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Regeneration of *Eurca Sativa* L. Plants from Cell Suspension- Derived Callus with Assessment of Anatomical Variation

Sahla M. Alzeadan, Faiq H. Alradi and Jamella H. Rasheed

Biology department, Education college for Pure Sciences, Mosul University, Mosul. IRAQ

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ABSTRACT

Background: The study succeed in establish a fine cell suspension derived from callus of rocket plant (*Eurca sativa* L.) stem explant in liquid MS medium supplemented with 1.0 mg/L NAA + 1.5 mg/L 2,4-D. **Objective:** the cells enhanced the first division within 24h., and continued their subsequent division to produce 5.2×10^3 cell/ml after 5 days of culture and the total number of colonies was 152, which produced callus after 4 weeks. **Results:** Regenerated shoots were rooted in MS medium; plantlets survived and showed new branch development under *in vitro* conditions. **Conclusion:** This study also finds out changes in anatomical & structural characterization of stem.

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INTRODUCTION

Eurca sativa L. plants are annual or biannual, that belong to the family Brassiaceae which is the biggest family and containing 3500 species (Bender & Kumar, 2010; Kumar & Andy, 2012). The genus of this family showed best results in tissue culture techniques (Cardoza & Stewart, 2004; Das *et al.*, 2010), and gave high percentage of callus formation from all parts of mustard plant when cultured on MS media with different combination of auxins & cytokinins. Application of *in vitro* regeneration techniques in *Eurca sativa* has been reported from cotyledonary nodes (98%) leaf (75%) and cotyledon (80%) other (65%). Somatic embryogenesis achieved in several *E. sativa* (Sharma *et al.*, 2013). Therefore this family indicated the regeneration ability of rocket plants callus tissue (Munir *et al.*, 2008; Hussein *et al.*, 2010). Density & viability Of cell suspension is important to determine cells division, expansion of cells and growth which followed by specialization, this technique also pursuit study metabolic compounds and activities of enzymes stimulation & gene expression (Pec *et al.*, 2010; Bonfill *et al.*, 2011; Veerashree *et al.*, 2012). Anatomical properties are very important character in most plant to illustrate the difference in structure of cells & tissues.

The purpose of the present work is to study internal structure & anatomy of primary tissue of stems derived from callus of cell suspension & seed plants.

MATERIALS AND METHODS

Production of axenic seedlings:

Surface sterilized seed of *Eurca sativa* L. were cultivated on the surface of 25 ml of agar-solidified MS (Murashige & Skoog, 1962) medium at ratio of three seeds/flask. They were kept in culture room in dark for 3 days and after germinated they transferred to light/dark regime and light intensity of 1500 lux. Three weeks old axenic seedlings were utilized for stem explants.

Establishment of cell suspension culture:

Stem explant cultured on the surface of agar-solidified MS medium supplied with 1.0 mg/L NAA + 1.5 mg/L 2,4-D, for callus initiation. One gram piece of friable and young stem callus 21 day age placed in 50 ml of MS liquid medium supplemented with 1.0 mg/L NAA+ 1.5 mg/L 2,4-D using 100 ml volume conical flasks. They were kept in rotary shaker (Shaking incubator. New Bruniswic U.S.A.), at velocity of 150 rpm., $28 \pm 2^\circ\text{C}$ in dark (Morris & fowler, 1980). The culture was passed through sterile plastic sieve of 46 μm pores, which allow only the passage of fine cells (Gresshoff, 1980). The culture was kept in the previous condition. These

Corresponding Author: Faiq H. Alradi, Biology department, Education college for Pure Sciences, Mosul University, Mosul. IRAQ.
Tel: 009647701790100; E-mail: faiq_alradi73@yahoo.com.

suspensions were regularly- sub- cultured every 2 days by removing the medium and replaced with same volume of fresh medium.

