Production and Analysis of Somatic Hybrids between *Lycopersicon esculentum* x *L. peruvianum* and *Solanum lycopersicoides*

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by

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ABBREVIATIONS

µm	micrometer
mm	millimeter
cm	centimeter
m	meter
µl	microliter
ml	milliliter
BA	benzyladenine
NAA	α-naphthaleneacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
GA_3	gibberellic acid
IAA	indole-3-acetic acid
ZR	zeatin riboside
VAC	volts of alterating current
var.	variety
cv.	cultivar
°C	degree centigrade
%	percent
rpm	revolutions per minute
mg/l	milligram per liter
kV/cm	kilovolt per centimeter
µs	micro second
sec	second
min	minute
h	hour
ng	nano gram
mg	milligram
g	gram
mM	millimolar
M	mole
mU	milliunit
NaAC	sodium acetate
EDTA	disodium ethylenediamine-N,N,N',N'- teteraacetic acid
TE buffer	Tris-EDTA buffer

TAE buffer	Tris-acetic acid-EDTA buffer
cm^2	square centimeter
SSC	saline sodium citrate
SDS	sodium dodecyl sulfate
N	normal
LB medium	Luria-bertani-broth medium
kb	kilobase
ϕ	diameter

1. INTRODUCTION

1.1 Introduction of Tomato

1.1.1 Tomato

Tomato is among the most popular fruits or vegetables. More than one and a half billion tons of tomatoes are produced commercially every year in the world (Smith 1994). Its origin was not resolved until the middle of the 20th century . The account that emerged was that the tomato originated in the coastal highland of western South America, where Mexico is especially considered to be the original site of domestication according to the balance of evidence (Esquinas-Alcazar 1981). The cherry tomato (*Lycopersicon esculentum* var. *cerasiforme*) which is two-celled is known to be the direct ancestor of the modern cultivated tomato. A genetic mutation occurred, developing a multi-celled fruit, which was large and lumpy. Currently large, smooth-skinned fruits are probably crossed between the large, lumpy-skinned mutations and the smooth-skinned cherry tomato (Rick 1956).

Firstly, tomato was propagated to Spain from original region, to Italy secondly, and finally spread to another European countries in Europe. Although tomato was considered to have been cultivated in continental Europe since 1540s, they were not grown in England until the 1590s. While, cultivation of tomato had been seen in Carolina in the British North American colonies in the early seventeenth century according to the first known reference. It was probably the first time that tomato was introduced to America. Besides, the country which has the oldest history of tomato cultivation is Italy. In 1811, tomato was cultured prosperously in Sicily which is the biggest island in the Mediterranean. Those tomatoes were shipped to Rome and Naples at that time.

According to some specialists, it was in early 18th century that tomato was introduced to Japan and its cultivation also started. However, a sketch of tomato in 1668

was found on a sketch book, which means that tomato had already introduced late 17 century. Tomato was considered as an ornamental plant rather than a plant for food at the beginning and became more and more popular since 1930s. According to the state of agriculture in Japan, the crop yield in 1995 amounted to about 753 000 tons under field and green house condition in Japan. It is remarkable that tomato contains important source of minerals and vitamins. Indeed, tomato stands at the top as regards to the total amount of minerals and vitamins available in a vegetable. In recent years, they are not used only in salads but also in making soups, juices, ketchaps and so on. Tomato has been ranked on important vegetable in Japan as well as in other countries.

1.1.2 Taxonomic status of the tomato

Lycopersicon is a comparatively small geneus in family *Solanaceae*. It shows typical unique anther morphology. The flower normally has five anthers, the stamens are joined together to form a flask-shape anther cone. All species in *Lycopersicon* genus have a chromosome base number of x=12 and identical genome formula 2n=2x=24, which is remarkable uniformity.

This genus consists of 9 species and those are classified into 2 main groupes, one is *esculentum*- complex, the other is *peruvianum*-complex. Rick (1976) carried out these classifications based on the crossing relation with commercial tomato. The *esculentum*- complex includes 7 species, *L. esculentum* (the present botanical name of tomato , it was getting popular in early 1980s after much argument (cited in Taylor 1986)), *L. pimpinellifolium*, *L. cheesmanii*, *L. parviflorum*, *L. chmielewskii*, *L. hirsutum* and *L. pennellii*, and those species can be crossed with the tomato easily. On the other hand, *peruvianum*-complex contains 2 wild species, *L. peruvianum* and *L. chilense* which cannot be crossed without any techniques or treatments. Distribution of 9 species in the genus *Lycopersicon* is shown in **Table 1-1**.

Table 1-1. Distribution of species in the genus *Lycopersicon* (adapted from Esauinas-Alcazara 1981).

Species	Native gengraphical area
L. esculentum var. *cerasiforme*	Tropical area, Peru, Ecuador
L. pimpinellifolium(Jusl.) MILL.	Coastal and northern regions in Peru, Ecuador, Andes
L. cheesmanii RILEY	Galapagos Islands
L. chmielewskii RICK, KEAICKI, FOBES and HOLLE	Central Peru
L. parviflorum	Central and Northern Peru, Ecuador
L. hirsutum HUMB. and BONPLL.	500-3300 m above sea level, Central Peru, Northern Ecuador
L. peruvianum (L.) MILL.	Peru, Northern Chile
L. chilense DUN.	Southern Peru, Chile
L. pennellii D'ARCY.	Western region in Central Peru, well drainage area

1.1.3 Breeding and biotechnology of tomato

Tomato genetics and breeding have resulted in great mutual benefit and much research has bridged both area. Many useful materials in tomato genetics have been contributed by tomato breeders, and conversely studies in basic tomato genetics and its techniques developed by geneticists have permitted much progress in tomato breeding.

Work to develop new varieties of improved tomato started more than two centuries ago (Stevens and Rick 1986). During the recent past tremendous breeding efforts have been made in several countries including Japan to develop potential varieties suited to different conditions. Generally, simple selection from the naturally existing chance variants within the varieties was being practiced for improvement before 1925. Later, controlled hybridization with selection followed to develop new varieties have led to increasing the yield, quality and resistance traits considerably.

Tomato is one of the most agriculturally important. In that case, why tomato is also one of the most genetically important and advantageous vegetable? Because the tomato plant is an excellent genetic material for classical breeding. For instance, it is easy to grow, a short life cycle, high self-fertility and many other traits offering a numerous advantage to genetic research.

In recent years, the wild relatives of tomato which still remain a virtually untapped reservoir of genetic potential have extensively been used as genetic sources for genetic improvement of tomato. A number of agronomically important traits such as disease resistance, fruit characteristics, high level of soluble solids and stress tolerance have already been introgressed into tomato from the other wild *Lycopersicon* species (Kalloo 1991, Rick *et al.* 1987). Assuredly, the genetic resources embodied in the wild species will generously provide important traits to the future generations of tomato cultivars.

