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Regeneration of leaf mesophyll protoplasts of tomato cultivars (*L. esculentum*): factors important for efficient protoplast culture and plant regeneration

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ABSTRACT

Conditions were established for efficient plant regeneration from four freshmarket cultivars of *Lycopersicon esculentum*. In order to increase the yield of viable protoplasts which are able to sustain cell divisions, the donor plants are preconditioned by incubation at 25°C in the dark for 18 hours, followed by a cold treatment at 4°C in the dark for the last 6 hours, prior to protoplast isolation. Browning of the dividing cell colonies can be prevented by culturing protoplasts in 100 µl droplets of low-melting agarose, surrounded by liquid medium. Alternatively, protoplasts can be cultured in liquid medium. In both procedures the plating efficiencies and percentage of shoot regeneration are increased, only when dilutions were performed with auxin-free culture medium. Shoot regeneration is obtained by using a two step procedure: initiation of greening of microcalli on a medium containing 0.2 M mannitol and 7.3 mM sucrose, which is followed by shoot development on a mannitol-free medium containing 0.5 M sucrose. In this way, plants can be regenerated within 3 months from the hybrid cultivars Bellina, Abunda, Sonatine and also from the true seedline Moneymaker. The latter one showed the highest regeneration frequency (30%).

Abbreviations: BAP, 6-Benzylamino purine; 2,4-D, 2,4-dichlorophenoxy acetic acid; IAA, indole acetic acid; MES, 2-(*N*-morpholino)- ethane sulfonic acid; NAA, naphthalene acetic acid; PE. plating efficiency.

INTRODUCTION

In recent years tomato breeding has been concentrated on obtaining increased yields, improved fruit quality, altered plant growth and disease and pest resistance (Rick,1982). The incorporation of such desirable traits into the cultivated tomato is usually attempted by crossing with wild species. However, interspecific incongruity between many of these species limits the value of sexual hybridization as a tool for the introduction of important traits from wild species. Somatic hybridization or somatic cybridization techniques could be a useful alternative in overcoming the incompatibility barriers. This approach however, requires that plants can be regenerated from protoplasts. Until recently, tomato protoplasts were regarded as recalcitrant in tissue culture, because of the lack of efficient and reproducible culture and regeneration methods. In this paper we describe some of the conditions necessary to obtain high viability and plant regeneration of *L. esculentum* protoplasts. These conditions are: the preconditioning of the donor plants, an alternative method of culturing tomato protoplasts, and a two step regeneration procedure. The combination of these conditions has resulted in a rapid and reproducible method for the regeneration of the tested tomato cultivars. We also have compared our procedure in combination with other protoplast culture media which were recently developed (Shahin, 1985; Niedz et al., 1985; Adams and Townsend, 1983). Our goal is to have a reliable protoplast system for genetic manipulations, such as DNA transformation and somatic cybridization between tomato and its wild relatives (Tan et al., 1986).

MATERIALS AND METHODS

Plant materials

L. esculentum cultivars used in this study were: cultivar Moneymaker, provided by the Institute for Horticultural Plant Breeding (IVT), Wageningen, F-1 hybrid cultivars Sonatine and Abunda, provided by the seed company Ruyter & Zonen in Naaldwijk, and F-1 hybrid cultivar Bellina, provided by the seed company Rijk Zwaan, De Lier, The Netherlands.

Plant growth conditions

In vitro grown plants. Seeds were surface sterilized by briefly dipping in 70% ethanol followed by incubation in 10% commercial bleach (equivalent to 1% NaOCl) for 10 minutes. The seeds were then washed three times in sterile distilled water, placed on MS-medium (Murashige and Skoog, 1962) containing 2% sucrose and 0.8% Difco agar, in sterile plastic containers, and were allowed to germinate at 25°C. After approximately 7 days, seedling shoot tips were excised and propagated on the same medium. These shoots were grown in a controlled environment chamber (16h photoperiod, 2000 lux, 20-25°C and 60% relative humidity)

In vivo grown plants. Tomato seeds were sown and the seedlings were grown in the greenhouse (18-22°C) until the third leaf appeared (5-6 weeks).

