

Heat Shock Stimulate Division of Hypocotyle Suspension Culture of *Dianthus caryophyllus* L.

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الخلاصة

نجحت الدراسة الحالية في إنشاء مزارع المعلقات الخلوية المشتقة من السيقان تحت الفلجية لبادرات القرنفل *D. caryophyllus* L. باستخدام وسط MS السائل المدعم باضافة 0.5 ملغم/لتر 2,4-D و 0.1 ملغم/لتر BA , وباشرت خلايا هذه المعلقات انقسامها الاوّل بعد 24 ساعة , واستمرت في انقساماتها المتتالية لتصل كثافتها 142 × 10³ خلية/سم³ بعد خمسة ايام من تحضينها, واعتمد في هذه الدراسة تعريض مزارع المعلقات الخلوية قبل زراعتها الى درجات حرارية مختلفة 20, 25, 30, 35, 40, 45, 50 م° ولفترات قصيرة المدى 5 , 10 دقيقة واخرى طويلة المدى 15-20 دقيقة واطهرت نتائج تعريض المعلقات الخلوية المزروعة بتقانة الطمر في قطرات الاكار التأثير التحفيزي للمعاملة الحرارية في زيادة انقسامات الخلايا وتكوين المستعمرات الخلوية لتصل إلى 959 مستعمرة عند تعرضها للمعاملة الحرارية قصيرة المدى 35°C/5/min واتصف الكالس بلونه الاخضر وبنيته الهشة.

Abstract

The study succeeded in establishing a fine cell suspension derived from callus of carnation, *Dianthus caryophyllus* L. Hypocotyle explants in liquid MS medium supplemented with 0.5 mgL⁻¹ 2,4-D and 0.1 mgL⁻¹ BA. The cells begin its first division within 24 h. These cell were continued their subsequent divisions to produce 142x10³ cell/ml culture after five days of initiation. Results indicated that exposure of cell suspension to 20, 25, 30, 35, 40, 45, and 50°C short-term 5, 10 min. and long-term 15-20 min. stimulate the division of cells suspension in all treatments. Heat shock sustained the division of cell cultured in multiple drop array (MDA) technique and increase the formation of cellular colonies which produced a numerous of calli primordia. The total number

of colonies was 959 developed when cells exposed to with the short-term 35°C/5 min heat shock. The produced calli was green in colour and friable in texture.

Introduction

Many studies reported that heat treatment have positive effects on different plant since it stimulate synthesis of novel protein named heat shock protein (HSP). Moreover, heat shock accelerate oxidation processes and help in some enzymes production (1). The same effect occur in yeast (2), and pathogenic fungi (3). Other investigators found that heat shock treatment increase the elasticity of plant cell wall and protein synthesis in cell cytoplasm of maize plant (4) and cucumber (5). Such study explained that exposure of plant cells to 45°C for one minute increase calcium ion Ca^{+2} in cell coupled with increasing building up phosphate lipids (6). In *Lycopersicon peruvianum* L. Heat shock stimulates synthesis of proteins and forming granular aggregates in cytoplasm of tomato culture (7). Other study observed that exposure of cell suspension of *Helianthus annuus* L. to 40°C for 10 minute promoted the division of cells as well as increased the production of cellular colonies after days of culture (8).

A study indicated that exposure of cell suspension of *Medicago sativa* to low temperature decrease the uptake of ion and soluble molecules in cells which resulted hardness of cell wall (9, 10) they reported that exposure of cell suspension of *Lycopersicon esculentum* to 35°C for 5 minute, result in a rapid and reversible decline of study level and mitotic index. The aim of this study is to recognize the effect of heat shock in division of cell suspension and their development to primordia of callus.

Materials and methods

*** Production of axenic seedlings:**

Surface sterilized seeds of carnation, *Dianthus caryophyllus* var. G.J. Sim were cultivated on the surface of 25 ml of agar-solidified MS medium (11) at ratio of there seeds/flask. They were kept in culture room in dark for 3 days and after germination they transferred to light/dark regime and light intensity of 3000 Lux. Three weeks old axenic seedlings were utilized for hypocotyle explants.

*** Establishment of cell suspension culture:**

Hypocotyles explants were cultured on the surface of agar – solidified MS medium either supplied with the addition of 0.5 mgL⁻¹ 2,4-D and 0.1 mgL⁻¹ BA (12) or with 1.0 mgL⁻¹ IAA and 1.0 mgL⁻¹ kinetin

(13) for callus initiation. One gram pieces of friable and young hypocotyle calli were placed in 50 ml of the liquid culture medium MS + 0.5 mgL⁻¹ 2,4-D and 0.1 mgL⁻¹ BA 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±2oC in dark. The cultures were passed through sterile plastic sieve of 46 μm pores (PGM.G, U.K.) which allow only the passage of fine cells (14). The cultures were kept in the previous condition. These suspensions were regularly sub-cultured every four days by removing the medium and replaced with same volume of fresh prepared medium.

*** Viability and density of cell suspension determination:**

Viability of cell suspension was carried out by mixing 0.1 ml of Evan blue solution (Chemical Ltd., Poole, England) with 1.0 ml sample of cell suspension and examined microscopically (15).

This following formula was used to calculate the percent of viable cells

$$\% \text{ viability} = \frac{\text{Total number of viable cells}}{\text{Total number of cell}} \quad (16)$$

Determination of cell suspension density was performed (17) by taking 0.1 ml samples from culture after 24, 48, 72, 96, 120, 144 and 168 h. of incubation. Cells were counted in each sample of each interval by heamocytometer (Labsco, W. Germany).