1.2 Introduction of This Study

The wild species in the genus *Lycopersicon* are important for tomato breeders because its presents enormous storage of potencially valuable traits (see section 1.3.1). These species constitute an extreamely valuable collection of useful genetic variation, including disease resistance (Boukema and Den Nijs 1984), nematode resistance (Lobo *et al.* 1988), insect resistance (Rick 1982), stress tolerance (Boukema and Den Nijs 1984, Bennetzen and Adams 1984), and fruit quality (Saccardo *et al.* 1981). In *L. peruvianum,* available traits such as resistance to root knot nematode (McFarlane *et al.* 1946), mosaic virus (Alexander 1963), bacterial spot, leaf mold, fusarium wilt, and septoria and alternaria blights (Alexander *et al.* 1942) should be noteworthy. Production of interspecific hybrids between tomato and *L. peruvianum*, one of the members of *peruvianum* -complex, is difficult because of embryo abortion in the seeds of F_1 hybrids during fruit ripening (Barbano and Topoleski 1984). Different techniques such as embryo culture (Smith 1944), chronic gamma irradiation of pollen of *L. peruvianum* (Yamakawa 1971), embryo cullus culture (Thomas and Pratt 1982), ovule culture (Imanishi 1988, Chen and Imanishi 1991), ovule selection method (Imanishi *et al.* 1993), the hormonal treatment and bud pollination (Gradzial and Robinson 1991) have been formulated to develop hybrids between tomato and *L. peruvianum*. In earlier reports, F_1 hybrids were sterile (Lesley 1950, Rick 1963), but these techniques effectively allowed the development of bridge lines possessing high cross ability that could be backcrossed with *L. esculentum* (Poysa 1990). While, Vulkova and Sotirova (1993) developed three-genome hybrids of tomato, *L. chilense* and *L. peruvianum* var. *humifusum*, through the sexual crossing using tomato as pistillate parent with *L. chilense* and subsequent crossing of the resulting F_1 with the pollen of *L. peruvianum.*

Solanum lycopersicoides is another wild relative of tomato possessing potentially valuable traits for tomato improvement, especially cold tolerance (Rick 1988,

see section 1.3.2). It is the only one which has been directly crossed to tomato among the four tomato-like nightshades. First sexual hybrid with tomato was reported by Rick (1951) 36 years ago and he suggested that the hybridization between tomato and *S. lycopersicoides* via embryo culture was considerably easier than that between tomato and *L. peruvianum*. Sexual hybrid can readily be produced using tomato as pistillate parent, but male sterility of F_1 hybrids causes a difficulty in backcrossing to tomato (Bills and Martin 1962). To break this problem, Stoeva *et al.* (1990) performed a hybridization between F_1 (*L. esculentum* var. *cerasiforme* x *L. esculentum*) and *S. lycopersicoides*. The hybrids were successfuly produced by embryo culture method, however, sterility of hybrids could not be overcome. Consequently, the backcrossing to tomato was impossible. Different approaches have been used to overcome this deadlock. Robinson and Phills (1979) developed two F_2 plants, one of which was backcrossed to tomato. Gradziel and Robinson (1989) reported that F_1 plants could be backcrossed to tomato either by selecting the individuals with sufficient pollen fertility or by applying a variety of pollination techniques to bypass the unilateral incompatibility which is normally expressed by F_1 hybrids towards tomato pollen. On the other hand, another strategies asisted to overcome the barriers. Chatelat *et al.* (1989) and Chatelat *et al.* (1997) obtained a large BC_1 population by using a bridging line bred from *L. pennellii*. However, the hybrids derived from the crosses of tomato with wild *Lycopersicon* and *Solanum* species generally contain cytoplasm from the tomato because of unilateral incompatibility, or preferably incongruity (Hogenboom 1972) at any rate.

During the recent years, somatic hybridization has been used as an alternative to overcome the limitation of classical sexual hybridization between tomato with wild *Lycopersicon* and *Solanum* species (See reviews Lefrancois *et al.* 1993, Wolters *et al.* 1994). Considerable numbers of symmetric and asymmetric somatic hybridization between tomato and *L. peruvianum* have been reported (Kinsara *et al.* 1986, Han San *et al.* 1990, Wijbrandi *et al.* 1990, Derks *et al.* 1991, Sakata *et al.* 1991). Handley *et al.*

(1986) developed allopolyploid somatic hybrids by fusing mesophyll protoplasts of tomato and protoplasts derived from suspension of *S. lycopersicoides*. Although chilling tolerance has been detected in these hybrids (Ball and Sink 1988) further hybridization was not reported. Hossain *et al.* (1994) developed fertile tetraploid somatic hybrids by the electrofusion of mesophyll protoplasts that could be backcrossed with tomato pollen. However, reports on the development of hybrids by the process of sexual and somatic cell fusion involving the three genomes of three different species are very limited. Guri *et al.* (1991) reported trigenomic somatic hybrids by the fusion of mesophyll protoplasts of tomato x *L. pennellii* with the callus derived protoplasts of *S. lycopersicoides*. Using the same process, McCabe *et al.* (1993) developed asymmetric somatic hybrids by the fusion of the mesophyll protoplasts of the F_1 of Tomato x *L. pennellii* with callus-derived protoplasts of *S. lycopersicoides*. However, in both the cases, the somatic hybrids were sterile. Electrically induced fusion of mesophyll protoplasts have been reported as a more efficient method for the production of higher rate of balanced fertile hybrids than chemically induced fusion (de Vries *et al.* 1987, Sihachakr *et al.* 1988, Han San *et al.* 1990, Naton *et al.* 1992, Hossain *et al.* 1994). In addition, introduction of the valuable traits separately from *L. peruvianum* and *S. lycopersicoides* into tomato would be a time consuming and tedious process that requires reiterated backcross with tomato. Production of fertile somatic hybrids that contains the genomes of the both wild species would be a time saving process that would allow the transfer of the desirable traits together into tomato.

The present study aimed to produce and characterize somatic hybrids between F_1(tomato x *L. peruvianum*) and *S. lycopersicoides* obtained through the electrofusion of mesophyll protoplasts.

1.3 Material Introduction

1.3.1 Lycopersicon peruvianum var. *humifusum* (LA2153)

L. peruvianum is a highly polymorphic species composed of more than 30 races (Rick 1986, Warnock 1988). The northerly races, *L. peruvianum* var. *humifusum*, which is found in the region around Cajamarca in Peru, morphologically distinct and have barriers restricting their intercrossing with the majority of other *L. peruvianum* races (Rick 1963). This group was separated from the typical forms of the speciecs on the basis of its short, dense, non-glandular hairs, its thin procumbent stem, and its small simplified leaves.

L. peruvianum var. *humifusum* retains many valuable traits such as resistance to *Clavibacter michiganensis* subsp. *michiganensis* (Volkova and Sotirova 1993), high regeneration ability (Imanishi 1991), and sucrose accumulation ability (Stommel 1992). However, sexual hybridization between *L. esculentum* and *L. peruvianum* var. *humifusum* is the most difficult work because embryos of this hybrids normaly abort very early that it can not be rescued by embryo culture (Taylor 1986). Nevertheless, Imanishi *et al.* (1996) obtained 23 germinated ovules from hybrids according to the ovule selection method (Imanishi *et al.* 1993). All of five hybrids tested were more than 20 % in pollen fertility and self-incompatible. The self-incompatibility expressed in all plants was derived from *L. peruvianum* var. *humifusum*. Using those pollen, they obtained a number of backcrossed progenies.