Protoplast isolation

Seven to ten days prior to protoplast isolation, the *in vivo* grown plants were transferred to a controlled environment chamber and grown under 2000 lux, short photoperiod (8 h), 18-20°C, and 60% relative humidity. Then, the plants were incubated in the dark for 18 h followed by an incubation at 4°C for 6 h in the dark. Young, fully expanded leaves of 3-5 cm were cut off and sterilized in 10% bleach (1% NaOCl). Alternatively, leaves of *in vitro* grown plants that were 3-4 weeks old were used, after the same pretreatment in the dark and in the cold. The leaves were cut in small strips and preplasmolyzed in a solution of CPW-salts (Frearson et al., 1973), supplemented with 9% mannitol and 3mM MES (pH 5.8) at 25°C, in the dark for one hour. The medium was then replaced by the preplasmolysing solution supplemented with 0.6% Cellulysin and 0.1% Macerase (Calbiochem). Incubation was performed in the dark at 25°C for 18 h.

The digested leaves were diluted with an equal volume of rinse medium (consisting of CPW salts supplemented with 20 g/l KCl) and filtered through a 70 µm nylon sieve. The filtrate was

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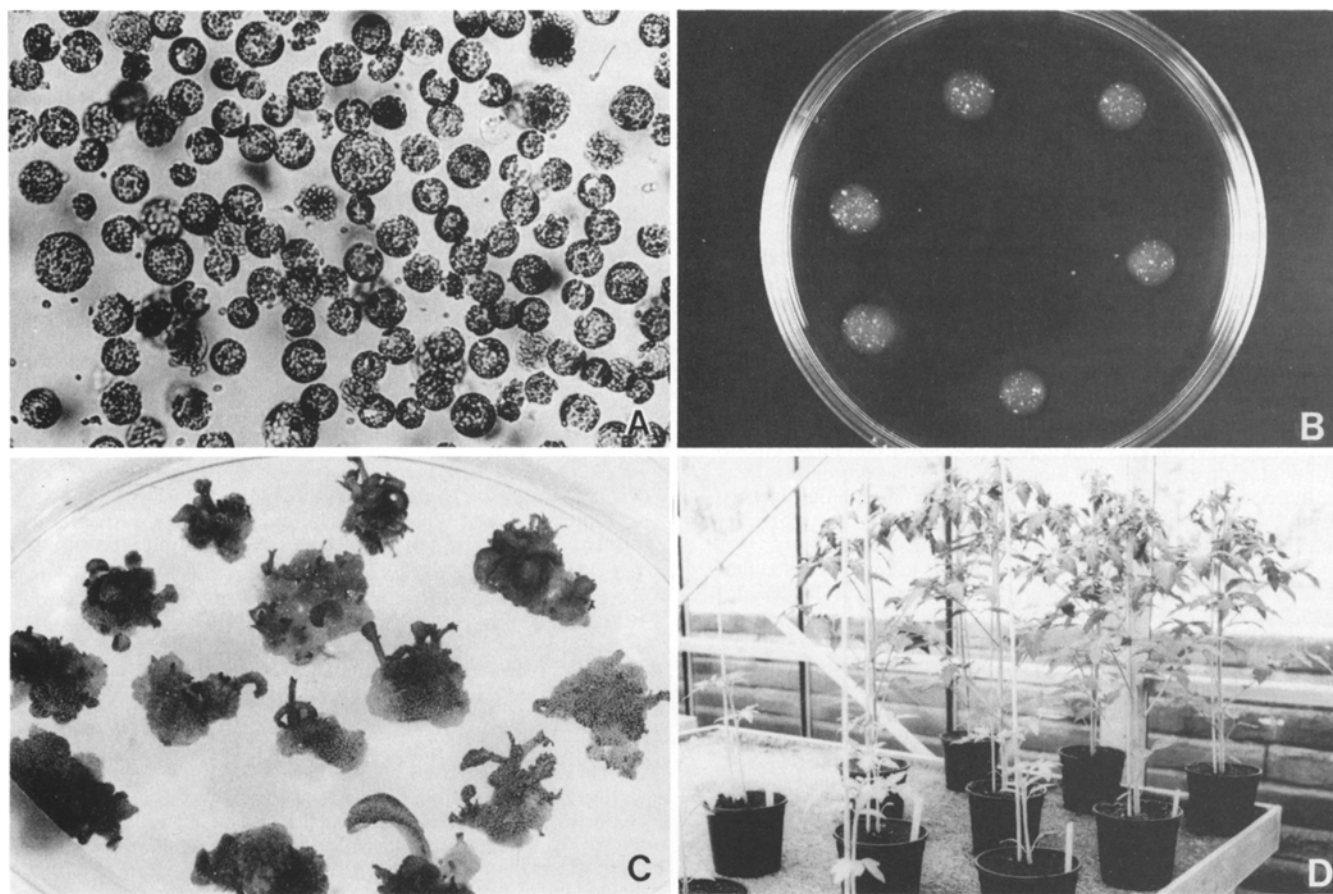

