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Regeneration of Seeda Tomato Plants from Isolated Protoplasts

Praparat Kalayawudhipong Supat Attathom and Rongrong Visessuwan

ABSTRACT

Greenhouse grown and tissue cultured Seeda tomato plants were used as leaf tissue source for protoplast isolation. Yields of 4.39×10^6 and 8.3×10^4 protoplasts/g fresh tissue were obtained from greenhouse grown and tissue cultured plants respectively. Only protoplasts from field grown plants divided, developed microcalli and regenerated into the whole plant.

INTRODUCTION

Protoplasts isolated from various plants have been used intensively for research in Plant Molecular Biology like the study of plant-virus interaction and gene transfer. Cocking (1977) reported the DNA transfer in tomato through protoplast fusion. Melchers *et al.* (1978) successfully fused potato and tomato protoplasts and regenerated into whole plant. David (1981) produced tobacco plants resistant to virus disease by protoplast fusion of 2 wild tobacco species. There were reports of successful protoplast fusion in petunia (Power *et al.*, 1976), rice (Yang *et al.*,1988) and some legumes (Sexen *et al.*, 1986).

At Plant Genetic Engineering Unit, Kasetsart University we are developing the biotechnological methods to produce tomato plants resistant to tomato yellow leaf curl virus. Since tomato becomes more and more economically important to Thailand, we have started the project to isolate protoplasts and regenerate plants of Seeda tomato, the most commonly used local Thai tomato variety, for experimental uses in the process of developing virus resistant plant.

MATERIALS AND METHODS

Plant Samples

Seeds of Seeda tomato variety were used as source of experimental plant samples. Greenhouse grown plants were obtained by planting germinated seeds in glasshouse at 25°c for 25 to 30 days. Plants grown *in vitro* were obtained from seeds, surface sterilized in 70% ethanol for 2 min and 20% chlorox for 10 min followed by three rinses with sterile distilled water. They were then germinated on MS medium (Murashige and Skoog, 1962) containing 2% sucrose and kept at 25°c with 2000 lux, 16 hrs per day illumination. After germination, shoot tips were excised and subcultured onto the same medium for experimental uses.

Protoplast Isolation and Purification

Young leaves, third from the top, were excised from greenhouse and *in vitro* grown plants. They were surface sterilized in 5% chlorox for 10 min and washed three times in sterile distilled water. Lower epidermal layers were removed with a tweezer and the leaves were soaked in washing medium containing 190 mg/l KNO_3, 44 mg/l $CaCl_2 . 2H_2O$, 37 mg/l $MgSO_4 . 7H_2O$, 17 mg/l KH_2PO_4 and 91.1 g/l mannitol in the dark for 1 hr. After removal of the washing medium, leaves were digested in enzyme solution containing 1% cellulase Onozuka R-10 and 0.5 % macerozyme R-10 dissolved in washing medium. The ratio of leaf and enzyme solution was 1 g/10 ml. After digestion for 2 hrs in the dark, protoplasts were filtered through 62-82 µm nylon sieve, centrifuged at 500 rpm for 3 min and washed twice in washing medium. Protoplasts were centrifuged at 500 rpm for 3 min and resuspended in 5 ml washing medium containing 15% sucrose with no mannitol. They were

Plant Genetic Engineering Unit, Kasetsart University, Kamphaengsaen Campus, Nakhon Pathom 73140, Thailand.

then purified by layering 1 ml of washing medium over and centrifuged at 500 rpm for 5 min. Protoplasts floating between these two layers were collected by using Pasteur pipette and washed twice in washing medium followed by centrifugation at 500 rpm for 3 min.

Protoplast cultures

Concentrated protoplast suspension was diluted to 5 x 10^4 - 10^5 cells/ml with modified Gamborg's culture medium (Gamborg *et al.*, 1981) containing 1.0 mg/l 2,4-D, 1.0 mg/l NAA, 0.1 mg/l BA; 1% coconut juice, 7% mannitol, 1 % sucrose and 0.5 % glucose. Two ml sample was withdrawn with Pasteur pipette and placed in 5 cm in diameter petri dish. Protoplasts were cultured at 22-26°C in the dark for 6 days and then transferred to 1000-2000 lux light, 16 hr day length. Each dish was supplied every four days with 0.4 ml of modified Gamborg's medium with 0.75 mg/l BA and no auxin. After 3 weeks of culture, microcalli were transferred to culture medium containing MS micro/macro nutrients, Nitsch vitamins (Nitsch and Nitsch, 1969), 0.2 M mannitol, 7.3 mM sucrose, 0.5 mg/l BA, 0.05 mg/l NAA and 0.2 % w/v gelrite.

