A NEW *In Vitro* REGENERATION PROTOCOL IN TOMATO (*Lycopersicon esculentum* Mill.)

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ABSTRACT. A protocol has been established for a rapid, high frequency plant regeneration of normal tomato plants. Dried mature seeds of tomato (*Lycopersicon esculentum* Mill.) from four cultivars were used to obtain adventitious shoot buds. Sterilised seeds were sown on filter paper that had been wet with water and precultured from zero to three days in the light at 25ºC. Precultured seeds were cut into two parts; the portion consisting of the proximal part of the hypocotyls was cultured on a medium with Murashige and Skoog salts, Gelrite 2g.L^{-1}, Mio-Inositol 100 mg.L^{-1}, Tiamine 4 mg.L^{-1} , 3 % commercial sucrose, without growth regulators. After two weeks, more than 60 % of the elongated shoots were excised individually from the explants and subcultured on the same medium for rooting. At the same time, calli were subcultivated on a fresh medium to obtain more adventitious shoots. Morphological characteristics of regenerated plants and fruits were similar and they set normally shaped fruits with mature seeds; on the other hand, 40 regenerated plants showed no variations in chromosome number (2n=24). The advantages of this regeneration method are: it does not employ exogenous growth regulators, it is feasibly handled, regenerated plants are obtained in a short time and also whole plants were obtained in other four cultivars applying this simple procedure. This paper reports an efficient system for plant regeneration from adventitious shoot buds.

Key words: tomatoes, *Lycopersicon esculentum*, revegetation, organogenesis, tissue culture

RESUMEN. El objetivo de este trabajo fue establecer un protocolo que permitiera una rápida y alta frecuencia de regeneración *in vitro*. Para obtener brotes adventicios se usaron semillas maduras de cuatro variedades de tomate (*Lycopersicon esculentum* Mill). Las semillas esterilizadas se colocaron en papel de filtro humedecido con agua y precultivadas de cero a tres días a la luz a 25ºC. Las semillas precultivadas se cortaron en dos partes; la porción de la parte próxima al hipócotilo se cultivó en un medio con sales Murashige y Skoog, 2 g.L^{-1} Gelrite, 100 mg.L^{-1} Mio-Inositol, 4 mg.L^{-1} Tiamina, 3 % de azúcar comercial, sin reguladores del crecimiento. Después de dos semanas, más del 60 % de los brotes elongados fueron escindidos individualmente de los explantes y subcultivados en el mismo medio para enraizar. Al mismo tiempo, los callos fueron subcultivados en medio fresco para obtener más brotes adventicios. Las características morfológicas de las plantas regeneradas y los frutos fueron similares a las que le dieron origen, produciendo frutos normales con semillas maduras; 40 plantas regeneradas no mostraron variaciones en su número cromosómico (2n=24). Las ventajas de este método de regeneración son: el no empleo de reguladores del crecimiento exógeno, su fácil manipulación, el corto tiempo para la obtención, y aplicando este simple procedimiento se obtuvieron plantas completas de otros cuatro cultivares. Este trabajo presenta un eficiente sistema de regeneración de plantas a partir de brotes adventicios.

Palabras clave: tomate, *Lycopersicon esculentum*, regeneración de plantas, organogénesis, cultivo de tejidos

INTRODUCTION

The successful application of plant tissue culture presupposes the establishment of an efficient culture system, consisting of a competent genotype and explant source as well as optimal culture conditions. Tomato (*Lycopersicon esculentum* Mill) is an important horticultural crop; thus, a number of investigations report the use of plant tissue culture technique in tomato breeding programs (1).

Most techniques for genetic regeneration depend on the use of plant growth regulators in complex and nearly empirical combinations adapted to each particular situation. Development of protocols independent of exogenous plant growth regulators could help standardise techniques for different species and cultivars, thereby, reducing problems of regeneration efficiency and elongation of regenerated and abnormal shoots (2).