1.3.2 Solanum lycopersicoides (LA2386)

Solanum is a closely related genus to *Lycopersicon*. In the tomato- like nightshades, a series in the *Solanum* species consists of *S. juglandifolium*, *S. ochranthum*, *S. sitiens* and *S. lycopersicoides* which especially shows the greatest affinity to the tomato. *S. lycopersicoides* is found at around 3000 m above sea level in the south of Peru and Northern Chile which are higher locations than the altitude of any of the *Lycopersicon*

species. One of the most interesting traits of *S. lycopersicoides* for tomato breeding is its cold tolerance. Introgression of this trait to cultivated tomato lowers the green house cost for energy production. Robinson and Phills (1977) reported that *S. lycopersicoides* and the intergeneric hybrid grew vigorously and flowered profusely at 10°C, whereas tomatoes were stunted, chlorotic and produced only a few sterile flowers. Accession LA2386 which was used in the present study also produced flowers in winter without heating the greenhouse. *S. lycopersicoides* possesses another available traits such as disease resistances for cucumber mosaic virus, tomato mosaic virus (Phills *et al.* 1977a), Fusarium wilt (Phills *et al.* 1977b), bacterial canker, Phytophthora root rot and gray mold (Gradziel and Robinson 1989) and insect resistance which may be related to its exceedingly high leaf glycoalkaloid content (Oleszek *et al.* 1986). Remarkable monogenic characters (white anther, sensitivity of flowering to long day), woody shrub habit (Rick and Yoder 1988) are also useful traits as phenotypes .

2. ESTABLISHMENT OF PROTOPLAST ISOLATION, CULTURE AND PLANT REGENERATION OF F1 (*L. ESCULENTUM* X *L. PERUVIANUM*)

2.1 Introduction

For an efficient production of somatic hybrids, several cell biological procedures have to be optimized before starting the cell fusion experiment. These include the isolation and culture of parental protoplasts and plant regeneration. These steps in the case of *L. esculentum* have extensively been studied and systematized (Zapata *et al.* 1977, Muhlbach 1980, Morgan and Cocking 1982, Adams and Townsend 1983, Shahin 1985, Neidz *et. al.* 1985, Chen and Adachi 1994, Hossain *et al.* 1995). However, it is not certain if the efficient methods in the production of somatic hybrids in *L. esculentum* is applicable to another species.

Protoplast isolation and plant regeneration in *L. peruvianum* have also been attempted and optimized by many researchers (Zapata *et al.* 1977, Muhlbach 1980, Tal and Watts 1979, Imanishi and Hiura 1983, Lefrancois and Chupeau 1993). Compared to *L.esculentum*, *L. peruvianum* is much easier to regenerate from protoplasts and also callus growth is abundant (Zapata *et al.* 1977, Muhlbach 1980). Koornneef *et al.* (1987) presented that this favorable cell-culture traits could be bred into *L. esculentum*. The method of plant regeneration from mesophyll protoplasts of F1 plants between *L. esculentum* cv. Early Pink and *L. peruvianum* var. *humifusum* (LA2153) has not also been established yet. It is likely that the same nature as cell-culture traits might be observed in the F1 plants.

In this section, alternative enzyme combination for protoplast isolation and suitable medium and combination of plant growth regulators for plant regeneration in F1 were investigated. The reproducible process would apply to the plant regeneration from fused protoplsts.

2.2 Materials and Methods

2.2.1 Plant materials

The seeds of *L. peruvianum* var. *humifusum* LA2153 (denoted Lp) were obtained from Professor C. M. Rick, Tomato Genetic Resource Center, University of California, Davis. F_1 hybrids of *L. esculentum* cv. Early Pink (denoted Le) x *L. peruvianum* var. *humifusum* (LA2153)(denoted F_1) developed through ovule selection method (Imanishi *et al.* 1996) were propagated *in vitro* culture. One to 2 cm length of shoot tips were excised and put into growth regulator-free MS medium (Murashige and Skoog 1962) containing 3% (w/v) sucrose and 0.8% agar in 300 ml conical flask. Sun cap sheet (Iwaki Glass) was used as a cap for conical flask to control air circulation and humidity. The shoots were cultured in a growth cabinet with 2500 lux (16h day length) at 25°C. Two to 3 days after culture, roots developed. *In vitro* grown F_1 plants are shown in **Fig. 2-1 A.**

2.2.2 Protoplast isolation

(1) Preparation of enzyme solutions

The following enzymes were used for the isolation of F_1 protoplasts: 0.3 % of Macerozyme R10 (Yakult Manufacturing Co. Ltd., Japan) as pectinase and a combination of two out of five cellulases which are Meicelase P1 (Meiji Seika Kaisha Ltd., Tokyo, Japan), Driselase (Kyowa Hakko Kogyo Co. Ltd., Japan), Cellulysin (Calbiochem, U.S.A.), Cellulase Onozuka R10 and Cellulase Onozuka RS (Yakult Honsha Co. Ltd., Japan). The enzymes were dissolved in CPW salts (Frearson *et al.* 1973) solution containing 9% mannitol as an osmotic stabilizer (CPW9M). The pH of the enzyme solution was adjusted to 5.7 with HCl or NaOH solution and centrifuged at 3000 rpm for 10 min. Finally, the supernatant was sterilized through 0.2 μm pore membrane filter and the enzymes were stored at -20°C until required.

(2) Protoplast isolation methods

Three to four weeks after *in vitro* shoot culture of F_1 plants, fresh and green leaves were selected for protoplast isolation. Detached leaflets were notched in small strips with a midrib, floated on CPW9M solution in plastic petridish (50 x 12 mm, Falcon plastic Ltd.), and incubated for 1 h in the dark for preplasmolysis at room temperature. After plasmolysis, CPW9M solution was carefully replaced by an enzyme solution. Digestion was completed after 17 h in the dark at 26°C. The enzyme solution was carefully replaced by about an equal volume of CPW9M solution. The digested leaves were gently squeezed and sieved through a stainless mesh (200 μm pore size) with a pipette. The suspension including protoplasts and comparatively big debris were centrifuged for 4 min at 650 rpm. The green pellet was resuspended with CPW21S (21% sucrose in CPW salts solution), and centrifuged for 8 min at 650 rpm to obtain only clean protoplasts. The floating protoplasts were collected with a pipette and transferred into new centrifuge tubes with CPW9M solution for washing. At this stage, a minimum volume of CPW21S with protoplasts was collected. The protoplasts were resuspended in a certain volume of fresh CPW9M solution (e.g. 10 ml) after washing. A drop of protoplast suspension was transferred to a modified Fuchs Rosenthal haemocytometer and the number of protoplasts in five triple lined squares was counted. The total yield of protoplasts was calculated using formula:

Total yield = number of protoplast x 10^3 x volume of protoplast suspension

2.2.3 F1 protoplast culture

(1) Preparation of culture media

TM-2 (Shahin 1985), half concentration of MS and 8E (Zapata *et al.* 1981) liquid media were used as the basic media. Organic components of B5 medium (Gamborg *et al.* 1968)

were also combined with 1/2MS medium. Each medium with or without a plant growth regulator was prepared. Three plant growth regulators (BA, NAA and 2,4-D) were filter-sterilized through 0.2 μm pore size membrane filter (Toyo Roshi kaisha, Ltd., Japan) and added in the media which were sterilized by autocraving. The culture media were stored at -20°C until required.