Plant Regeneration

Microcalli with the diameter of 4-5 mm were transferred to greening medium consisting of MS micro/macro nutrients, Nitsch vitamins, 0.2 M mannitol, 7.3 mM sucrose, 2 mg/l zeatin and 0.1 mg/l IAA. Green and compact calli were then transferred to shoot regeneration medium containing MS medium, 2 % sucrose, 3 mg/l zeatin and 0.2 mg/l IAA. Regenerated shoots, 3-4 cm in height were excised from green calli and transferred to rooting medium consisting of MS medium and 2 % sucrose. Rooted plants were transferred to 1:1 vermiculite and burn husk mix and maintained in a growth chamber at 22-26°C for 1 week prior to field planting.

RESULTS AND DISCUSSION

Protoplast Isolation

Cut leaf tissues with lower epidermal layers removed (Fig. 1 a) were suitable for enzyme digestion. After 2 hrs of digestion, most of protoplasts were released. Leaves collected from field grown plants yielded 4.39x10^5 protoplasts/g fresh tissue whereas cultured plants yielded 8.31x10^4 protoplasts/g fresh tissue (Table 1). Also preparation of protoplast released from field grown plants contained cells with size larger than those of cultured plants (Fig 4b, 4c). Therefore, field grown tomato leaves seemed to be a good source for protoplast isolation. The problem that researcher should be aware of is the severe epidemic of tomato yellow leafcurl disease. If there is no effective diagnosis for tomato yellow leaf curl virus (TYLCV), one may obtain infected protoplasts which will not be desirable for further uses.

Low speed centrifugation with 15 % sucrose cushion can effectively separate protoplasts from cell debris (Fig. 1d, 1e). The percentage of protoplast viability was not yet determined in this study.

Protoplast Culture

Protoplasts isolated from field grown plants grew well on culture medium as shown by cell division and callus formation (Fig. 2a-2e). No callus formation was observed in protoplast isolated from tissue cultured plants.

Cell division of protoplast was observed 4-6 days after isolation (Fig.2a). Cluster of cells resulting from cell division was pronounced in 25 days (Fig.2b). After that the increase of BA concentration and the decrease of 2,4-D and NAA are necessary for the development of microcalli (Fig.2c, 2d). Tan *et al.* (1987) reported that the constant level of auxin in the medium caused the browning of microcalli resulting in cell death within 7-10 days.

Table 1 Yields of protoplasts isolated from leaves of tissue cultured and field grown Seeda tomato plants.

Leaf Source	Yield (protoplasts/g fresh tissue)			
	1	2	3	Average
Field grown	5.23x10^6	4.53x10^6	3.40x10^6	4.39x10^6
Tissue cultured	1.30x10^5	4.17x10^4	7.70x10^4	8.31x10^4