Several protocols have been published for *in vitro* plant regeneration of *Lycopersicon* species. Methods previously reported are in general tedious and time consuming, with variable efficiencies and high production

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costs. In all cases, regeneration systems involved media containing growth regulators. Although tomato adventitious shoot regeneration is not considered a real problem, the interest to obtain more efficient, reliable, simple, rapid and universal methods for genetic engineering is well documented in literature (2, 3, 4, 5, 6).

In the present study, a protocol has been established for an efficient, rapid and high frequency plant regeneration method of normal tomato plants, independently of exogenous growth regulators in culture medium. It also reports the absence of morphological and chromosomal variations among regenerated plants.

MATERIALS AND METHODS

Plant material. Dried mature seeds of tomato (*Lycopersicon esculentum* Mill.) from commercially available four cultivars (Campbell-28, Amalia, Lignon and Floradel) were used as explants. These were obtained from the germplasm collection of the tomato breeding program at The National Institute of Agricultural Sciences (INCA).

Culture media and conditions. Seed surface was cleaned with Tween 80 % for 20 seconds, washed by sodium hypochlorite 3 % for 10 min and rinsed three times in sterilised water. After sterilization, seeds were sown on filter paper previously wet with sterilized water and precultured from zero to three days, with a photoperiod of 16 hours per day (65 $\mu mol.m^{-2}.s^{-1}$) at 25°C. Precultured seeds were cut into two parts, the portion consisting of the proximal part of the embryo hypocotyls (7) was cultivated on the following shoot culture medium (SCM): Murashige and Skoog (8), Mio-Inositol 100 $mg.L^{-1}$, Tiamine 4 $mg.L^{-1}$, commercial sugar 30 $g.L^{-1}$, and pH adjusted to 5,8 before adding Gelrite 2$g.L^{-1}$ and sterilising by autoclave at 121°C. Each glass container with five explants was incubated in a growth room at 25±2°C, with a photoperiod of 16h/day (65 $\mu mol.m^{-2}.s^{-1}$).

Plant growth and regeneration. After two weeks, the number of explants with regenerated adventitious buds and/or callus as well as the number of adventitious buds per explant were counted. Elongated shoots were individually excised from the explants and subcultured on a fresh SCM for rooting. The remaining calli were excised and subcultured on an SCM medium; after one week, adventitious buds emerged and shoots were subcultured for rooting. All experiments were repeated twice under the same conditions.

After rooting, vitroplants were acclimatized under greenhouse conditions for 20 days. Plantlets were transplanted to pots with a mixture of Litonite and soil (1:1) for growing. Plant morphology, fruit setting and seed maturity were evaluated after three months.

Data analisis. Data of 10 explants were recorded after four weeks of culture, scoring the number of shoots per explant. Each one was repeated at least once with similar results and representative experiment data presented here are the mean values. Data generated during the study were statistically analysed including means, SE and c^2 test. Means were separated by Duncan's test. In tables, means with the same letters indicate no significant differences between treatments.

Histological observations of adventitious buds, cv. Amalia *through optical microscopy*. Fresh sections from adventitious buds at different developmental stages were cut with a microslicer. Sections were stained with a solution of Safranin 0.5 %. A Leica microscope was used for sample visualization and photography.

Scanning electron microscopy. Fresh sections of apical regenerated explants from cv. Amalia at different developmental stages were fixed with glutaraldehyde 2.5 % and 0.1 M phosphate buffer (pH 7.3), by gently shaking at a temperature of 4°C for one hour. Materials were rinsed repeatedly with the above buffer and postfixes in Osmium tetraoxyde 1 %. Next, the sections were dehydrated with a graded ethanol series. The dehydrated materials were treated with isoamyl acetate and critical-point dried with gold by an ion sputtering equipment, Tousimis Samdei-PVT-3B, and examined with a scanning electron microscope, Jeul-Jeeni JMS-T330.