(2) Culture of F_1 protoplast

The density of protoplast in each of the medium maintained above were adjusted to 1 x 10^5/ ml. Plant growth regulators were combined, i.e. NAA, BA and 2,4-D in 8E medium and NAA, BA and ZR in 1/2MS and TM-2 medium. Final density of protoplast was adjusted to 5 x 10^4/ml and 2.5 ml of protoplast suspension was placed in plastic petri dish (50 x 12 mm, Falcon Plastic Ltd.) sealed with Parafilm (American National Can, USA). The cultures were kept at 25°C in the dark without shaking until cell division was observed (2 or 3 days later). When cell wall synthesis and then favorable cell division started, the cultures were transferred to a low light condition. When active protoplast division was observed with a white substance, 1.0 ml of fresh growth regulator free medium was added into the culture every after 2 days. The content of one petri dish was divided into 2 dishes, when the volume of culture exceeded over 2.0 ml. Ten days after initial culture, the number of divided protoplasts was counted and the plating efficiency (number of dividing protoplasts/total number of protoplasts) was calculated.

2.2.4 Callus development and plant regeneration

(1) Preparation of callus development and plant regeneration medium

TM-3 basic medium (Shahin 1985) supplemented with BA (0.5 mg/l) and 2,4-D (0.2 mg/l) was solidified with 0.9 % Difco Bacto agar and sterilized by autocraving. This medium was utilized for callus development. For plant regeneration, MS medium

containing ZR (1.0 or 3.0 mg/l), GA_3 (0.1 mg/l) and 20 g/l sucrose was prepared and solidified with either 0.9 % Difco Bacto agar or 0.9 % agarose. MS semi-solid medium with GA_3 was autocraved, while ZR was filter-sterilized through 0.2 μm pore size membrane filter (Toyo Roshi kaisha Ltd., Japan) and added in an auto craved medium. Each medium was plated in plastic petridishes (50 x 12 mm, Falcon Plastic Ltd., USA).

(2) Plant regeneration

Ten-day-old colonies were transferred into a glass centrifuge tube containing the culture medium. The suspension was kept for 10 min and the colonies were made to settle down in the bottom of tubes. The culture medium was replaced with fresh TM-2 medium without growth regulator using a pipette. The density of colony suspension (number of colonies per 1 ml medium) was adjusted to 3×10^2. One ml of the suspension was spread on TM-3 callus development medium or directly onto MS regeneration medium (first plating). The lid of the petridishes were sealed with Parafilm. All the colonies which grew on TM-3 and MS regeneration medium without the callus developing green spots (shoot primordia) were transferred onto MS regeneration medium 10 days after first plating (second plating). The cultures were kept under 2500 lux (16h day length) at 25°C. Shoots of 1 to 2 cm in length originating from callus were cultured and rooted on MS (0.1 mg/l IAA) agar medium.

2.3 Results and Discussion

Protoplast isolation

Total yields of protoplast isolated through different combination of enzyme treatments were determined. The result is shown in **Table 2-1**. The highest yield of protoplast s was obtained in the enzyme solution which consisted of Meicerase P1, Cellulase Onozuka R10 and Macerozyme R10 with a yield of more than 25 x 10^6 protoplasts from 1 g of fresh leaves. The concentration of Meicerase P1 between 0.5 and 1.0 % revealed no significant difference in the yield of protoplasts. However, protoplast yield was reduced when Meicerase P1 concentration was increased to 2.0% and combined with Cellulase Onozuka R10.

The enzymatic isolation of protoplasts was first reported by Cocking (1960) from tomato seedling root tips. Takebe *et al.* (1968) succeeded to apply commercially available cell wall-degrading enzymes in tobacco and large amount of protoplasts from a wide variety of plant tissues were isolated. Enzymes for protoplast isolation are classified into three categories; cellulases, hemicellulases and pectinases by their catalytic action. Combination of these enzymes and development of isolation procedures have made protoplast isolation from any plant tissues easier. The concentration of enzyme required is also an important factor because it affects the yield of protoplasts and their viability. Normally, protoplasts lose their viability during the process of enzyme digestion. If the concentration of enzyme is too high, the tissues are digested too much. In fact, the yield of F_1 protoplasts was decreased when higher concentration of Meicerase P1 was applied. Another factor which influences the isolation of viable protoplasts is the condition of the materials used. For example, old cell suspensions yield very little or no protoplast at all, whereas young cultures, especially embryogenic tissues are easily degraded (Abdullah *et al.* 1986). Likewise young *in vitro*-grown plants require low concentrations of enzymes and relatively short period of treatment as compared to large leaves from old or from mature plants (Bajaj 1972). A manipulation of young tissue, pure enzyme and the

reduction in the period of incubation often result in higher viability and plating efficiency. It is likely that when the plant materials are grown under conditions such as low light intensities, *in vitro* or when the tissues are young, the cells tend to synthesize thinner cell walls with low concentrations of peculate (Cassels and Barlass 1976). Consequently, lower concentrations of enzyme solution will be required and viable protoplasts therefore yielded. According to the previous works, young leaves from *in vitro* cultured F_1 plants used were apparently the appropriate material to obtain viable protoplast. It was also a better point to use *in vitro* grown materials than those from *in vivo* cultures since the leaves needed no sterilization with hydrochloric acid solution, hence less damage to the plant materials. Isolated protoplasts are shown in **Fig. 2-1 B**.

One-month-old leaves from *in vitro* cultured F_1 plants were digested by the enzyme solution which consisted of 0.5 or 1.0 % of Meicerase P1, 1.0 % of Cellulase Onozuka R10 and 0.3% of Macerozyme R10 for 17 h at 26°C in the dark. It was proved that approximately 25×10^6 protoplasts from 1 g fresh leaves could be isolated. The yeild was equivalent to the amount of protoplasts for 25 times of electrofusion experiments.

Table 2-1. Effect of enzyme combination upon the number of isolated F1 protoplasts.

	Cellulysin	Meicelase P1			Driselase
	1.0%	0.5%	1.0%	2.0%	0.3%
Cellulase Onozuka RS 1.0%	13.8×10^6	13.5×10^6	11.0×10^6	12.7×10^6	6.7×10^6
Cellulase Onozuka R10 1.0%	14.4×10^6	25.3×10^6	25.0×10^6	17.7×10^6	8.6×10^6

*0.3% of Macerozyme R10 (Pectinase) was added to all enzyme solutions.

**Leaves were digested for 17h at 26 ℃ in the dark.

*** Protoplasts were isolated from 1g fresh leaves.

Protoplast culture and plant regeneration

Plating efficiencies in the three different culture media supplemented with plant growth regulators were investigated. The basic 8E medium with NAA 0.3 mg/l, BA 0.3 mg/l and 2,4-D 0.3 mg/l induced 6.1 % of plating efficiency (**Table 2-2**). Other combinations of plant growth regulators gave less than 1 % of plating efficiency. 1/2MS medium did not induce active cell division, i.e. protoplasts divided only few times or did not divide at all (**Table 2-3**). Protoplast division in TM-2 medium seemed to be comparatively stable. The highest plating efficiency (7.58 %) was observed in TM-2 medium containing NAA 1.5 mg/l and BA 1.5 mg/l (**Table 2-4**).