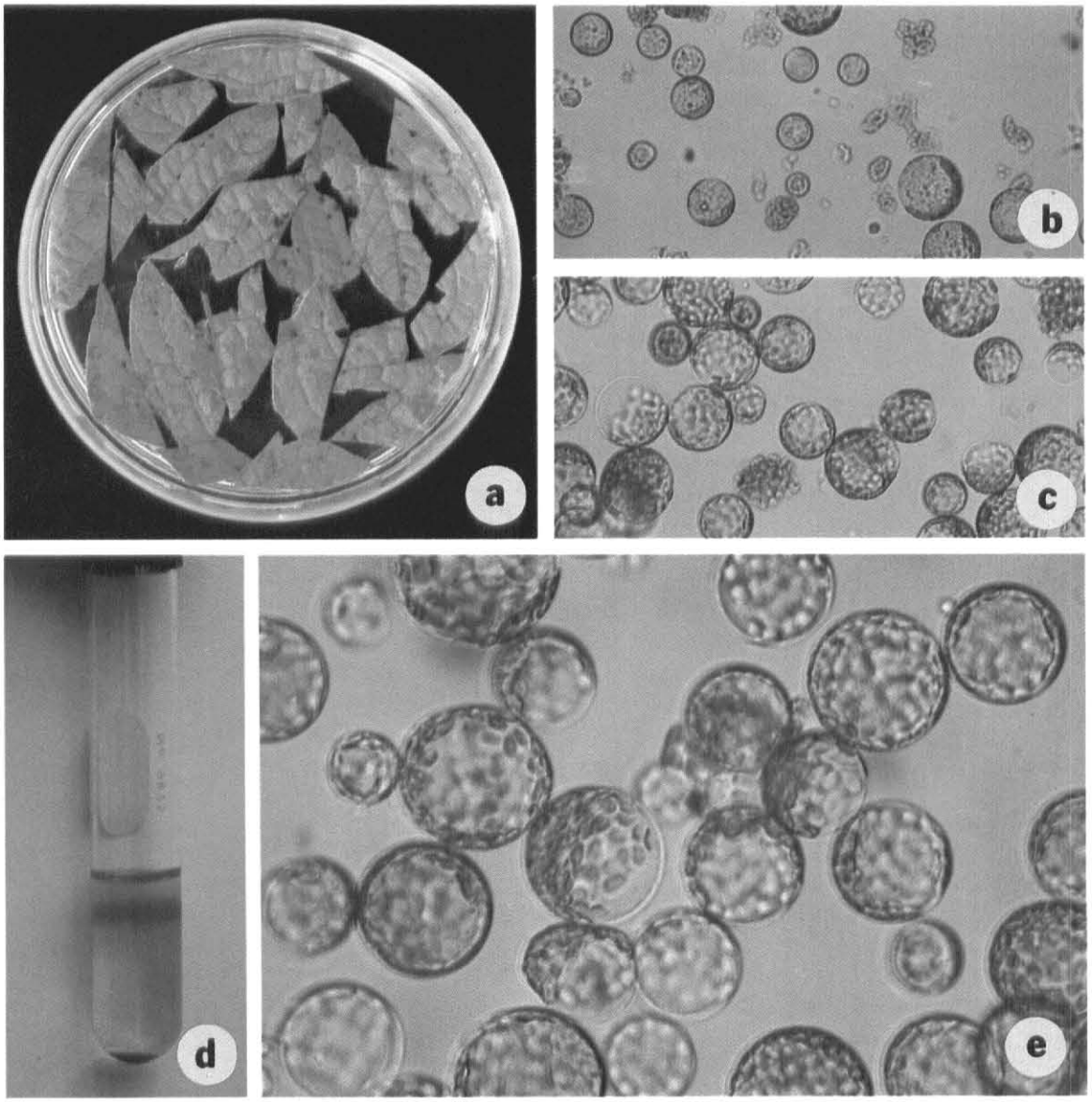

Fig. 1 Isolation and purification of proplasts of Seeda tomato
a. Seeda tomato leaves, with lower epidermis removed, in enzyme solution
b. protoplasts released from tissue cultured plant
c. from field grown plant
d. protoplast layer after low speed centrifugation in 15 % sucrose
e. purified protoplasts