Fig.1: Plant regeneration from leaf mesophyll protoplasts of *L. esculentum* cv. Moneymaker. **A.** Freshly isolated leaf mesophyll protoplasts, **B**. Culture method of protoplasts in agarose droplets. **C**. Shoot regeneration from microcalli. **D**. Regenerated tomato plants grown in the greenhouse

centrifuged for 3 min at 45 x g. The protoplast pellets were resuspended in 10 ml of rinse medium and washed by centrifugation for 3 min at 45 x g. The protoplasts were resuspended in 9 ml of 15% sucrose in CPW salts, topped with 1 ml rinse medium and recentrifuged for 7 min at 45 x g. Viable protoplasts were collected at the interface and washed twice by centrifugation in rinse medium before they were resuspended in culture medium. The number of protoplasts was determined using a hemocytometer.

Protoplast culture

The isolated and resuspended protoplasts (Fig.1a) were cultured, unless stated otherwise, in 1.6 ml liquid Lycopersicon Culture Medium (LCM) at a density of 10^5 pps/ml. This medium consists of B 5 micro- and macro nutrients (Gamborg et al., 1968) but without NH_4NO_3 supplemented with 0.88 mg/l folic acid, KM-8P vitamins (Kao and Michayluk, 1975), 1 g/l myo-inositol, 100 mg/l casamino acids, 3 mM MES (pH 5.8), 7% mannitol, 1% sucrose, 0.5% glucose, 1 mg/l NAA, 0.5 mg/l 6-BAP and 0.5 mg/l 2,4-D. The osmolarity of the medium was ± 10 530 mOsm/kg. They were cultured in 6 cm petri dishes, sealed with Nescofilm. After 4 days of incubation at 25° C in the dark, cell wall synthesis and the first cell divisions could be observed (Fig. 1c). Subsequently, 0.4 ml of fresh culture medium containing 0.75 mg/l 6-BAP, but no auxins, was added every 4 days. Each time when the total volume of the protoplast cultures had doubled, the cultures were divided over 2 dishes. In the case where the protoplasts were cultured in agarose droplets (Fig. 1b), freshly isolated protoplasts were added to the LCM medium which was solidified with 0.5% Sea Plaque LMT agarose (Marine Colloids, USA), to give a final density of 10^5 pps/ml. Six droplets (100 µl) of this mixture was pipetted into a 9 cm petri dish. After gelling of the agarose, 6 ml of liquid LCM medium was added. After four days of incubation at 25°C in the dark, the dilution of the surrounding medium was performed twice a week by replacing 1.0 ml medium with the dilution medium, which contained 0.75 mg/l 6-BAP, but no auxins.

Plating efficiency (number of dividing protoplasts/total number of protoplasts) was determined ten days after protoplast isolation. After 25 days, when the microcalli have reached the size of about 1-2 mm, they were transferred to greening medium consisting of MS micro/macro nutrients, Nitsch vitamins (Nitsch and Nitsch, 1969) 0.2 M mannitol, 7.3 mM sucrose, 0.5 mg/l 6-BAP, 0.05 mg/l NAA and 1% Sea Plaque agarose. The osmolarity of the medium was 250 ± 10 mOsm/kg.