Viability & density of cell suspension determination:

Viability of cell suspension was carried out by mixing 0.1 ml of Evan blue solution (chemical Ltd., Pool, England) with sample of cell suspension and examined microscopically (Kanai & Edward, 2002). This following formula was used to calculate the perceive of viable cells.

$$\% \text{ Viability} = \frac{\text{Total cells} - \text{Dead cells}}{\text{Total cells}} \times 100 \text{ (Paul, 1970)}$$

Determination of culture density of cell suspension was performed (Hinton & Moulood, 1979), by taking 0.1 ml from culture after 24, 48, 72, 96, 120, 144 and 168 h. of incubation. Cells were counted in sample of each interval by heamocytometer (Labsco, Germany).

Culture of cell suspension multiple drops arrays technique:

All cell suspension of 0.1 ml volume was cultured by embedding in agar (Dixon, 1985). The procedure involved the mixing of 0.1 ml of cell suspension with 1.0 ml 3% agar autoclaved and molten solution kept in waterbath at 40°C, the produced mixture was dripped into identical drops on the bottom of 9.0 diameter plastic petri-dish at ratio of 8 drops/dish. They kept open in the cabinet for solidification then 4 ml of fresh liquid MS medium which used in the establishment of cell suspension was added to each dish, and dishes closed by nescofilme. The specimens kept in culture room at condition of 25 °C, 500-700 diffused illumination 16 band/8h. dark. Cells cultures in drops were examined microscopically every day to detect their division. These cultures were subcultured every 4 days by removing the liquid medium & replaced with the same volume of fresh medium.

Development and transfer callus primordia:

After a series of cell division numbers of small size callus primordia were developed into the agar drops. These small size primordia were picked up (Rasheed, 2002) transferred to 100 ml vial containing 25 ml of agar-solidified MS medium supplemented with 1.0 mg/L NAA+ 1.5 mg/L 2,4-D, which used for callus initiation. Specimens were kept in culture room at the same condition explained above.

Results:

Initiation of cell suspension from stem calli:

The results expressed that the friable callus induced on agar solidified MS medium provided with 1.0 mg/L NAA+ 1.5 mg/L 2,4-D (fig.2-A) was suitable to produce fine and homogenize cell suspension. Growth of these culture sustained viability of single cells and enhance their division leading to produced high densities of viable cell culture. (table-1).

Table 1: Density and viability of cells forming suspension culture derived from stem of *Eurca sativa* L.

Incubation period (h.)	Culture density (cell)	Cell viability (%)
72	3.4×10^3	38
96	4.6×10^3	65
120	5.2×10^3	53

Liquid MS media encouraged the division of cell division to produce high density which is 5.2×10^3 cell/ml after five days of culture, while the viability of cell suspension was 65% after four days of culture (fig. 1).

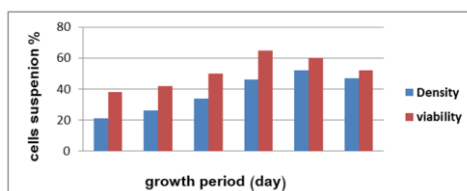


Fig. 1: The density and viability of cells suspension.

Culture of cell suspension in agar drops:

Preliminary experiment showed that density 4.6×10^3 cell/ml is the most convenient to culture. Samples of cell suspension, embedded in agar showed that the most cell still alive and starting their first division after the third day of culture (fig.2-B) and forming two daughter cells (fig.2-C), the second division after 24 h. from the first division to produce four similar cells (fig.2-E), and these cells continuing division producing cells colonies

within 14 days (fig.2-F), the total number of colonies was 170 after four days of culture (table-2), and formation of callus primordia involved 21 day, but they varied numbers of colonies and calli primordia.

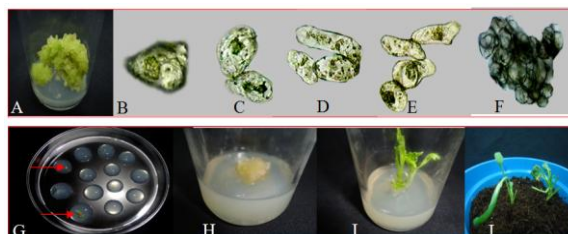


Fig. 2: Establishment the cell suspension from callus derived of rocket's stem.