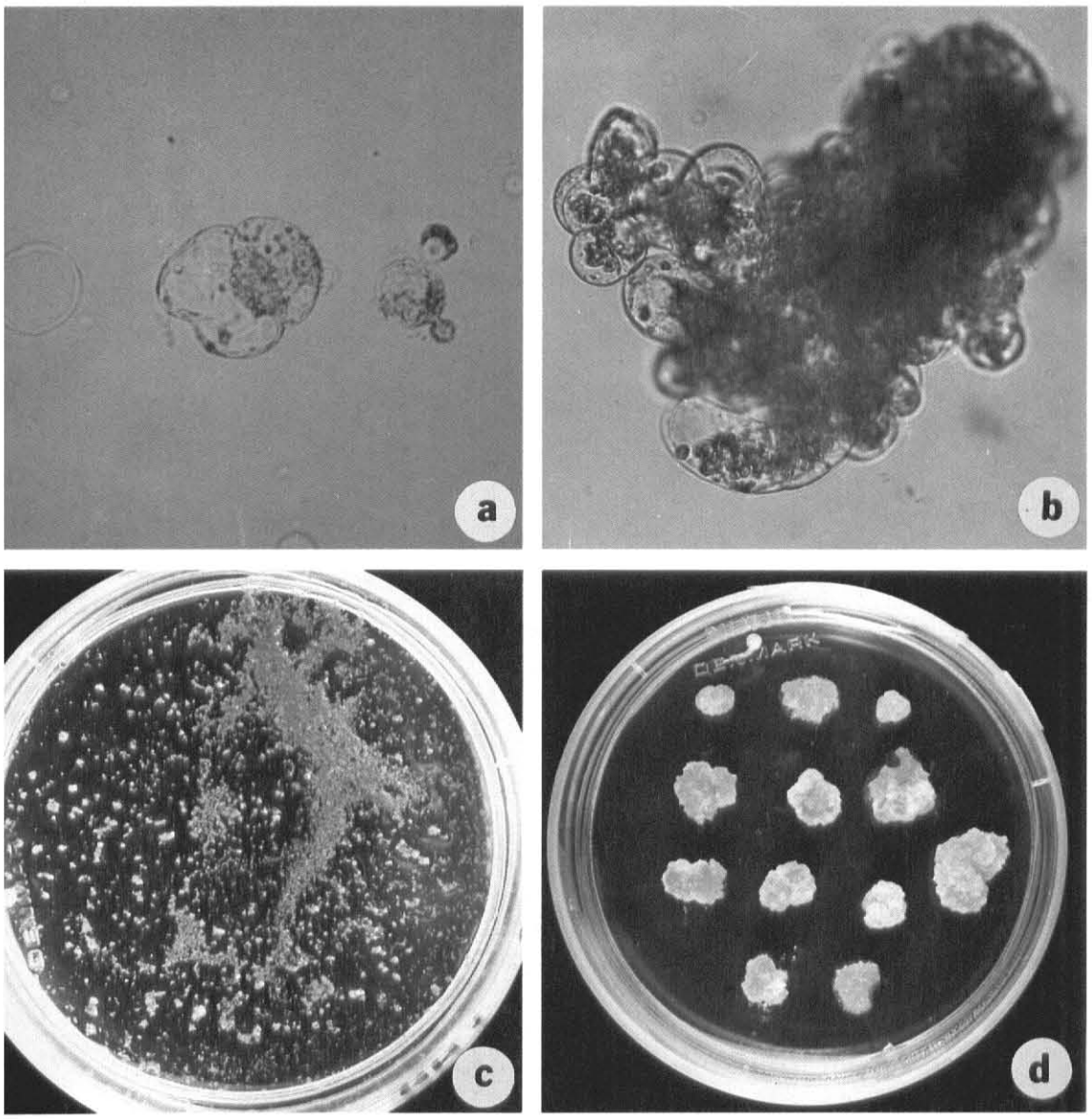

Fig. 2 Protoplast culture
a. divided cell after 6 days in modified Gamborg's medium
b. 25 days old culture showing cluster of cells
c. microcalli plated on MS micro/macro nutrients containing Nitsch vitamins.
d. microcalli on greening medium