Some key factors for successful *L. esculentum* protoplast culture were considered. Firstly, Hanson *et al.* (1989) suggested that low NH_4^+ ion concentration in initial media were required. Handly and Sink (1985) also reported that high concentration of NH_4NO_3 was toxic to the protoplasts. MS medium contained NH_4^+ ion. On the other hand, 8E and TM-2 did not contain it. However, elimination of NH_4NO_3 from the culture medium gave no benefit in the protoplast division of *S. lycopersicoides* (Zapata *et al.* 1981). Therefore, the effect of NH_4^+ ion seems to depend on the plant genotype. Secondly, some vitamins probably have influenced good protoplast division. TM-2 medium which provide much higher plating efficiency than 8E medium was enriched with vitamins and first introduced by Shahin (1985) in the tomato protoplast culture medium. Tan *et al.* (1987) also succeeded in developing plants from tomato protoplasts using the medium fortified with rich vitamins. However, as the number of vitamins was increased in culture medium, for example, 18 organic components in TM-2 medium, it was not clear which organic component was essential or if all were required. If an important organic component was obvious, a simple medium for protoplast culture could be organized. Thirdly, it was suggested that rapid dilution of protoplasts was important (Hanson *et al.* 1989, Hossain *et al.* 1995). During initial culture, the medium gradually became impure with whitish substance. Hossain (1995) suggested that the presence of

some detrimental substances might have caused the cell browning and colony death. The addition of a fresh medium did not only reduced concentration of whitish substances but also the density of the cell colonies. This method has been employed in inducing sufficient plating efficiency in other studies (Shahin 1985, Sakata *et al.* 1987).

More than half of the callus turned green after transfer directly from the initial culture medium onto the MS regeneration medium (**Fig. 2-1 C**). Shoots were only induced from the callus cultured on MS + ZR (1.0 mg/l) + GA_3 (0.1 mg/l) medium and subsequently on MS + ZR (3.0 mg/l) + GA_3 (1.0 mg/l) medium. For well shoot formation, comparatively high concentration of ZR might be necessary. However, the callus via culture on TM-3 medium (0.5 mg/l BA and 0.2 mg/l 2,4-D) seemed to be dry and powdery, and it did not develop adventitious shoots even transferred onto the regenration medium with 3.0 mg/l of ZR (**Table 2-5**).

For the plant regeneration from protoplast, it is considered that initial culture should be done in the medium containing high concentration of auxin and low concentration of cytokinin and transferred the callus onto/into the medium containing high concentration of cytokinin and low concentration of auxin. Moreover, osmotic pressure of medium was required to decrease gradually (Shahin 1985, Sakata *et al.* 1988).

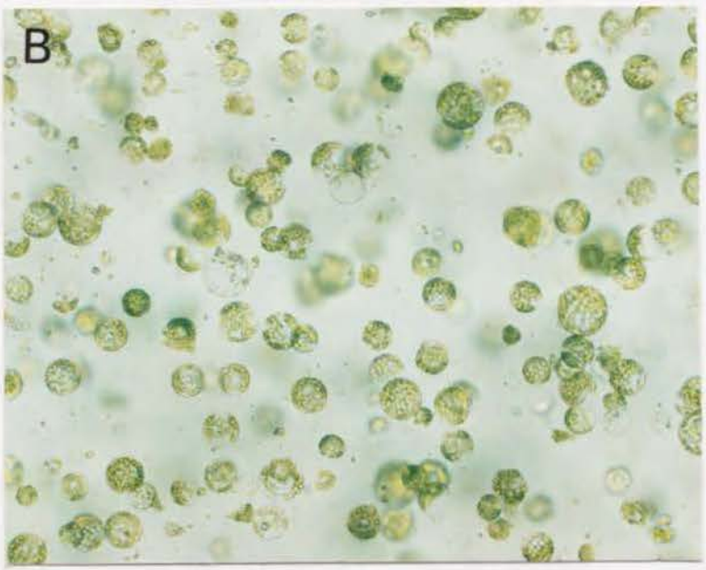
Short period of culture for callus development is one of the critical points for efficient adventitious shoot regeneration. Nishio *et al.* (1988) reported that only a few shoots were developed from callus which took long time to be ready for transfer in shoot regeneration medium. In tomato, a similar tendency was also observed by Shahin(1985) and Sakata *et al.* (1988).

One of the important factors affecting plant regeneration from protoplasts is the plant genotype. Some lines belonging to the *peruvianum*-complex have higher regeneration capacity, and this desirable trait has been introduced into tomato from *L. peruvianum* (Koornneef *et al.* 1987). Chen and Imanishi (1991) reported that the plant regeneration ability from leaf segments of F_1 between tomato cultivar and *L. peruvianum*

was obviously greater than tomato cultivar and less than the *L. peruvianum*. Superior regeneration capacity from protoplasts of *L. peruvianum* was also proved by Zapata et al. (1977), Muhlbach (1980) and Koornneef *et al.* (1987). In fact, the regeneration of plants from F_1 protoplasts in the present study was faster and its maintenance was much easier than the *L. esculentum*. It is then posssible that the high regeneration capacity observed in the F_1 plants was introduced from the *L. peruvianum* var. *humifusum* (LA2153) by sexual hybridization.

Fig. 2-1A. *In vitro* cultured F_1 plants between *L. esculentum* cv. Early Pink and *L. peruvianum* var. *humifusum* LA2153 in MS agar medium. **B.** Freshly isolated F_1 protoplasts in CPW9M solution. **C.** F_1 callus derived from a protoplast on MS regeneration medium (second plating). Bar = 10 mm.


A


B


C

Table 2-2. Effect of combination and concentration of plant growth regulators in 8E medium upon plating efficiency of F_1 protoplasts.

Sucrose (%)	Glucose (%)	Mannitol (%)	BA (mg/l)	NAA (mg/l)	2,4-D (mg/l)	Plating efficiency (%)
1.0	0.5	7.5	0.3	0.3	0.3	6.10
1.0	0.5	7.5	0.5	0.5	0.5	0.90
1.0	0.5	7.5	0.5	0.5		0.96
1.0	0.5	7.5	0.5		0.5	1.11

Table 2-3. Effect of organic components and plant growth regulators in 1/2MS medium upon plating efficiency of F1 protoplasts.

Organic components	Sucrose (%)	Mannitol (%)	BA (mg/l)	NAA (mg/l)	2,4-D (mg/l)	Plating efficiency*
Based on	3.0	7.2	0.5	0.5	0.5	+
1/2 MS medium	3.0	9.0	0.3	0.3	0.3	-
	3.0	9.0	0.5	0.5	0.5	-
	3.0	9.0	1.0	1.0	1.0	-
	3.0	9.0	0.5	0.5		-
	3.0	9.0	0.5		0.5	-
Based on						
B5 medium	3.0	7.2	0.5	1.0		-

* -: no division of protoplasts.

+: a few times division of protoplasts.

Table 2-4. Effect of combination and concentration of plant growth regulators for plating efficiency in TM-2 medium.

