Mesopo. Environ. j., Special Issue E : 107-114 , 2018

ISSN 2410-2598

proceeding of 2nd International conference of science and Art
University of Babylon and Liverpool John Moores
University, UK

Mesopotemia Environmental journal

journal homepage:www.bumej.com

Obtaining heterokaryons following electrical fusion between mesophyll and transformed hairy roots protoplasts of sugarbeet

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To cite this article:

AL-Nema, Q. S. and AL-Mallah, M. K., Obtaining heterokaryons following electrical fusion between mesophyll and transformed hairy roots protoplasts of sugarbeet, *Mesop. environ. j.*, 2018, Special Issue E.;107-114.

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Abstract

Eleven enzyme mixtures consisted from various cell wall degrading enzymes were succeed in isolation of protoplasts from leaf mesophyll of axenic seedlings of sugarbeet, *Beta vulgaris* L.. Also, some of these enzymatic mixture support the isolation of protoplasts from the adventitious hairy roots produced as a result of sugarbeet explants inoculation with *Agrobacterium rhizogenes* R1601. Somatic hybridization between the two types of protoplasts took place through electrofusion and approach 39% under conditions of 1MHz, 1000 Vcm^{-1}, 2 pulses, 1.5 msec./pulse

Keywords: protoplast, electrical fusion, *Agrobacterium rhizogenes*, hairy roots, sugar beet.

Introduction

Fusion of plant is one of the important possibilities to produce hybrid plants of interested characters. This is particularly in plant species facing problem in their breeding program [1]. Electrical stimulus to induce protoplasts fusion of *Rauvolfia serpentia* plant species [2]. Somatic hybridization between plant protoplasts through fusion technology represent an efficient experimental system to obtain genetically modified plants. The economical value of this approach lie in many applied field

Mesopotamia Environmental Journal
ISSN 2410-2598
Mesop. environ. j. 2018, Special Issue E.;107-114
(proceeding of 2nd International conference of science and Art –University of Babylon and Liverpool John Moores University, UK).

such as the transfer of herbicides resistance to cereals for example rice [3]. Also, the production of cybrids as a result of fusion between *Citrus sinensis* L. and *C. paradisi* L. [4], and the production of potato plants resistant to fungal blight disease [5]. A study reported to improve the plant species *Beta vulgaris* L., sugarbeet through the production of somatic hybrid cells following the electrical fusion between protoplasts of two different varieties [6]. This study aimed to produce genetically modified plants of sugarbeet probably of high sucrose content.

Materials and Methods

Plant material

Seeds (var. Baraka) of sugarbeet were (obtained from the General Enterprise for Sugar Industry, Mosul) cultivated in vermiculite. Plantlets were kept under growth room condition [6]. Other group of the same seeds were surface sterilized [7] and sown on agar-solidified MSO [8]. Speciueus were incubated in culture room.

Inoculation of leaves with *Agrobacterium rhizogenes*

Sugarbeet leaves were excised from field-grown plants, surface sterilized by immersing in 3% solution of NaOCl then washed thoroughly by autoclaved water [6].

Leaf explants were inoculated with *Agrobacterium rhizogenes* R1601 (Supplied by Professor E. W. Nester, Washington Univ. U. S. A) grown in APM [9] liquid medium provided with 100 mgL^{-1} of each kanamycin and carbencillin. Inoculated plants were placed on he surface of 15 ml of agar solidified MS medium in 9.0 cm diam. Plastic Petri-dishes which covered by lids and sealed with nescofilm strips.

Establishment of hairy root culture

Young hairy roots developed on inoculated leaves were cut and placed in 9.0 cm diam. plastic Petri-dishes containing 15 ml of agar solidified MS medium. When bacterial growth was noted, hairy roots were transferred sequentially to MS medium supplied with gradual conc. 50, 100, 150 and 200 mgL^{-1} of cefotaxime. They stayed on each conc. 2 wks until clean culture was produced, [10] and sub cultured routinely.

Transgenesis of hairy roots

Conservation of genetic marker on Ri-plasmid of *A. rhizogenes* R1601 was carried out through the spread of antibiotic saturated paper disc on the surface of agar-solidified APM medium previously streaked with 0.1 ml of bacterial suspension [11]. Detection of agropine in these tissue was carried out following the procedure mentioned previously [12].