Transfer of the rapidly growing microcalli was performed twice a week, by cutting them into 2-3 pieces. After 14 days they were transferred on the same medium, but with 2 mg/l zeatin and 0.1 mg/l IAA instead of the usual hormones. When the bright-green or yellow-green calli showed green bud primordia, they were transferred to shoot induction medium (MS medium, 2% sucrose, 2 mg/l zeatin and 0.1 mg/l IAA). Light intensities were increased gradually throughout the regeneration procedure from 500-1000 lux (on the greening medium) to 3000 lux (on the shoot induction medium). Plates were observed periodically for the appearance of shoots (Fig. 1c). Shoots were excised from the callus and transferred to test tubes with rooting medium (MS medium, 2% sucrose). The plants were potted in soil and after being adapted gradually to a lower humidity (by increasing the opening of the lids of the pots), they were transferred to the greenhouse (Fig. 1d).

RESULTS AND DISCUSSION

Preconditioning and source of the donor plants

Previous studies on the culture of protoplasts of *Solanum tuberosum* and *Lycopersicon esculentum* (Shepard, 1977; Cassels & Barlass, 1978) showed that the use of donor plants at the right physiological stage is a prerequisite for obtaining satisfactory yields of viable protoplasts which are able to divide and regenerate into plants. The exposure of the donor plants to low light intensities (1500-2000 lux) and to short photoperiods (6 h light) was beneficial for obtaining consistently high yields of viable protoplasts from *Solanum* (Tan et al., 1987; Hassanpour-Estahbanati and Demarly, 1985) as well as *Lycopersicon* species (Tabaeizadeh et al., 1984). An explanation for this could be that when plants, such as tomato, are grown under low light intensities, the leaves tend to synthesize thinner cell walls with low concentrations of pectate (Cassels & Barlass, 1976). As a consequence, such leaves will require lower concentrations of cell wall degrading enzymes and therefore, will yield protoplasts that are more viable. In view of this, we have also grown our tomato plants (either *in vitro* or *in vivo*) prior to protoplast isolation under low light intensities at 2000 lux for at least 7 days.

Cold conditioning of plant material was used to enhance the stability of *Solanum* and *Lycopersicon* protoplasts (Haberlach, 1985, Tan et al., 1987). The beneficial effect of cold treatment is not yet fully understood, but it may shut down the physiological stress response by lowering the enzyme activities, thus reducing possibly harmful oxidation processes during cell wall digestion and protoplast isolation. In general, this pretreatment could induce the plant to adapt to stress conditions which are imposed during the protoplast isolation procedure.

We have developed a procedure in which donor plant material from tomato cultivars is grown under standard conditions (*in vivo* as well as *in vitro*), preconditioned at low light intensity and pretreated at 4°C for 6 hours in the dark, prior to protoplast isolation. Pretreatment of the donor plants at low temperatures lowers the yield of protoplasts isolated from leaves of the tested tomato cultivars, but the obtained protoplasts are more viable and show sustained divisions. Pilot studies have shown that there are no differences between pretreatment at 10°C for 12 h or at 4°C for 6 h, so for practical reasons we have chosen the last treatment. Chilling injury was observed when the plants were treated much longer at 4°C, or at lower temperatures. Shorter incubation periods or pretreatment at higher temperatures gave lower plating efficiencies and resulted in micro calli which rapidly turned brown.

Table 1: **Plating efficiencies and frequency of shoot regeneration from leaf mesophyll protoplasts of different tomato cultivars.**

cultivar:	pps/g leaf	PE[a] (%)	no. of tested calli	% shoot regeneration[b]
Moneymaker	1.7x105	35 ±5	210	30%
Bellina	$1.9x10^5$	30 ±6	113	22%
Sonatine	$2.1x10^5$	13 ±4	108	9%
Abunda	$1.8x10^5$	5 ±3	100	4%

a All protoplasts of the tomato cultivars were cultured on LCM-medium.

b Shoot formation is expressed as the percentage of microcalli that gave at least one shoot

Table 2: **Plating efficiencies (%) of protoplasts of tomato cultivars on different culture media.**

	AT	SCM	TM-2	M-8E	LCM
Money-maker	22.5 ±5	6.0 ±4	4.0 ±4	12.5 ± 5	35.0± 5
Bellina	25.0 ±4	20.0 ±5	4.0 ±2	10.0 ±4	25.0± 5

All media were adjusted to the carbohydrate and hormone concentrations as used in LCM medium.