BA(mg/l)	ZR(mg/l)	NAA (mg/l)	Plating efficiency (%)
0	-	0	0
0.25		0.25	2.18
		0.50	0.39
		1.50	6.55
0.50		0.25	3.08
		0.50	1.93
		1.00	3.92
		1.50	3.34
1.00		0.50	5.26
		1.00	2.44
		1.50	6.29
1.50		0.25	4.37
		1.00	7.31
		1.50	7.58
	0.25	0.25	1.16
		0.50	0.39
	0.50	0.25	2.18
		0.50	2.70
		1.00	4.11
	1.00	1.00	2.05

Table 2-5. Effect of culture media and shoot regeneration from the calli derived from F1 mesophyll protoplasts.

First plating[1)]					Second plating[1)]			Number of		
Medium	BA (mg/l)	2,4-D (mg/l)	ZR (mg/l)	GA_3 (mg/l)	Medium	ZR (mg/l)	GA_3 (mg/l)	Cultured calli	Green calli	Shoot formation[2)]
TM-3	0.5	0.2						20	0	0
TM-3	0.5	0.2			MS	1.0	0.1	22	4	0
TM-3	0.5	0.2			MS	3.0	0.1	11	11	0
MS			1.0	0.1	MS	1.0	0.1	22	22	0
MS			1.0	0.1	MS	3.0	0.1	31	21	17

1) First and second platings indicate callus transfer at 10 and 20 days after initial protoplast culture in TM-2 medium, respectively.

2) Shoot formation was investigated at 60 days after initial protoplast culture.

3. SOMATIC HYBRIDIZATION BETWEEN F1 AND *S. LYCOPERSICOIDES* THROUGH ELECTROFUSION OF MESOPHYLL PROTOPLASTS

3.1 Introduction

The production of somatic hybrid circumvents sexual crossing barriers and provides new materials (Shepard *et al.* 1983, Kumar and Cocking 1987). Consequently, the cell fusion process will result to: (1) the combination of two complete genomes; (2) the partial genome transfer from a donor to a recipient protoplasts; (3) and the transfer of organelles (chloroplast and mitochondria) in relation to various properties. Hence, many reseachers have attempted to produce interspecific and intergeneric somatic hybrids and various regenerated plants have been obtained in *Petunia, Nicotiana, Hyoscyamus, Solanum, Citrus, Brassica, Medicago, Trifolium*, and so on (see review Bajaj 1989). Likewise, in *Lycopersicon,* many somatic hybrids between cultivated tomato and another species or genus have been developed for the introduction of their valuable genes to tomato (See review, Lefrancois *et al.* 1993).

The beginning of protoplast isolation from leaves was developed in the 1960s (for review see Chupeau and Bourgin 1980). Following this, chemical methods such as $NaNO_3$ (Power *et al.* 1970, Kameya and Takahashi 1972), a high pH and high calcium ion solution (Keller and Melchers 1973), polyethyleneglycol (PEG) (Kao and Michayluk 1974), dextran and dextran-sulfate (Kameya 1975, 1979) and polyvinyl alcohol (Nagata 1978) methods were reported as protoplast fusion in 1970s. PEG method is the most commonly used procedure on chemical fusion experiment and many somatic hybrids above mentioned were also obtained through this procedure. However, the chemical fusion was reported to give undesirable effects to the protoplasts (Binding *et al.* 1987, Han San *et al.* 1990). Prolonged experiments were also required and it was not suitable for large scale treatment. On the other hand, electrofusion was used in the 1980s. Senda

et al. (1979) with the aid of a micromanipulator brought two electrodes into contact with adjoining protoplasts and, after applying a brief electrical pulse, induced fusion. Subsequently, Zimmermann and Scheurich (1981) established the electrofusion method using the dielectrophoresis. This method was considered safer to the protoplasts, required less skill and take less time than the chemical fusion method. It has provided many somatic hybrids in *Solanum* (Puite *et al.* 1986, De Vries and Tempelaar 1987, Sihachakr *et al.* 1989), *Ipomoea* (Sihachakr *et al.* 1988), *Petunia* (Taguchi *et al.* 1993) and *Brassica* (Nishio *et al.* 1987). On the other hand, not many somatic hybridization by electrofusion in *Lycopersicon* have been reported except several studies (Han San *et al.* 1990, Wolter *et al.* 1991, Hossain *et al.* 1994). Hossain *et al.* (1994) indicated that somatic hybridization between tomato and *S. lycopersicoides* was effectively carried out through electrofusion and fertile somatic hybrids could be produced at a high rate.

The objective of this section was to establish somatic hybridization by electrofusion in the genus *Lycopersicon* and to produce somatic hybrids between Le x Lp and Sl using the fusion of mesophyll protoplasts.

3.2 Materials and Methods

3.2.1 Plant materials

Fresh and green leaves of *in vitro* F_1 hybrids were used for protoplast isolation (see Chapter 2, Section 2.2). The seeds of *S. lycopersicoides* (LA2386) were obtained from Professor C. M. Rick (Tomato Genetic Resource Center, University of California, Davis). The seeds were sown in a commercial potting soil (Soil compost, Kurea Chemical Co.) in clay pots and maintained in the greenhouse.

3.2.2 Protoplast isolation

F_1 mesophyll protoplasts were isolated according to the procedure described in CHAPTER 2 (Section 2.2.2). The enzyme solution used consisted of 0.3 % Macerozyme R10, 0.5 % Meicelase P1 and 1.0 % Cellulase Onozuka R10 in 9 % mannitol and 10% CPW salts (pH 5.7). *S. lycopersicoides* plants were propagated by cuttings and maintained in a growth chamber at 20°C with 16 h/day illumination and 75 % humidity. Three-week-old plants were incubated in the dark at 20°C for 48 h before protoplast isolation. The fully expanded young leaves (3rd and 4th leaves) were surface sterilized for 20 min in a solution containing 3% commercial bleach (Sodium hypochloride) and a few drops of Tween-20. The leaves were rinsed 5 times with sterile tap water and notched in small strips with the midribs. To preplasmolyse the cell, the leaflets were floated abaxially on CPW9M solution in plastic petridish (90 x 12 mm, Falcon Plastic Ltd.), and incubated for 1 h in the dark at room temperature. After plasmolysis, CPW9M solution was carefully replaced by an enzyme solution containing 1 % Cellulysin (Calbiochem), 0.5 % Macerase (Calbiochem), 0.01 % Pectolyase Y-23 (Seishin), 9 % mannitol and CPW salts. The cultures were kept in the dark for 17 h at 25°C (Hossain *et al.* 1994). The enzyme solution was carefully replaced with an equal volume of CPW9M solution. The digested leaves were gently squeezed and sieved through a stainless mesh (68 μm pore size) with a

pipette. The protoplast suspension was poured into a glass centrifuge tube and washed by centrifugation at 800 rpm for 4 min. The supernatant was replaced with CPW23S (23 % sucrose in CPW salt solution) and centrifuged at 800 rpm for 7 min after resuspended carefully. The floating protoplasts were collected with a pipette and transferred into a clean centrifuge tube with W5 solution (Menzel and Wolfe 1984).

3.2.3 Electrofusion procedure

Isolated parental protoplasts were suspended in a fusion soluion (2.5 mM $CaCl_2$ and 9 % mannitol) and washed once. Both parental protoplasts were adjusted to a density of 1 x 10^6/ml with the fusion solution and mixed together at 1:1 retio. One ml of the protoplast mixture was transferred into a sterilized fusion chamber (FTC-03). The electrofusion treatment was carried out by Shimazu Somatic Hybridizer SSH-2 (Shimazu, Kyoto, Japan). The electrofusion condition were as follows; 40 VAC, VAC initial time of 15 sec., 1.5 kV/cm, pulse width of 30μs and two times at room temperature. Fused protoplasts were allowed to stand for 10 min after electrofusion.

