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ISOLATION AND CULTURE OF TOMATO MESOPHYLL PROTOPLASTS

PIERRE J. G. M. DE WIT

Laboratorium voor Fytopathologie, Landbouwhogeschool Wageningen

SUMMARY

Viable tomato mesophyll protoplasts were isolated by using the non-phytotoxic macerating enzyme PATE instead of the generally used Macerozyme. To obtain the osmotic conditions required during isolation, salt solutions, containing 2.5% (w/v) KCl and 1% (w/v) $MgSO_4 \cdot 7H_2O$, were used. From studies in different culture media it appeared that, under optimum conditions, cells regenerated a wall and divided three to four times. After about three weeks of incubation cell division stopped and cells started to degenerate.

1. INTRODUCTION

In earlier publications (OTSUKI & TAKEBE 1969, WAKASA 1973) methods for isolation of mesophyll protoplasts of several plants species including tomato have been described. The yield of intact tomato mesophyll protoplasts was sometimes very low (OTSUKI & TAKEBE 1969), sometimes rather high, but protoplasts were always very vulnerable (WAKASA 1973). Tomato mesophyll protoplasts isolated according to TAKEBE et al. (1968) were usually non-viable and died a few hours after isolation. In this article, isolation and culture of viable tomato mesophyll protoplasts is described.

2. MATERALS AND METHODS

2.1. Plants

Tomato seeds (*Lycopersicon esculentum* Mill, cvs Purdue 135 and Vetomold (supplied by Mrs. I. Boukema, I.V.T., Wageningen) were sown in trays with peat soil (Trio No. 17); seedlings were transplanted in pots (diam. 18 cm) after two weeks. Plants were kept in a greenhouse between 20 and 25°C and at 60% relative humidity. Light intensity was kept between 10000 and 15000 lux for 12 hours each day by shadowing the greenhouse in the summer if necessary and supplementing the light during the winter (HPRL-400 W, Philips). Usually, fully expanded third leaves and sometimes fourth of fifth leaves of plants of 40–50 days old were used for protoplast isolation.

2.2 Isolation of protoplasts

Leaves were surface-sterilized for 15 min in 1% (w/v) sodium hypochlorite solution and subsequently rinsed 5 times in sterile distilled water. As soon as

leaves were dry, the lower epidermis was removed and pieces of stripped leaves were pre-incubated in 0.7 M mannitol for 30–60 min. For isolation of protoplasts two methods were employed:

Method I

According to a slightly modified procedure of MEYER (1974) stripped leaves were incubated, lower surface downwards, in Petri dishes (diam. 15 cm), containing 0.05% (w/v) polygalacturonic acid transeliminase (PATE) (Hoechst A.G., Frankfurt: supplied by Dr. Y. Meyer, Botanisches Institut, Universität Heidelberg F.R.G.) in 0.7 M mannitol pH 7 for 90 min. After removal of the enzyme solution the leaf pieces were incubated in a salt solution, containing 2.5% (w/v) cellulase (Onozuka R-10 Kinki Yakult Mfg. Co., Ltd., Nishinomiya, Japan) pH 5.5. Every 30 min the Petri dishes were shaken very gently. After 30–45 min of incubation the first released protoplasts were observed; incubation was completed after 90–120 min. The incubation solution containing non-digested leaf material and released protoplasts, was then filtered through a 100 nm mesh stainless steel filter. Protoplasts were washed twice by suspending in the same salt solution and centrifuging at 100 × g for 2 min. Finally, these protoplasts were resuspended in the following culture media:

Medium A, (R 0.6), according to MEYER & ABEL (1975a)
Medium B, according to NITSCH & OHYAMA (1971)
Medium C, according to HARADA (1973)
Medium D, according to UPADHYA (1975)
Medium E, according to COCKING & POJNAR (1969)

Method II

According to a slightly modified method of TAKEBE et al. (1968), stripped leaves were incubated in 100-ml Erlenmeyer flasks with 25 ml of a 0.7 M mannitol solution, containing 0.5% (w/v) Macerozyme (R-10 Kinki Yakult Mfg. Co., Ltd., Nishinomiya, Japan) and 0.1% (w/v) potassium dextran sulphate (Meito Sangyo Co., Ltd., Nagoya, Japan) pH 5.8. The enzyme solution was infiltrated in vacuo and the Erlenmeyer flasks were incubated on a rotary shaker at 30°C and 80 rev/min. After 30 min the enzyme solution was removed, the leaf pieces were washed with 0.7 M mannitol, and incubated in 0.7 M mannitol containing 1% (w/v) cellulase (Onozuka R-10 Kinki Yakult Mfg., Co., Ltd., Nishinomiya, Japan) pH 5.5; the flasks were incubated at 20°C and 80 rev/min for 2 hours. The incubation medium, containing the released protoplasts, was filtered through a 100 nm mesh stainless steel filter and washed twice by suspending in 0.7 M mannitol and centrifuging at 100 × g for 2 min. After the last centrifugation, the protoplasts were resuspended in the five culture media A, B, C, D, and E.