Isolation of protoplasts

Leaves were excised from 6 wks old axenic seedlings with the removal of lower epidermis. Peeled leaves were cut into small portions and inoculated into 10 ml CPW 13M solution for one hour in dark [13]. Different enzyme mixtures (Table 1) were tested for isolation of protoplasts.

Mesopotamia Environmental Journal
Mesop. environ. j. 2018, Special Issue E.;107-114
ISSN 2410-2598
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Table (1): Enzyme mixtures used in isolation of protoplasts from leaf mesophyll of sugarbeet (*Beta vulgaris* L.) seedlings.

Enzymes	Enzyme solutions (%)								
	I	II	III	IV	V	VI	VII	VIII	IX
Cellulase R10	2.0	2.0	0.5	1.0	0.0	0.0	0.0	0.0	0.0
Cellulase RS	1.0	1.5	0.5	0.0	1.0	1.0	2.0	2.0	0.5
Cellulysin	0.0	0.0	0.0	0.0	0.1	0.5	0.1	0.5	0.0
Driselase	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0
Hemicellulase	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0
Macerozym R10	0.5	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pectinase	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.0
Pectolyase Y-23	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.1
Mannitol	9.0	9.0	4.0	4.0	9.0	9.0	9.0	9.0	13

Enzyme mixture containing the released protoplasts were passed through nylon sieve (80 µm, PGMG, Nott. Univ., UK) and then placed in test tubes and centrifuged at 100 g for 5.0 min. to the precipitated protoplasts, 5.0 ml of CPW 13M was added, this step was repeated three times and protoplasts resuspended in 2.0 ml of liquid KM8p [14] medium.

In the same manner protoplasts was isolated from transformed hairy roots following the steps used previously [15] utilizing several enzyme mixtures (Table 2).

Table (2): Enzyme mixtures used in isolation of protoplasts from transformed hairy roots of sugarbeet (*Beta vulgaris* L.).

Enzymes	Enzyme solutions (%)										
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Cellulase R10	0.5	1.0	0.0	0.5	1.0	0.0	0.0	1.0	2.0	1.0	0.0
Cellulase RS	0.5	0.0	1.0	0.5	0.0	1.0	1.0	0.0	1.5	1.0	0.0
Cellulase YC	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0	0.0	1.0	1.0
Pectolyase Y-23	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.1
Macerozym R10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0

Mannitol	4.0	4.0	4.0	5.0	5.0	5.0	5.0	4.0	0.0	4.0	13

Electrofusion of mesophyll and transformed protoplasts

Electrofusion between the two protoplasts was carried out as described [16] in Labs. of PGMG / Plant and Crop Sciences Dept., Nottingham Univ., UK. Finally resuspended the precipitated fused and non-fused protoplasts into 20 ml of liquid KM8p medium to be ready for culture.

Visual selection method [17] was followed in picking – up fused protoplasts using micromanipulator. This method depend on the size of fused protoplasts.

Results

Isolation of mesophyll protoplasts

The results indicate that leaf mesophyll tissues is a good source to obtain high yield of protoplast. Eight of nine enzyme mixtures were efficient in isolation of reasonable yield and viable protoplasts (Table 3).

Table (3): efficiency of enzyme mixtures used in isolation of protoplasts from leaf mesophyll of sugarbeet, *Beta vulgaris* L.

Enzyme mixture	Duration (h)	Yield ($\times 10^4$ prot. / ml)	Viability (%)
I	2.00	3.0	77
II	1.00	7.3	93
III	-	-	-
IV	4.00	1.6	73
V	1.30	1.8	92
VI	24.00	1.5	80
VII	2.00	1.3	54
VIII	3.30	1.08	67
IX	16.00	18	87

isolation failed.

Light microscope examination of this protoplasts referred to the spherical shape unvacuolated cells (Fig. 1, A) with regular distribution of chloroplasts (Fig. 1, B) and highly viable (Fig. 1, C).

