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Somatic Embryogenesis from *Lycopersicon peruvianum* Leaf Mesophyll Protoplasts*

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Summary. One to five percent of *Lycopersicon peruvianum* (L.) Mill. leaf mesophyll protoplasts undergo cell division and concomitant organization to form embryogenic-like structures when cultured in Murashige and Skoog medium (1962) containing 3% sucrose, 9% mannitol, 1.0 mg/l kinetin (K) and 1.0 mg/l naphthalene acetic acid (NAA) at pH 5.6-5.8 (medium A). These embryogenic structures, after passing through developmental stages similar to those observed in zygotic embryogeny, are capable of forming shoots on hormone-free medium A. In medium B, wherein 0.5 mg/l of 2,4-dichlorophenoxyacetic (2,4-D) replaced the hormones (K and NAA), embryogenic structures did not develop. However, callus originating in medium B retained morphogenetic capacity as was evidenced by subsequent shoot regeneration when they were transferred to medium A with K and NAA replaced by 1.0 mg/l zeatin (Z). The potential value of incorporating this regeneration trait into *Lycopersicon* species and cultivated lines for use in tissue culture programs is discussed.

Key words: *Lycopersicon* – Protoplasts – Somatic embryogenesis – Plant regeneration

Introduction

Plants of different species have been recovered following the isolation and culture in vitro of their protoplasts (Thomas et al. 1979). Thus, as successful regeneration is attained for a wider range of economic species, they may be increasingly utilized in somatic hybridization studies as well as in studies on plant somatic cell genetics related to plant breeding programs (Cocking 1977). Plant regeneration and protoplast fusion with protoplasts of species in the genus *Lycopersicon* are of particular importance since the recovery of sexual interspecific hybrid plants is often very low and difficult due to cross-incompatibilities (Rick 1973). Successful plant recovery from leaf mesophyll protoplasts of *Lycopersicon esculentum* and *L. peruvianum* has previously been reported (Zapata et al. 1977). The former species had a low regeneration capacity compared to *L. peruvianum*, which readily formed shoots. In addition to the low inherent regeneration capacity of *Lycopersicon esculentum*, and many other plant species, another limitation in protoplast culture methodology is that most species pass through a callus stage during culture which may result in chromosomal aberrants (Sink and Power 1977). One method to overcome this difficulty could be through somatic embryogenesis. The formation of embryoid-like structures directly from isolated protoplasts has been observed from callus and cell suspension cultures of the amphi-haploid and diploid *Nicotiana tabacum* cv. Samsum (Lorz et al. 1977), from leaf mesophyll and suspension cultures of haploid *Nicotiana sylvestris* (Facciotti and Pilet 1979), from suspension cultures of *Atropa belladonna* (Gosch et al. 1975) and *Daucus carota* L. cv. 'Royal Chantenay' (Dudits et al. 1976), from non-differentiating ovular callus of *Citrus sinensis* (Vardi et al. 1975), from embryogenic suspension cultures derived from immature embryos of *Pennisetum americanum* (Vasil and Vasil 1980), and from leaf mesophyll protoplasts in *Medicago sativa* (Kao and Michayluk 1980). This study reports the developmental stages of somatic embryo-like structures arising from *Lycopersicon peruvianum* leaf mesophyll protoplasts. This trait will be used in future experiments in the production of somatic hybdrids and in exploring the possibility of transferring this embryogenic trait to other *Lycopersicon* species and breeding lines.

Materials and Methods

Seeds of *Lycopersicon peruvianum* (L.) Mill. were kindly provided by Dr. S. Izhar (Division of Plant Genetics and Breeding, The Volcani Center, Bet-Dagan, Israel) and germinated on a soil-less plant-

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ing medium (V.S.P., Bay Houston Towing Co.) in a greenhouse under 550 $\mu Em^{-2}s^{-1}$ light intensity from fluorescent lamps (G.E. F96T12 CW). Seedlings were subsequently transplanted into 10 cm clay pots using the same cultural procedures. Fully expanded leaflets from 6 to 8 week old plants were surface sterilized, and all further steps were carried out using aseptic techniques: 20 min in an aqueous solution of 0.01% NaOCl (diluted commercial Clorox 5.25%), followed by 4 separate sterile distilled water rinses. Leaflets were allowed to become flaccid for approximately 20 min, and using fine jeweler forceps, the lower epidermis was removed. The leaf pieces were floated abaxial side down on an enzyme solution containing 1% (w/v) Cellulase R-10, (Yakult Biochemicals Co. Ltd., Nashinomiga, Japan), 0.5% (w/v) Macerase, (Calbiochem, San Diego, California, USA), 0.5% (w/v) Driselase (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) and 0.5% (w/v) potassium dextran sulphate (Calbiochem, San Diego, California, USA), in the presence of 8% (w/v) mannitol, and CPW salts (Cocking and Peberdy 1974) in mg/l: KH_2PO_4 (27.2), KNO_3 (100), $CaCl \cdot 2H_2O$ (1480), $MgSO_4 \cdot 7H_2O$ (246), KI (0.16) and $CuSO_4 \cdot 5H_2O$ (0.025), pH 5.6-5.8 using 0.1 N HCl for titration.