2.3 Culture of protoplasts

Two and a half ml of protoplast suspension (ca 5.10^4 protoplasts/ml) was incubated in small glass or plastic Petri dishes (diam. 5 cm). The Petri dishes were sealed with parafilm and placed in a growth chamber at 25°C for 3 days in

darkness; thereafter, incubation was continued under fluorescent light at 1000 lux (Philips 65 W/33).

2.4 Fluorescence microscopy

Cells were collected by centrifugation and then resuspended for 5 min in 0.7 M mannitol solution, containing 0.1% (w/v) Calcofluor White ST (American Cyanamid Co., Wayne, N.J., U.S.A.), centrifuged again and resuspended in 0.7 M mannitol. Fluorescence was observed with a Zeiss microscope, a HBO 200 W/4 illuminator (300–400 nm excitation) and UG 1/3 and LP 418 barrier filtes.

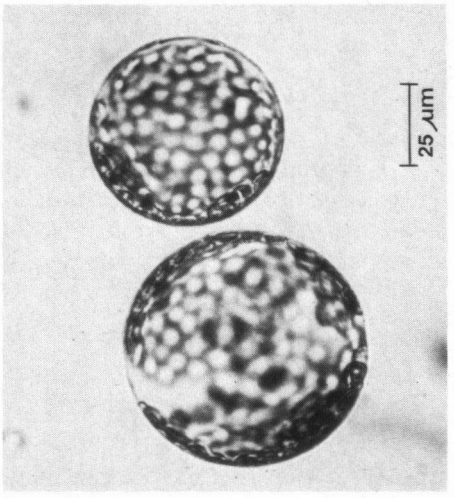
3. RESULTS

3.1 Isolation of protoplasts

Method I was found to be the best for isolating viable tomato mesophyll protoplasts. Essential contributions to this successful isolation are probably the use of the non-phytotoxic macerating enzyme PATE instead of the phytotoxic Macerozyme and the use of a salt solution instead of a sugar solution as plasmolyticum during cellulase incubation. PATE treatment was done in sugar solution because this enzyme is hardly active in salt solution. The yield of viable protoplasts was about 2.10^7/g leaf material (*fig. 1*) with third leaves. If fourth or fifth leaves were used, the number of protoplasts produced in the same incubation time was only half to one third of this yield. This difference is probably due to a lower digestion of the older leaves by the enzyme used. Fully expanded third leaves of plants of 40–50 days old gave the highest yields of protoplasts, which appeared also the best for cultivation. Isolation of protoplasts according to Method II was rarely successful. With this method, age and physiological condition of plants seemed to be more critical than with Method I. When isolation with Method II was successful and viable protoplasts were obtained, they remained alive for only 2 days. Very often, cells died during incubation with Macerozyme, which seemed to be toxic to tomato mesophyll protoplasts.

3.2 Culture of protoplasts

Protoplasts were cultured in different media. In *table 1* the effect of the isolation method and the culture medium on the development of protoplasts is presented. Only protoplasts isolated according to Method I could be cultivated. Media B and C are most suitable for sustaining cell-wall synthesis (*figs. 6, 7*) and cell division (*figs. 3, 4, 5*). In these media division occurred sometimes by cleavage but mostly by budding. In Media A and E division only occurred by budding. Medium D neither sustained cell-wall synthesis nor cell division. After 8 to 10 days of incubation in Media A and E protoplasts started to degenerate (*fig. 8*); cells stopped division and large vacuoles appeared. In Media B and C cell division continued up to three weeks after incubation. In these media after a few divisions sometimes cells were formed, which seemed



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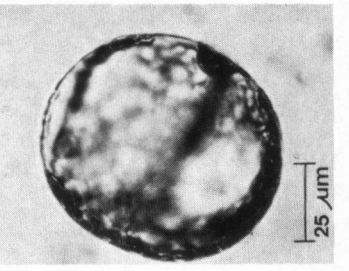

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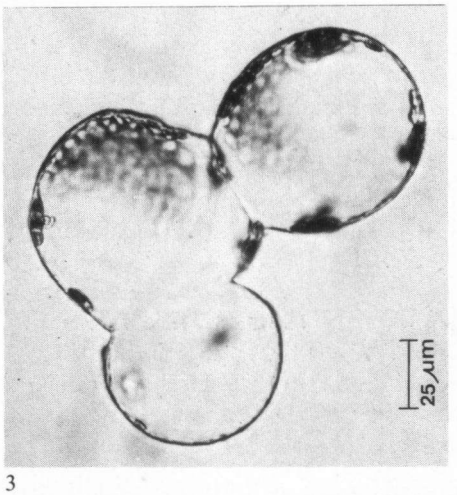