3.2.4 Culture of fused protoplast

One ml of TM-2 (MTM2, composed of 2 x concentrated of all components of TM-2 except that the $CaCl_2$ concentration was reduced from 440 to 170 mg/l) medium (Hossain *et al.* 1994) supplemented with 1.0 mg/l NAA and 0.5 mg/l BA were added to one ml of fused protoplast mixture. The density of protoplasts after fusion was adjusted to 5 x 10^4 with TM-2 medium with 0.912 % mannitol, 6.84 % sucrose, 1.0 mg/l NAA and 0.5 mg/l BA. Protoplast suspension (2.5 ml) was cultured in a 50 x 15 mm plastic petridish in the dark at 25°C. When cell wall synthesis and then favorable cell division were observed, the culture was transferred under light condition (aprox. 1000 lux). When active protoplast division was observed, 1 ml of fresh growth regulator free TM-2 medium was added to the culture every after 2 days. The content of one petri dish was divided into 2

dishes when the volume of the culture was exceeded over 2.0 ml. Ten days after the initial culture, the number of protoplasts undergoing division was counted and the plating efficiency was estimated.

3.2.5 Plant regeneration

Ten-day-old growing putative-fused colonies were collected and put into a clean glass tube and kept stationary for 10 min. The supernatant was removed and the colonies found at the bottom of the tube were resuspended with fresh TM-2 plant regulator free medium. One ml of colony suspension was first plated on either TM-3 medium (0.5 mg/l BA, 0.2 mg/l 2,4-D, 0.9 % bacto-agar) or MS regeneration medium (1.0 or 3.0 mg/l ZR, 0.1 mg/l GA_3, 3 % sucrose and 0.9 % agarose) (first plating). The culture was kept at 25°C under 2500 lux. Ten days after the first plating (20 days after initial protoplast cuture), calli without shoot primordia were transferred onto the MS regeneration medium. Adventitious shoots which grew more than 1.5 cm long were excised and transferred onto MS rooting medium (20 g/l sucrose, 0.1 mg/l IAA and 0.8% agar) in a 15 cm glass tube (ϕ 2.5 cm). When the shoots were sufficiently rooted, the agar medium was washed with tap water. The shoots were planted in clay pots containing a commercial potting soil (Soil compost, Kureha Chemical Co.). The pots were covered with plastic bags and placed under low light condition in the greenhouse for acclimatization. After 7-10 days, when the plants appeared to grow vigorously, the corners of the plastic bags were cut open to allow gradual airexchange. When the plants had finally aclimatized, plastic bags were removed.

3.3 Results and Discussion

After 15 sec of dielectrophoresis, protoplast pairs were observed. Several protoplasts exhibited a pearl chain formation (**Fig. 3-1 A**). Protoplast fusion was attained following the electric pulse application. In preliminary experiments, sufficient fusion events could not be confirmed with a single application of electric pulse. When double pulses were applied, fusion ratio was increased and many fused protoplasts were observed under a microscope (**Fig. 3-1 B**). Additionally, multikaryons, i.e. fusion of more than three protoplasts were also observed (**Fig. 3-1 B**).

Fused protoplasts were cultured without removing the fusion solution by centrifuge. The supplement medium was only added to fused protoplasts after fusion experiment. In fact, the fusion solution simply consisted of $CaCl_2$ and mannitol which also are contained in the culture medium. Besides, their concentrations were the same as or lower than TM-2 medium. Therefore, it was not necessary any longer that the fused protoplasts suffered from the conventional centrifuge step which results in the mechanical damage, if the supplement medium is applied after electrofusion. Hossain (1995) introduced this method and obtained a large number of somatic hybrid plants.

The earliest cell division was observed 3 days after the initial culture, but most of the putative heterokaryons started undergo division within 5 days (**Fig. 3-2 A**). However, the frequency of protoplast division was low. Ten-day old colonies (**Fig. 3-2 B**) were transferred onto TM-3 medium or directly onto the regeneration medium wherein they continued to grow rapidly and vigorously. Some of the calli turned green and developed bud primordia within 10 days after transfer onto the regeneration medium. The highest frequency of shoot regeneration (number of shoots forming callus/ number of cultured callus) was observed when colonies were cultured onto MS agarose medium containing 1.0 mg/l ZR and 0.1 mg/l GA_3, and subsequently transferred onto MS Bacto-agar medium containing 3.0 mg/l ZR and 0.1 mg/l GA_3(**Fig. 3-2 C**). Fourteen calli out of 52 (26.9 %) developed shoots when the calli were cultured directly onto MS agarose

medium containing 3.0 mg/l ZR and 0.1 mg/l GA_3 (**Table 3-1**). It is probable that this treatment induces rapid shoot regeneration when the calli are still small (2 to 3 mm in size) (**Fig. 3-3 A**). The period of shoot regeneration was approximately 20 days. On the other hand, it was previously reported that shoot regeneration from Sl protoplasts was attained 3 months of initial culture only. It was also suggested that the Sl protoplasts could not divide in TM-2 medium. Therefore, self-fused Sl protoplasts were inhibited to divide, while the heterokaryons and F_1 fused and unfused protoplasts could continue to grow in this culture. However, callus derived from the one-step transfer method to regenerate shoots produced only one shoot which appeared to show hyperhydricity.

Effective selection of the heterokaryon is one of the important steps in somatic hybridization. Regeneration capacity from one of the fusion parents has been used as a selectable marker and was reported to be utilized in protoplast fusion experiments in *Nicotiana* (Maliga *et al.* 1977), *Petunia* (Itoh and Futsuhara 1983), *Brassica* (Terada *et al.* 1987) and *Lycopersicon* species (Adam and Quiros 1985). In addition, vigorous growth (heterotic effect) of the callus resulting from the hybridity of the cells was also applied as a selection system of somatic hybrids (Shieder 1982, Schenck and Robbelen 1982). In this study, the period of initial culture and subsequent subculture or plating were comparatively short, then only vigorous callus were transferred onto the next culture or regeneration medium. Therefore, it is likely that the regenerated plants obtained were true somatic hybrids.

Finally, 85 regenerated plants from 46 out of 184 cultured calli were obtained and acclimatized in the greenhouse (**Fig. 3-3 B**). The hybridity of the plants were analyzed and confirmed using various methods described in CHAPTER4.

Table 3-1. Effect of culture media on shoot regeneration from the calli derived from putative fused protoplasts.

First plating[1]					Second plating[1]			Number of		
Medium	BA (mg/l)	2,4-D (mg/l)	ZR (mg/l)	GA_3 (mg/l)	Medium	ZR (mg/l)	GA_3 (mg/l)	Cultured calli	Green calli	Shoot formation (%)[2]
TM-3	0.5	0.2			MS	1.0	0.1	34	24	0 (0)
TM-3	0.5	0.2			MS	3.0	0.1	10	10	1 (10.0)
MS			1.0	0.1	MS	3.0	0.1	39	27	23 (60.0)
MS			1.0	0.1				49	25	8 (16.3)
MS			3.0	0.1				52	20	14 (26.9)

1) The first and second platings indicate callus transfer at 10 and 20 days after initial culture in TM-2 medium, respectively.