Production of transformed hairy roots cultures

The findings that adventitious hairy roots were developed from injected and not injected sites on leaf explants (Fig. 1, D). those hairy roots were separated and placed on cefotaxim supported MSO medium. After sequential transfer of these tissues

on the above medium, many cultures of bacterial hairy roots were obtained. Additionally, they were agropine positive and grow happily (Fig. 1, E).

Isolation of protoplasts from transformed hairy roots

Data indicate that protoplasts were isolated from transformed hairy roots using various mixture of enzymes (Table 2). The most active enzyme mixture consisting from 1.0 % Cellulase YC and 0.1 % Pectolyase Y23 in 13 % of mannitol. The optimal releasing time of these protoplasts were 16 hrs. Yield was 12 $\times 10^4$ prot.ml^{-1} and viability was 88 % of these protoplasts. The results referred to low yield of protoplasts obtained from transformed hairy roots (Fig. 1, F), and to the absence of chloroplasts in this protoplasts (Fig. 1, G). Viable protoplasts in the FDA stained preparation was of fluorescent green color when exposed to UV light (Fig. 1, H). Both types of protoplasts used in fusion experiments was accepted concerning their viability, nucleation and cell wall regeneration.

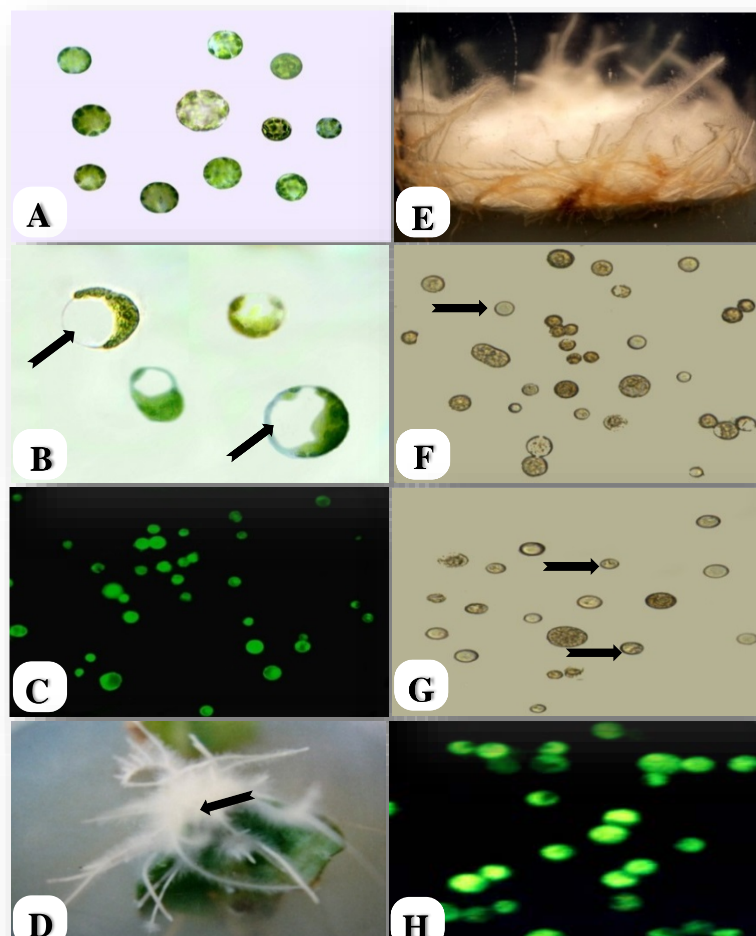

Fig. (1): protoplasts isolation from leaf mesophyll (A-C) and from transformed hairy roots (D-H) in sugarbeet, *Beta vulgaris* L.

A. Unvacuolated protoplasts of regular distribution of chloroplast.
B. Vacuolated protoplasts (arrowed).
C. Viable protoplasts stained with FDA under UV.
D. Hairy roots (arrowed) developed on leaf 15 days old.
E. Transformed hairy root culture (6 wks) grown on MSO medium free from bacteria.
F. Protoplasts isolated from hairy roots in (E).
G. Transformed protoplasts in (F), note the small size and absence of chloroplasts.
H. Fluorescent transformed protoplast in (F) stained with FDA and visualized under UV light.