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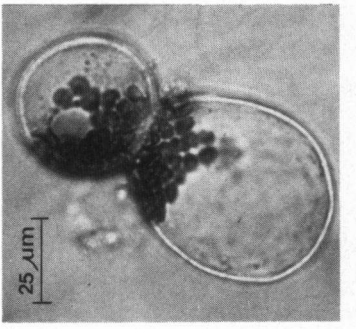

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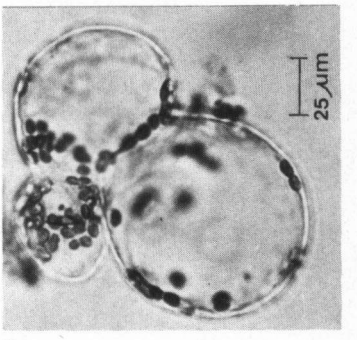

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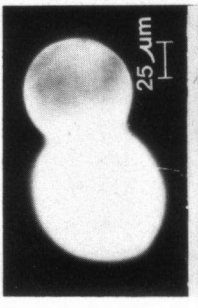

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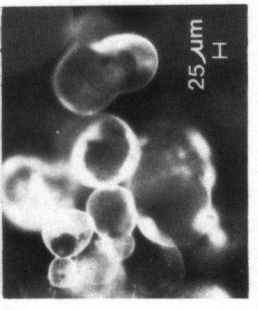

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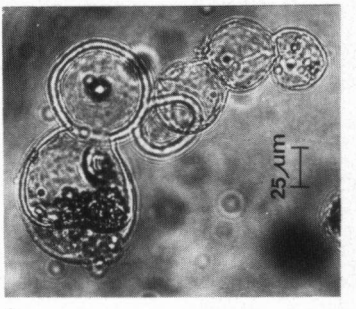

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Table 1. The influence of isolation method and culture medium on development of tomato mesophyll protoplasts[1].

culture medium	% of protoplasts alive on Day 2		% of protoplasts producing a cell-wall on Day 6		% of cells showing cell division on Day 10	% of cells showing more than one cell division
	Method I	Method II	Method I	Method II	Method I	Method I
A	60	20	20	0	10	0
B	80	20	60	0	20	10
C	70	10	40	0	20	10
D	20	0	0	0	0	0
E	50	10	40	0	10	0

[1] Results are the average of four experiments except those of Medium E, which are the average of two experiments.

to be without cell content while their wall stained positively with Calcofluor White. After about 3 weeks of culture in Media B and C cell division stopped and also here degeneration started. Mostly, results were similar for both tomato varieties.

4. DISCUSSION

Successful isolation of tomato mesophyll protoplasts could be achieved by using the non-phytotoxic macerating enzyme PATE instead of the generally used Macerozyme. With a salt solution the physiological condition of plants seemed less important than with mannitol. Favourable results of salt solutions have also been described by FREARSON et al. (1973), MEYER (1974), UPADHYA (1975), and COUTTS & WOOD (1975) for petunia, tobacco, potato, and cucumber, respectively.

WATTS et al. (1974) found that physiological condition and age of plants were very critical for successful isolation and culture of protoplats from tobacco, when sugars were used as plasmolyticum. By using salt solutions, as described in this paper, variation in protoplast viability between different isolations was reduced. For culture of tomato mesophyll protoplasts Media A, D, and

Fig. 1. Freshly isolated protoplasts.
Fig. 2. Protoplast after 3 days of culture in Medium B.
Fig. 3. Budding division after 8 days of culture in Medium A.
Fig. 4. Budding division after 8 days of culture in Medium B.
Fig. 5. Budding and cleavage division after 10 days of culture in Medium B.
Fig. 6. Protoplast showing budding division, after staining with Calcofluor White.
Fig. 7. A number of protoplasts showing budding divisions, after staining with Calcofluor White.
Fig. 8. Cells degenerating in Medium B, after 3 weeks of culture.

E were less favourable than Media B and C. In Medium A cells divided by budding and only pseudo-walls were formed. This phenomenon was also observed in the same medium by MEYER (1974) and by MEYER & ABEL (1975a, 1975b) with tobacco mesophyll protoplasts. Budding was also observed by POJNAR et al. (1967) with tomato fruit protoplasts cultured in Medium E. Protoplasts isolated and cultured as described above can be useful in studying interactions of plants with fungi (TOMIYAMA et al. 1974) and viruses (TAKEBE 1975). Mesophyll protoplasts are being used in current experiments on the interaction of various physiological races of the fungus *Cladosporium fulvum* with tomato plants which react differentially to these races.

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