After 4 hr incubation in the dark at 25°C, digestion was complete. The protoplasts were pelleted by centrifugation at 40 g for 5 min. The supernatant was removed and replaced by a solution of CPW salts containing 21% (w/v) sucrose, pH 5.6-5.8. The resuspended protoplasts were centrifuged at 80 g for 5 min and the floated protoplasts were collected, diluted and counted by using a haemocytometer (Modified Fuchs Rosenthal). The yield of protoplasts was approximately 2×10^6 per gm of leaf tissue. The isolated mesophyll protoplasts (Fig. 1) were cultured in liquid medium A or B at a final density of 5×10^4 protoplasts per ml in plastic Petri dishes (Falcon 1007, 60 × 15 mm), and wrapped with Parafilm. Plated protoplasts were incubated in the dark at 25°C for 8 days and then transferred to a light intensity of 15 $\mu Em^{-2}s^{-1}$ (16 h photoperiod) at 25°C. Every 8-10 days fresh medium with progressively lower mannitol concentrations (6, 3, 0%) was added in order to maintain cell division.

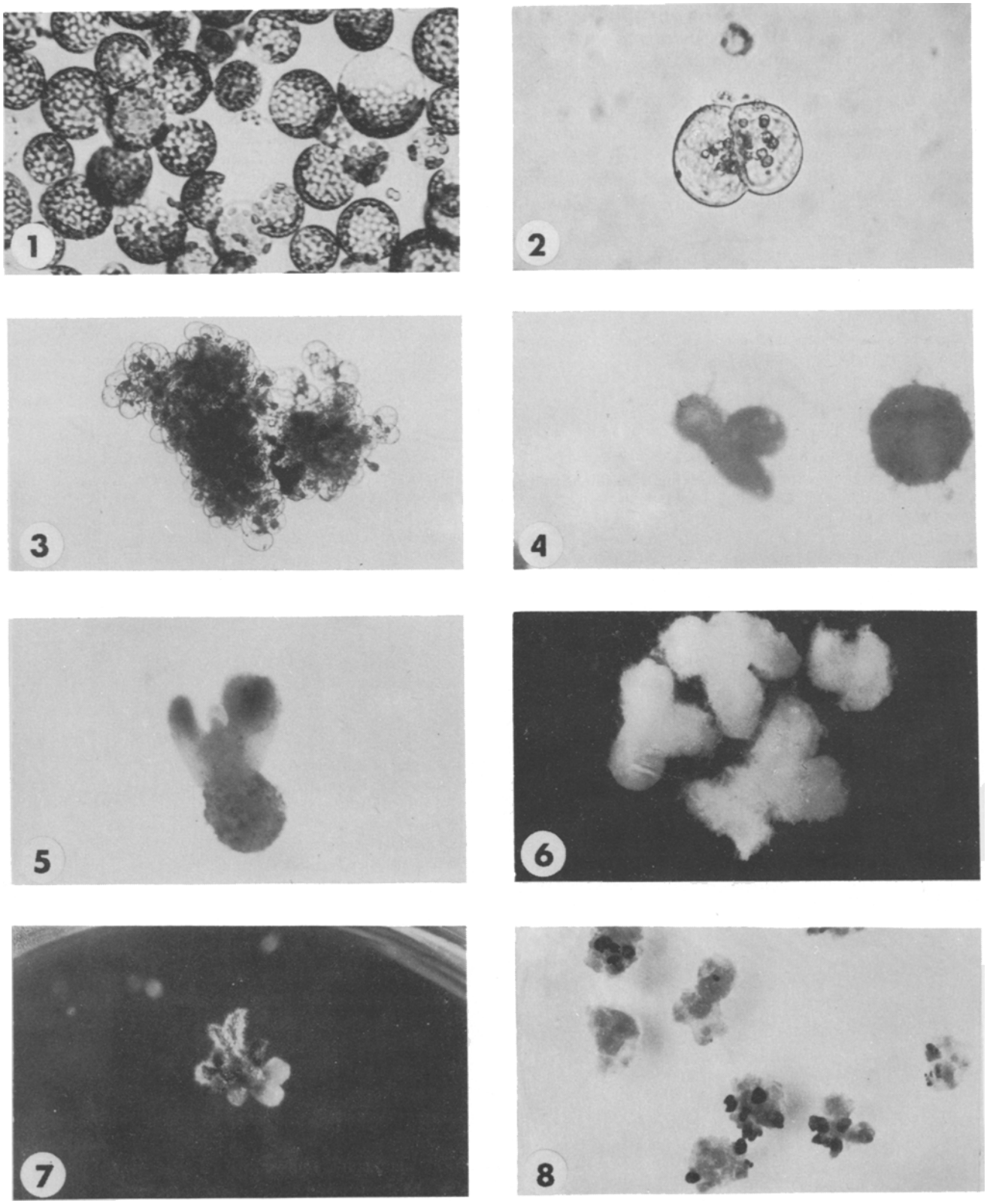
Results and Discussion

First cell division was observed in both media A and B after 2 days in culture (Fig. 2). Within 8 to 14 days in culture, plating efficiencies were calculated and found to be 1-5% in medium A and 20-30% in medium B. Small colonies were visible in both media (Fig. 3) after 15-20 days in culture. Cells of colonies in medium A observed under light microscopy appeared small with dense cytoplasm and starch accumulation. The cells of the colonies in medium B were usually highly vacuolated and larger. After one month, structures very similar to the embryogenic globular (Fig. 4), and cotyledonary (Figs. 5, 6) stages were visible to the naked eye. These separate stages could be observed simultaneously since the developmental pattern from isolated protoplast to cotyledonary stage was not synchronized in any isolation. Even so, only embryogenic-like structures occurred in medium A. This observation is in contrast to what has been observed in *Nicotiana sylvestris* where a mixed population of callus and embryogenic developmental patterns occurred (Facciotti and Pilet 1979). The structures at macroscopic size (Figs. 5, 6) were transferred to medium A, solidified with 0.8% agar and without growth regulators, where they continued to develop into tomato shoots (Fig. 7). Adventitious roots often developed on the basal portion of these shoots which readily facilitated transfer to the soil-less planting medium. If they were kept in medium A, callus formed and multiple shoots were observed (Fig. 8).

In order to study the factors controlling somatic embryogenesis, the colonies growing in medium B were transferred to medium A. To date, the attempt to induce embryo-like structures in this manner has not been successful, although when callus from medium B was cultured on Murashige and Skoog medium (1962) containing 1.0 mg/l zeatin, shoots were regenerated. From our experiments, the induction of embryo-like structures appeared to require both an auxin and cytokinin, as found in medium