Most of callus primordia developed with 10-14 day after culture having the green color (fig.2-G), callus primordia increased in size and transferred to solid MS media to grow (fig.2-H). Finally the callus was differentiated to shoot & roots (fig.2-I). Plantlets have been acclimatized to greenhouse condition (fig.2-J)

Table 2: Culturing of cell suspension derived from stem of *Eurca sativa* L. in MS supplemented with 1.0 mg/L NAA+ 1.5 mg/L 2,4-D.

The cultured density	The cultured drops	Cellular colonies	Callus primordia	Callus stimulation	% Callus stimulation
Comparison	110	----	----	----	----
3.4×10^3	160	136	42	77	48.12
4.6×10^3	148	170	86	96	64.86
5.2×10^3	164	152	68	84	51.21

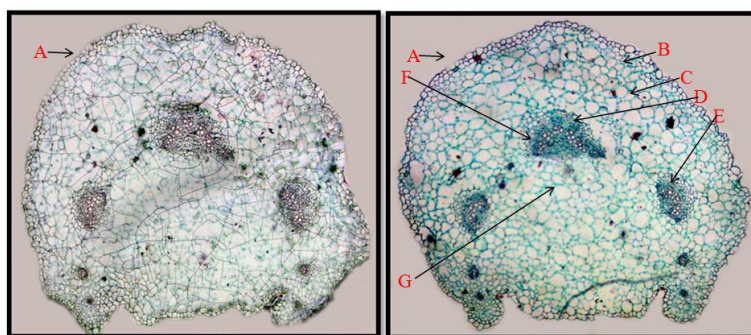


Fig.3: C.S Rocket's stem of seed plant.

Fig. 4: C.S Rocket's stem derived from cell Suspension.

The anatomical characteristics of *Eurca sativa* stems, showed changes in internal structure of cross sections of rocket seed plants (fig.3-A), and sections of stems derived from callus of cell suspension (fig.4-A), the epidermal cells being tubular in shape and the size of the epidermis was 11.0 μm compared with 10.0 μm and plasmodesmata are readily seen with light microscope, which to be connected with material transport and conditions of stimuli, these cells much deeper (fig.4-B), than cells in seed plants. The cortex contain 7 rows of parenchyma cell, which is kind of storage cells commonly have thin primary walls, and the carbohydrate develops in these cells (fig.4-C), parenchyma also prominent a big intercellular spaces, the pericycle is uniseriate consist of thin walled parenchyma (fig.4-D). Stem of rocket plant contain 14 open collateral vascular bundles (fig.4-E), as compared with 12 collateral V.B. in seed plants, each bundle has cambium produced a few cells between xylem & phloem tissue, the diameter of vessel in xylem tissue in stem sections of rocket plant was 23 μm (fig.4-F), while it was 20.25 μm in seed plants, the vascular bundles were separated by parenchyma rays which expand to the center of the stem to form pith (fig.4-G).

Discussion:

Cell suspension provided a biological system to study the behavior of single cell in tissue culture which indicated features of cells such as stages of division, enlargement, specialization & growth (Moscotiello *et al.*, 2013). It is demonstrated that changes in growth regulators affected divisions of cells which reflex on the density & viability of cells in culture media (Silveira *et al.*, 2009). The presence of growth regulators in perfect concentration it doesn't mean positive role to increased cell division & initiation of the primordia, but the effects refer to genes inside the cells & the environmental conditions & physiological functions related to the

wall structure & implied in water movements through it, this fact has been established by measurements of vessels & sieve tubes diameters. The results show the most important variation between stems of rocket plant derived from callus of cell suspension & seed plants concerned, the number of vascular bundles and layers of cortex & accumulations of starch grains which raised in stems of callus derived from cell suspension, these changes may be related to the increasing of cell wall permeability (Gupta *et al.*, 1988), or to the enhancement of specific protein synthesis & other phenolic compounds in cell suspension of rocket plants (Erich *et al.*, 2002).

Therefore cell suspension culture by embedding in agar behaved normally in its growth, divisions & forming colonies which developed to calli primordia, this related to the viability & biological activities of cell suspension (Tan *et al.*, 2010). This experiment induced a friable callus & differentiated to plants (Al-Alaaf, 2013), therefore anatomical changes in internal structure of plants derived from callus of cell suspension & seed plants (Alradi, 2013).

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