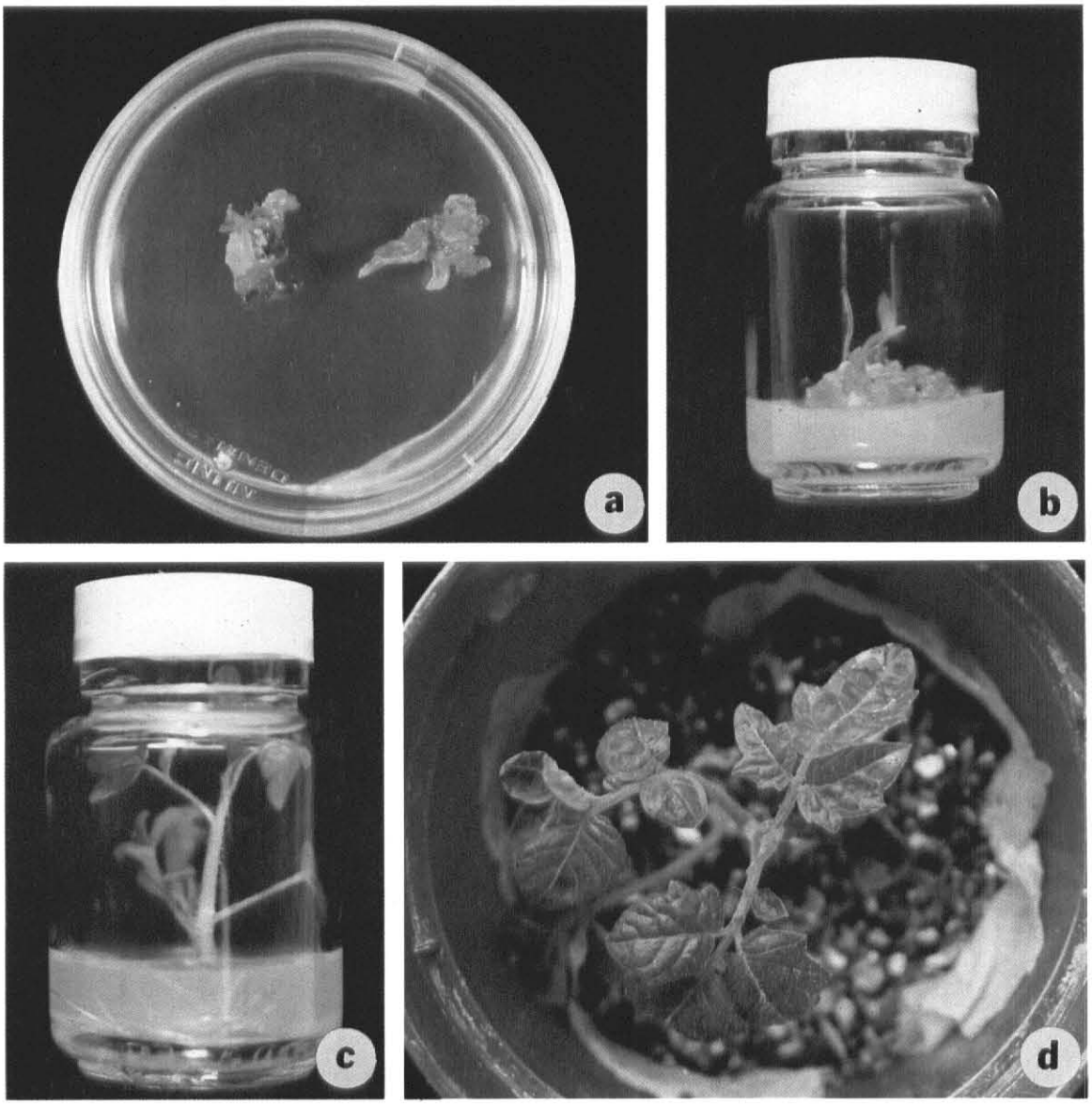

Fig. 3 Plant regeneration of isolated protoplasts
a-b, microcalli plated on modified shoot regeneration medium showing shoot formation.
c. plant development of regenerated shoot in rooting medium
d. regenerated Seeda tomato plant on pot culture

Plant Regeneration

Gradual reduction of osmotic pressure was important to microcallus development. Therefore greening medium with 0.2 M mannitol is a necessary step prior to culture in shoot regeneration medium which contains no mannitol. Similar result was observed by Tan *et al.* (1987) who regenerated plant from leaf mesophyll protoplasts of tomato cultivars. We failed to regenerate Seeda tomato plant by using shoot regeneration medium (Tan *et al.* 1987) containing 2 mg/l zeatin and 0.1 mg/l IAA. Successful shoot regeneration was obtained from modified shoot regeneration medium containing 3 mg/l zeatin and 0.2 mg/l IAA (Fig.3). This may simply reflect the different tomato cultivars being used in the studies.

We reported here the procedure for isolation, culture and plant regeneration of protoplasts of Seeda tomato. We believed that this work will be useful to the future tomato development program in Thailand.

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