A, for a certain period of culture time in order to induce the formation of embryogenic clumps. When the auxin source was changed, as in medium B, a different developmental pattern occurred. Embryos were not formed in the presence of 2,4-D in medium B when the colonies were transferred from medium B to A. Thus, 2,4-D may suppress the embryogenic potential of the cells by inhibiting the expression of those genes involved in somatic embryogenesis and consequently divert the embryogenic tomato cell into callus formation. Tisserat and Murashige (1977) found that 2,4-D at 0.1 mg/l completely suppressed embryogenesis in carrot callus.

Butenko et al. (1967) reported the necessity of reduced nitrogen for somatic embryogenesis in carrot cells while Lorz et al. (1977) showed that tobacco protoplasts cultured at increased concentrations of NH_4^+ formed embryo-like structures more quickly. The MS culture medium used in our experiments is rich in reduced nitrogen and as Reinert (1973) has discussed, there is the possibility that the conversion of a somatic cell to an embryo is not only due to the presence of reduced nitrogen but to an increase in the ratio of nitrogen to auxin. Based on our observations, formation of *Lycopersicon* embryogenic cells is dependent on the interaction of growth substances which may regulate totipotent expression and thus direct a somatic cell(s) to become embryogenic. Somatic embryogenesis decreases the required in vitro culture time from isolated protoplast to regenerated shoot when compared to the conventional method used in tissue culture. Thus, the occurrence of adverse genetic changes should be minimal.

Until now the utilization of tissue culture technology for improvement in *Lycopersicon* has been hindered due to the difficulty in regeneration capacity of both cultivated varieties and some wild species. For example, shoot regeneration from *L. peruvianum* callus derived from leaf mesophyll protoplasts was high and repeatible (Zapata et



Figs. 1-8. 1-freshly isolated leaf mesophyll protoplasts from *Lycopersicon peruvianum*; 2-first division of mesophyll protoplast two days after plating; 3-small cell colony, approximately 300 cells, 15 to 20 days in culture; 4-globular embryogenic stage; 5, 6-cotyledonary embryogenic stage; 7-early shoot development on MS basal medium; 8-callus and shoot development when cotyledonary stage was retained in medium A (Magnification: **Fig. 1** 100X; **2** 1200X; **3** 600X; **4** 750X; **5** 750X; **6** 750X; **7** 15X; **8** 15X)

al. 1977) but in callus obtained from leaf mesophyll protoplasts of seven cvs. of *L. esculentum*, only one cv., 'Ailsa Craig', infrequently produced shoots (Zapata, unpublished results).

Hence, successful sexual transfer of the somatic embryogenic trait reported herein in *L. peruvianum*, into cultivated and wild species of *Lycopersicon* may be the key step to securing efficient plant regeneration and thus enable breeder application of somatic cell techniques in tomato.

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