Chromosome numbers. Root tips of 20 regenerated plants from cv. Amalia were randomly collected and incubated an 8 Hidroxiquinoline solution for four hours, washed with distilled water and fixed in ethanol and acetic acid (3:1). They were dipped in 1N HCL at 60°C for about two hours. Chromosomes were stained with chromic hematoxilin for one hour and squashed with a drop of 45 % acetic acid. Chromosomes were counted on intact metaphase plates.

RESULTS AND DISCUSSION

Plant regeneration. Seeds of tomato, cv. Amalia started to germinate in a few days on SCM. After a week, the young seedlings grew with root but without apical part. Two weeks later, emerged elongated hypocotyls of explants and adventitious buds differentiated around the cut surfaces, in all treatments (Figure 1A). These adventitious buds differentiated when explants were precultured for zero to three days (Table I).

The number of explants with regenerating adventitious buds and of adventitious buds per explant were counted (Table I). In all treatments, the percentage of explants forming buds surpassed 60 % and the treatments with three preculture days presented the highest percentage. As culture continued, such percentage increased. This result represents a good frequency of regeneration for this species (9), which suggests that morphogenetic activity takes place in these explants in absence of growth regulators.

Table I. Effects of preculture on adventitious bud development in immature tomato explants (cv. Amalia)

Duration of preculture (days)	Explants forming buds (%)	Number of buds/explant	Explants forming buds after subculture (%)	Number of buds/explant after subculture
0	69	1.75±0.07b	0b	0±0.04d
1day	63	2±0.07b	100a	2.81±0.04a
2days	100	1.58±0.06b	100a	2.41±0.05b
3days	81	2.57±0.06a	100a	1.94±0.04c
ESx±	0.10 ns	**	0.11***	***

Means with the same letters in the column are significantly different at *** p<0.001 and ** p<0.01

On the other hand, the number of differentiated adventitious buds obtained from immature plant parts was greater than in previous explant reports (10, 11, 12). All treatments exhibit organogenic ability ranging from 1.58 shoots per explant for two preculture days to 2.57 for three preculture days (Table I). Here, shoot yield per explant was similar to other studies using a more complex system of culture medium, explants and plant growth regulators (5, 11, 12, 13, 14).

The results presented in Table I showed that only two weeks later, the rate of differentiation of adventitious buds was higher than in the first culture and shoots elongated from all subcultured explants. The explants that had not been precultured did not differentiate adventitious buds. These results showed the importance of preculture time and its influence on shoot bud induction. Different explant sources have been used for shoot formation in cultivated and wild tomato species, such as leaf, stem, root, cotyledon or hypocotyls (2, 5, 6, 10, 11, 12, 14), but in our experiments, a reasonable regeneration rate was obtained using immature plant parts. This investigation also revealed the important role of explant source and age in the experiments. The system permits to obtain normal plants in only four weeks, reducing production costs and manipulating effects in a feasible handling way.

Histological, morphological and chromosomal observations. In order to know how cells from the cut surface of explants differentiated into buds, we performed histochemical analyses of the time-course evolution of this zone. Adventitious meristems and shoots emerged from the epidermal cells located around the cut surface of explants. In other cases, we could observe that meristems regenerated from both the callus and cells surrounding the cut surface (Figure 1B). This observation confirms other results (2, 7).

Scanning electron microscope allowed us to examine the cut surface of a wide range of explants and confirm the origin of plant regeneration process via organogenesis. Thus, this additional histological information demonstrates that the unique structures obtained by pre-treatment have highly meristematic regions, which are thought to have the potential to regenerate plantlets and identify differentiating cells during organogenesis process (Figure 1C).

Plantlets were easily acclimatized (Figure 1D). When 20 regenerated plants were grown in a greenhouse, the morphology of all plants was normal and all of them set normally shaped fruits with mature seeds. The number of chromosomes in the root tip cells from 20 regenerated plants was 2n=24 (Figure 1F), which corresponds to the original number in this species.