Electrical fusion between mesophyll and transformed protoplasts

The results pointed out to the successful fusion between mesophyll protoplasts and transformed protoplasts isolated from transgenic hairy roots. Protoplast density had an important role in fusion process and general fusion percent ranged between 12-39 % affected by fusion conditions (Table 4).

Table (4): Fusion products obtained from electrical fusion between mesophyll protoplasts and transformed protoplasts of sugarbeet, *Beta vulgaris* L.

Protoplasts densities ($\times 10^4$ cell ml^{-1})	Fusion conditions	Fusion (%)	No. of fused cells
.0 2	**1MHz, 1000 Vcm^{-1}, 2 pulses, 1.5 msec./pulse**	12	54
3.0	**1MHz, 1000 Vcm^{-1}, 2 pulses, 1.5 msec./pulse**	23	59
5.0	**1MHz, 1000 Vcm^{-1}, 2 pulses, 1.5 msec./pulse**	39	73
Aggregate of fused cells			186

Photographs picked up through fusion process exhibit the arrangement of protoplast in chains when exposed to AC current (Fig. 2, A) and fused together when DC current pulse pass through (Fig. 2, B). Unfortunaty, those fusion products failed to divide in culture.

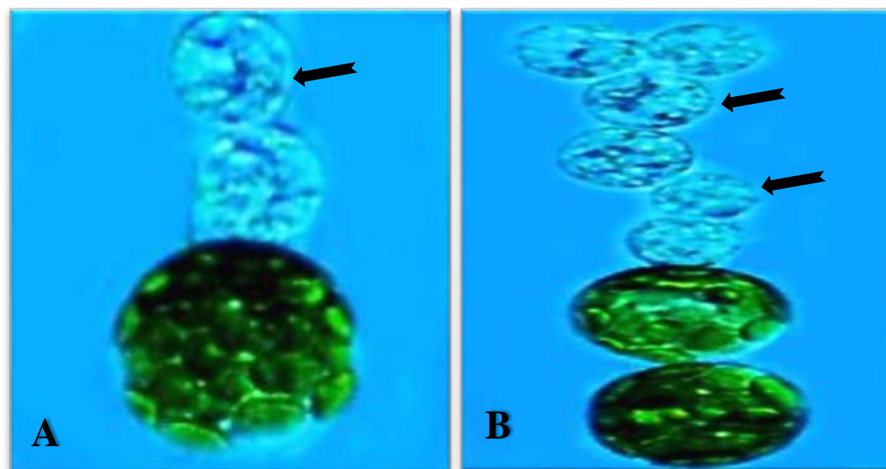

Fig. (2): Steps of electrofusion between mesophyll protoplasts and transformed protoplsts of sugarbeet, *Beta vulgaris* L.

A. Produced short chains affected with a weak AC current (40 X), Note transformed protoplasts without chloroplasts (Arrowed).

Discussion

Most of breeding programs of sugarbeet *Beta vulgaris* L. were not efficient. The interesting technique followed for this industrial crop includes plant regeneration from protoplasts [18]. Generally, mesophyll protoplasts are difficult in culture whereas protoplast isolaton, culture and plant regeneration in other plant species considered easy such as in rice, *Oryza sativa* L. [19] and *Phaseolus vulgaris* L. [20]. The difficulties face mesophyll protoplast of sugarbeet probably due to the availability of specific nutrients. Therefore, most workers benefit from guard cell protoplasts which is unique to produce plant from this protoplasts [21]. It was found that addition of Phytosulfokin to medium promote protoplast division [22].

Protoplasts isolation from transformed hairy root is still determinant, this is might explained to bacterial contamination probabilities. Few workers succeed in isolation this protoplast from hairy roots of *Solanum dulcamara* plant [15].

The present study obtained well characterized protoplasts from both leaf mesophyll and transgenic hairy roots. This two parents were acclimatized to fuse together since protoplasts fusion represent one of transformation possibilities to produce genetically modified plants of interested characters [23]. The conclusion that protoplast fusion between these two types of protoplasts may offer an efficient pathway to produce sugarbeet plant of high sugar content or plant resistant to some fungal diseases.

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