Protoplast culture methods

Protoplasts are cultured in 1.6 ml liquid medium, in 6 cm petri dishes. To stimulate cell growth, an initial protoplast density of 0.5 - 2.0 x 10^5 protoplasts per ml was required, depending on the genotype. Higher numbers of protoplasts favoured initial cell division, but it caused aggregation of the protoplasts and increased the browning of cell colonies. The auxin 2,4-D appeared to be necessary to initiate cell division of the protoplasts as was stated earlier by Niedz et al. (1985). We have tested concentration range between 0.1 and 1.0 mg/l and found no differences in plating efficiencies between 0.5 and 1.0 mg/l 2,4-D. We have chosen the lower concentration because high auxin levels may inhibit shoot regeneration. However, if the auxin level remains the same in the culture medium, then the microcalli start turning brown after 7-10 days. Therefore, the dilution medium in our procedure contains no auxins. Another method which can be used to avoid browning of the microcalli is a culture method in which the protoplast are cultured in small agarose droplets, surrounded by liquid culture medium. This culture system was recently used to culture protoplasts of *Cucurbitaceae* (Brouwer and Dons, 1986). The method facilitates frequent addition of fresh liquid medium and removal of used medium, without damaging the protoplasts. Furthermore it can also be applied to culture manually isolated fusion products in smaller microdroplets. Both methods have proven to give the same plating efficiencies for the tomato cultivars tested in our laboratory. Data presented in this paper are based on the culture method in liquid medium.

Culture media

The Lycopersicon Culture Medium (LCM), which in our laboratory gave succesful plant regeneration from tomato protoplasts, was developed by combining the NH_4NO_3-free micro/macro salts of B5 (Gamborg et al., 1968), with the rich vitamins of Kao and Michayluk (1975). No significant differences were detected in plating efficiencies between protoplasts derived from *in vitro* and *in vivo* grown plants, provided that these plants were pretreated, and the protoplasts isolated and cultured, as was described in our procedure. However, when the osmolarity of the culture medium was reduced to approximately 400 mOsm/kg. (by lowering the mannitol concentration to 6%), protoplasts from *in vitro* grown plants divided more rapidly and reached a higher plating efficiency than those of the in vivo grown plants. Further experiments are under way to compare the regeneration responses of the protoplasts from these two types of explants. Optimal osmolarity for protoplasts from *in vivo* grown plants was found to be between 500-540 mOsm/kg, depending on the genotype. The addition of a buffer MES (3mM) seems essential to prevent a sudden decrease in pH during cell divisions.

To compare our culture medium with other, recently developed tomato protoplast media, we have also tested protoplasts of the tomato cultivar Moneymaker and the hybrid variety Bellina for their behaviour in different protoplast culture media: AT medium (Adams and Townsend, 1983), TM-2 medium (Shahin 1985), M-8E medium (Niedz et al., 1985) and the Solanum Culture Medium (SCM), which was used for wild *Lycopersicon* and *Solanum* species in our laboratory (Tan et al., 1987). In TM-2 medium the concentration of mannitol had to be increased to 0.3 M because our protoplasts did not divide in TM-2 medium with the original osmotic value (380 ± 10 mOsm/kg), which was originally described for protoplasts of *in vitro* grown plants. This may suggest that different types of explants may require different osmotic values of the media. The other media were tested without changes, except that the following hormone concentrations were used in all experiments: 1 mg/l NAA, 0.5 mg/l 6-BAP and 0.5 mg/l 2,4-D. Using our protoplast isolation and culture procedure, we have observed good plating efficiencies on AT medium and on LCM medium for the tested cultivars. Low plating efficiencies were obtained on TM-2 medium. Genotypic differences play an important role in the response of different culture media, as we have seen in studies with 11 cultivars (Tan et al., submitted). Furthermore, we could observe differences in the response of the cultivars Moneymaker and Bellina on SCM medium (Table 2) and also in plating efficiencies of the 4 tomato cultivars on LCM medium (Table 1). Therefore, these data indicate that one particular medium will not always result in optimal plating efficiencies for every tested tomato cultivar. Nevertheless, we have shown that in using the LCM medium in combination with the preconditioning of the donor plants, the protoplast isolation and the 2 protoplast culture procedures give satisfactory results for all tested tomato cultivars.