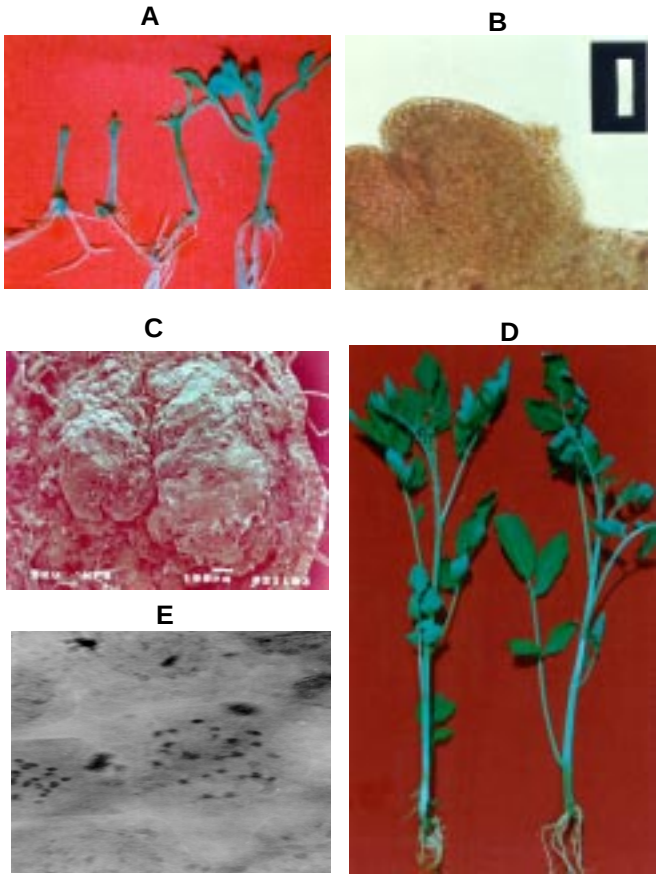

Figure 1. Regeneration of tomato plants (cv. Amalia). A: adventitious bud formed on the cut surface of a hypocotyl, growing adventitious bud, elongation of shoot from an adventitious bud; B: differentiating cells during organogenesis process; C: differentiating cells during organogenesis process (bar 100µm); D: acclimatised whole plant; E: chromosomes from a regenerated plant (2n=24)

Application to other cultivars. After two weeks of culture, explants from four cultivars differentiated adventitious buds. The percentage of explants with elongated shoots ranged from 54 to 81 %. The shoots rooted on SCM and regenerated whole plants were observed in all cultivars tested. Similar tendencies were recorded in each pair of duplicated experiments.

Table II. Differences in the ability to form adventitious buds and shoots among various tomato cultivars

Cultivar	Explants forming buds (%)	Number of buds/explant
Campbell 28	69ab	1.45±0.05
Amalia	81a	2.57±0.06
Lignon	69ab	1.55±0.06
Floradel	54b	1.88±0.06
ESx±	0.01***	0.06**

Means with the same letters in the columns are significantly different at *** p<0.001 and ** P<0.01

Genotypic differences for the number of regenerated shoots were observed in this study. The effect of genotypes on organogenic ability was significant and all the genotypes tested were regenerated. The Campbell 28 and Lignon cultivars presented the lowest percentage of explants with adventitious buds whereas cv. Amalia presented the highest value. In contrast with other culture methods, differences among genotypes were lower in the number of shoots per explant (2, 5, 6, 10). Shoots rooted on SCM regenerated whole plants in all cultivars tested.

These results showed an efficient, rapid and reproducible tissue culture regeneration protocol, independently of exogenous growth regulators. Our investigation has also revealed the determining role of genotypes in the efficiency of shoot formation. Moreover, tomato adventitious shoot capacity depends on explant source, thereafter on genotypes and finally on growth regulators. This method is applicable to many cultivars and it is free of genetic variation.

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