multiple protoplast division and callus formation. After 21 days, plating efficiencies that was calculated as the percentage of protoplasts to form microcalli of 16 cells or more between 1×10^3 and 1×10^4 density treatments are very close, but higher than that of low density 5×10^2 protoplasts per ml (Table 4).

Table 1. Influence of light and culture conditions on *Cucumis metuliferus* protoplasts division.[z]

Culture conditions	Light ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Chamber	Day 4 PD4-1[y]	Day 4 PD4-2[x]	Day 8 PD8-1[y]	Day 8 PD8-2[x]
Agarose	13.5	1	27	3.1	25	29
	-	2	27	2.2	37	21
Liquid	13.5	1	7	0.0	17	3
	-	2	7	0.1	12	2
LSD (5%)			4	1.3	6	6
CV (%)			13	51	17	27

[z,y] Percentages of protoplasts which divided once and more than once.

Table 2. Protoplast isolation yields of different genotype and tissues.[z]

Genotype	Tissue source	Protoplast yield ($x10^6$/g)[y]	Protoplast viability (%)
Cucumis metuliferus			
PI 482452	Cotyledon	4.1 ± 1.5[x]	66.3 ± 30.6[x]
PI 482461	Cotyledon	7.9 ± 0.4	73.4 ± 17.0
PI 482454	Cotyledon	7.5 ± 2.3	77.9 ± 16.3
PI 482454	Leaf	13.0 ± 2.0	69.2 ± 8.4
Cucumis sativus			
Sumter	Cotyledon	2.4 ± 0.5	63.7 ± 19.1
Wis. SMR 18	Cotyledon	9.1 ± 0.5	78.6 ± 8.1
Wis. SMR 18	Leaf	3.5 ± 1.1	62.3 ± 8.9

[z] Data are means of 3 replications.

[y] Viable protoplasts released/g fresh weight of material.

Table 3. Effect of center disc size and low protoplast densities on the percent multiple protoplast division 4 days after isolation (%).[z]

Disc size	Protoplast density (no./ml agarose)		
	$5x10^2$	$1x10^3$	$1x10^4$
10 µl	0.4	1.1	3.9
50 µl	0.4	3.0	4.2

[z] Data are means of 7 combinations of genotypes and tissue source (see table 2). Each combination has 3 replications, 3 samples and 2 subsamples.

Table 4. Effect of different densities with nurse cultures on the multiple protoplast division and plating efficiency in *Cucumis metuliferus* and *C. sativus*.[z]

Protoplast density (no./ml agarose)	Protoplast division(%)		Plating efficiency(%)
	Day 4	Day 8	Day 21
$5x10^2$	0.4	5.0	2.2
$1x10^3$	2.0	10.9	4.2
$1x10^4$	4.1	15.9	5.1
LSD (5% for column comparisons)	2.1	4.6	2.5

[z] Data are means of 14 treatments (7 genotype and tissue source combination with 2 disc sizes: 10 and 50 µl). Each treatment has 3 replications, 3 samples and 2 subsamples.

[y] Means with different letters are significantly different at 5%.

Table 5. Effect of different tissue source with nurse cultures on the multiple protoplast division and plating efficiency in *Cucumis metuliferus* and *C. sativus*.[z]

Protoplast source	Protoplast division(%)		Plating efficiency(%)
	Day 4	Day 8	Day 21
Cotyledon	2.9a[y]	13.6a	5.2a
Leaf	0.0b	3.0b	0.1b

[z] Data of leaf protoplast are means of 2 genotypes with two disc sizes and data of cotyledon protoplast are means of 5 genotypes with two disc size. Each treatment has 3 replications, 3 samples and 2 subsamples.

[y] Means with different letters are significantly different at 5%.

Moreover, great differences for multiple protoplast division were also seen between cotyledon and leaf protoplasts (Table 5). Generally leaf protoplasts that had not lysed by day four showed little or no cell-wall development or cell division for the duration of the experiment. Although an acceptable yield of protoplasts was obtained from all species and tissue types (Table 2), the culture technique used was not suitable for leaf protoplasts (Table 5). The current isolation technique or some aspect of the culture media could be the cause of the poor survival and division rate of leaf protoplasts although leaf protoplasts of *C. sativus* have been successfully cultured using agarose (2, 7).

Throughout this study, the densities between 1×10^3 and 1×10^4 protoplasts per ml with agarose nurse culture is an efficient technique for both *C. metuliferus* and *C sativus* protoplast isolation and culture. The importance of weekly replenishment of limiting nutrients and plant-growth regulators should not be ignored as a possible advantage of agarose over liquid culture. However, despite trials using many different media and growth regulator combinations, no shoot differentiation from the callus of either species has occurred to date. The regeneration technique need to be further studied.

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