Plant regeneration

Shoot regeneration was obtained 3 months after protoplast isolation for all 4 tested cultivars. To obtain efficient plant regeneration, the microcalli have to be cultured on medium containing zeatin (2 mg/l) and IAA (0.1 mg/l). When the microcalli are transferred directly from culture medium to this regeneration medium, they rapidly turn brown and die. It is important to gradually lower the osmolarity of the medium and to induce greening of the microcalli, (which both happen on the greening medium) before the microcalli could be induced to form shoots on regeneration medium. Sometimes certain genotypes will give microcalli, which will turn brown rapidly on the regeneration medium. By adjusting the osmolarity (by adding 0.1 M mannitol in the regeneration medium) this browning reaction can be reduced. In our procedure, the addition of gibberrellic acid did not give a better response in shoot regeneration as has been described earlier (Shahin, 1984), because it only stimulated callus growth.

In this paper we have shown that preconditioning of the donor plants, the genotype as well as the choice of culture medium and the culture method, all seem to be important for good plating efficiencies of tomato protoplasts. The cultivars Moneymaker and Bellina gave the highest regeneration frequencies, 30% and 22% respectively. Rapid plant regeneration can be obtained within 3 months with a two step culture method, by first inducing greening of the microcalli, and then by inducing shoots on regeneration medium, while gradually lowering the osmolarity of the medium.

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REFERENCES

Adams TL and JA Townsend (1983) Plant Cell Rep., *2,* 165-168.
Brouwer R and JJM Dons (1986) Acta Bot. Neerl., *35,* 45.
Cassels AC and M Barlass (1976) Physiol. Plant., *37,* 239-246.
Cassels AC and M Barlass (1978) Physiol. Plant., *42,* 236-242.
Frearson EM, JB Power and EC Cocking (1973) Dev. Biol., *33,* 130-137.
Gamborg OL, RA Miller and K Ojima (1968) Exp. Cell. Res., *50,* 151-158.
Haberlach GT, BA Cohen, NA Reichert, MA Baer, LE Towill, and JP Helgeson (1985) Plant Sci. Lett., *39,* 67-74.
Hassanpour-Estahbanati A and Y Demarly (1985) J. Plant Physiol., *121,* 171-174
Kao KN and MR Michayluk (1975) Planta *126,* 105-110.
Murashige T and F Skoog (1962) Physiol. Plant., *15* , 473-479.
Niedz RP, SM Rutter, LW Handley and KC Sink (1985) Plant Sci. Lett., *39* , 199-204.
Nitsch JP and C Nitsch (1969) Science *163,* 85-87.
Rick, CM (1982) In: Vasil, I.K., W.R. Scowcroft and K.J. Frey (eds): Plant Improvement and Somatic Cell Genetics pp. 1-28. Academic Press, New York.
Shahin EA (1985) Totipotency of tomato protoplasts Theor. Appl. Genet., *69* , 235-240.
Shepard JF and RE Totten (1977) Plant Physiol., *60* ,313-316.
Tabaeizadeh Z, C Bunniset-Bergounioux and C Perennes (1984) Physiol Veg. *22* (2), 223-229.
Tan MMC, F Van der Mark, J Hoogendijk, GAM Van Marrewijk and AJ Kool (1986) Acta Bot. Neerl., *35* ,45.
Tan MMC, HS Boerrigter and AJ Kool (1987) A rapid procedure for plant regeneration from protoplast isolated from suspension cultures and leaf mesophyll cells of three *Solanum* species. Plant Sci. Lett., (in press).