2) Number of shoot developed callus/number of callus cultured. Shoot formation was investigated at 60 days after initial culture.

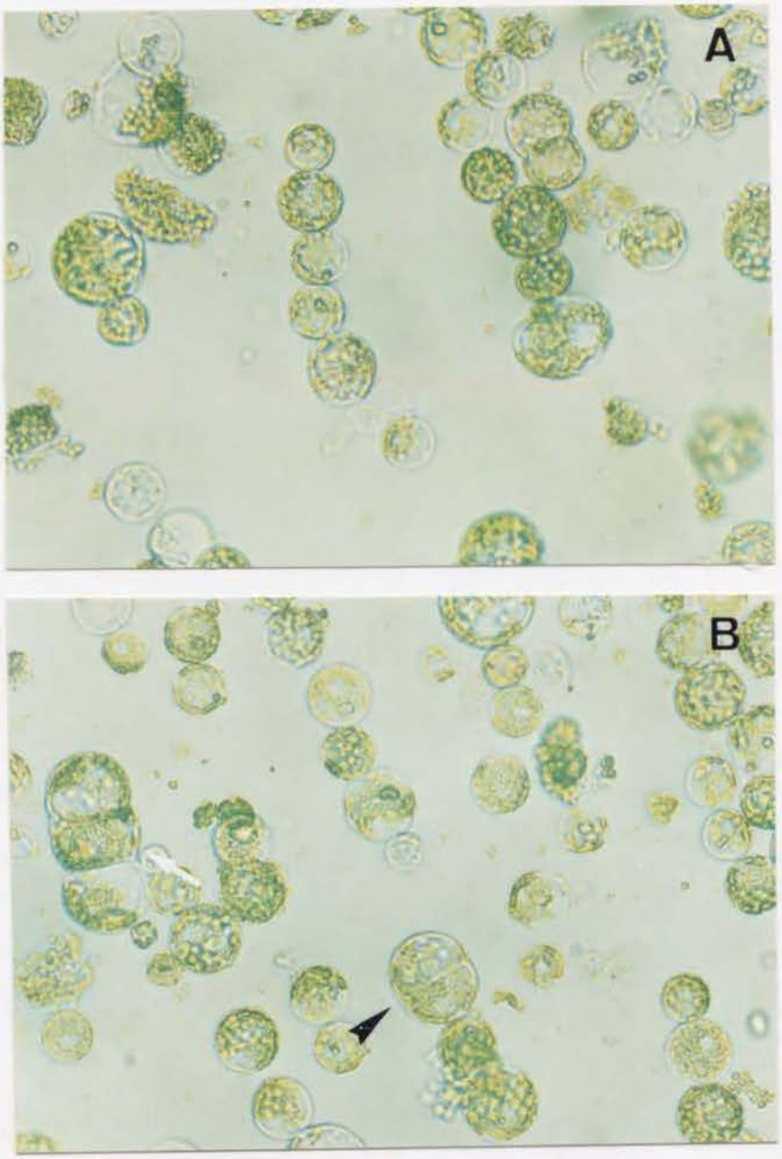
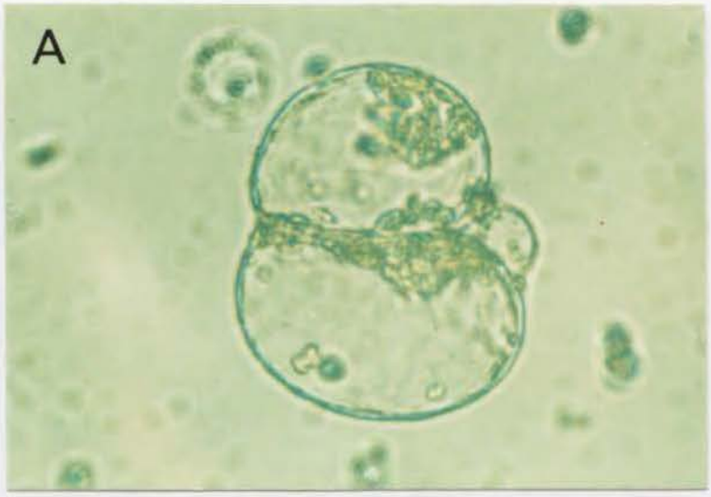


Fig. 3-1 A. Pearl chain formation of F1 (*L. esculentum* x *L. peruvianum*) and *S. lycopersicoides* protoplasts by dielectrophoresis. **B.** Protoplast fusion. Two protoplasts were fused (Black arrow). Three protoplasts were fused (white arrow).

Fig. 3-2 A. First division of a putative fused protoplast three days after initial culture. **B.** A colony derived from a putative fused protoplast one week after initial culture. **C.** Green calli on MS regeneration medium (second plating). Many adventitious shoots were observed.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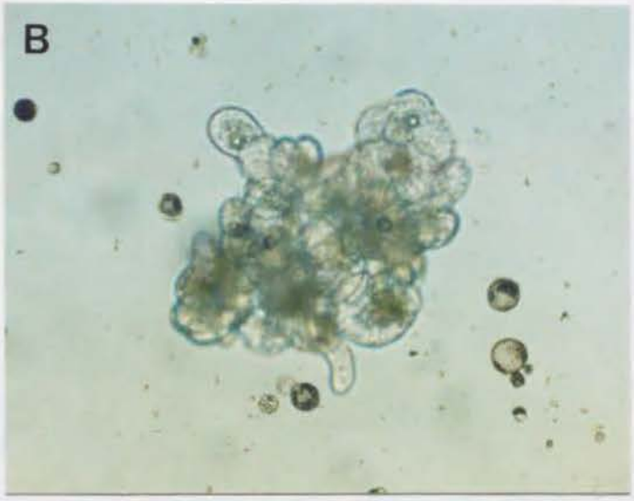
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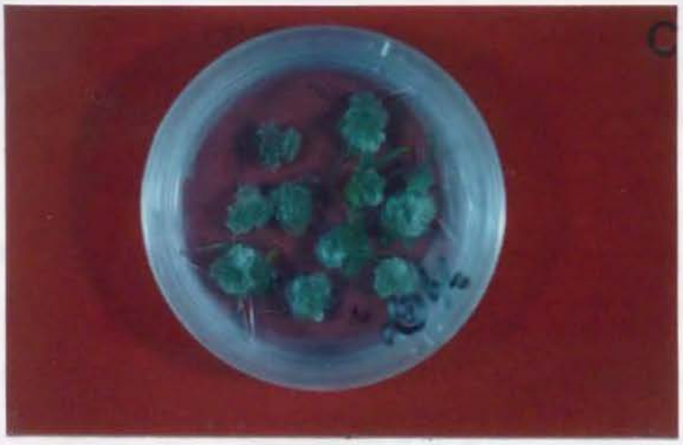
C

Fig. 3-3 A. Callus transferred directly from initial culture onto MS regeneration medium containing 3.0 mg/l of ZR and 0.1 mg/l of GA_3. Adventitious shoots tend to show hyperhydricity. **B.** Putative somatic hybrids at 2 months after acclimatization in the greenhouse.