*** Exposure of cell suspension to heat shock:**

Samples of 1.0 ml of cell suspension were taken at density of 1.42x10³ cell/ml and placed into 5.0 cm capacity sterile test tube. These samples were exposed to various temperature including 20, 25, 30, 35, 40, 45, and 50oC for 5.0 and 10 min/each to represents the short-term heat shock. Other samples were heat-treated using the same degrees but for 15 and 20 min/each representing the long-term heat shock. Each sample was treated by soaking the test tube into water bath previously fixed on the desired temperature for 10 min, then tubes were removed and immersed into ice bath to bring the degree to room temperature. After treatment all samples are ready for culture (18).

*** Culture of cell suspension by multiple drop array technique:**

All cell suspensions of 1.0 ml volume heat-treated and untreated were cultured by embedding in agar (19). The procedure involved the mixing of 1.0 ml of cell suspension with 1.0 ml of 3% agar autoclaved and molten solution kept in water bath at 40°C, The produced mixture was dripped into identical drops on the bottom of 9.0 cm diam. Plastic Petri-dish (Sterilin, UK) at ratio of 8 drops/dish. They kept open in the cabinet for solidification, then 5.0 ml of fresh liquid MS medium which used in the establishment of cell suspension was added to each dish. Lids were put and dishes closed by nescofilm. The specimens kept in culture room at condition of 25°C, 700-500 diffused illumination 16 h and 8 h dark. Cells cultured in drop were examined microscopically every day to detect their divisions. These cultures were subcultured every four days by removing the liquid medium and replaced with the same volume of fresh medium.

*** Development and transfer callus primordia:**

After a series of cell divisions numbers of small size callus primordia were developed into the agar drops. These small size primordia were picked up (20) and transferred to 100 ml vial containing 25 ml of agar – solidified MS medium supplemented with 0.5 mgL⁻¹ 2,4-D and 0.1 mgL⁻¹ BA which used for callus initiation. Specimens were kept in culture room at the same condition explained above.

Results

*** Initiation of cell suspension from hypocotyle calli:**

The results expressed that the friable callus induced on agar-solidified MS medium provided with 0.5 mgL⁻¹ 2,4-D and 0.1 mgL⁻¹ BA (Fig. 1,A) was suitable to produce fine and homogenize cell suspension. Growth of these culture continued their division producing high densities of viable cells (Table 1).

Table (1) : Density and viability of cells forming suspension culture derived from hypocotyle of *Dianthus caryophyllus* L.

Incubation period (h)	Cell density (x10 ³ cells)	Cell viability (%)
24	46	55
48	60	60
72	80	76
96	116	86
120	142	87
144	125	80
168	90	72

*** Culture of heat-pretreated cell suspension in agar drop :**

Preliminary experiment showed that the density 142×10^3 cell/ml is the most convenient to culture. Samples of cell suspension, pretreated with temperature of short-term and subsequently embedded in agar showed that most cells still alive and starting their first division after the third day of culture forming two daughter cells (Fig.1.B). The second division occur after 24 h from the first division to produce four cells stage (Fig.1.C). On the other word, these cell tolerated these degrees of temperature, since they continuing division producing cell colonies within 14 days (Fig. 1.D). The total number of colonies was 959 (Table 2) and formation of callus primordia involved 21 day. But they varied in their effects on the numbers of colonies and calli primordia. Most of callus primordia developed within 10-14 day after culture having the green colour but they differ in their sizes (Fig. 1.E).

Table (2): Effect of short-term heat shock on division of cell suspension of *Dianthus caryophyllus* and development of callus primordia.

treatment °C/min	First div. (d)	Formation of CC (d)	Development of CP	Number CP
Untreated (control)	6	102	56	6
20/5	4	236	49	10
/10	4	365	84	10
25/5	4	348	149	17
/10	4	242	174	19
30/5	4	711	511	22
/10	4	478	363	21
35/5	3	959	805	30
/10	3	745	465	29
40/5	4	227	182	11
/10	4	265	207	13
45/5	4	655	324	26
/10	5	419	301	12
50/5	5	104	85	12
/10	5	98	54	10

CC = Cellular colony

CP = Callus primordial

The results also observed that long-term heat shock enhanced cell division within 3-4 days , cells continued their division to form cellular colonies within 2 weeks from culture. The total number of colonies reached 518 (Table 3) when cell suspension treated with long-tem $35\text{C}^0/15$ min heat shock. These colonies developed to form numerous of

calli primordia within 25 days, the produced calli were dark green in color and compact in texture. (Fig. 1.F)

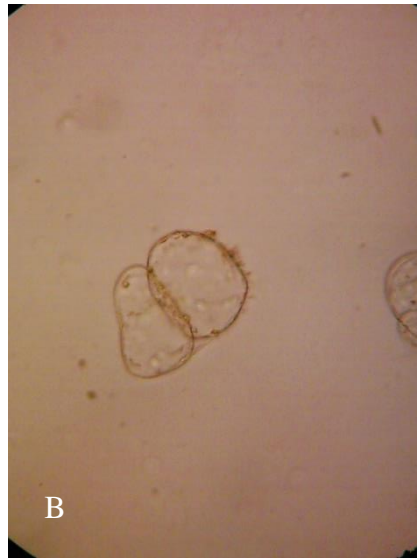
Table (3): Effect of long-term heat shock on division of cell suspension of *Dianthus caryophyllus* and development of callus primordia.

Heat treatment °C/min	First div. (d)	Formation of CC (d)	Development of CP	Number CP
Untreated (control)	7	211	215	17
20/15	3	180	135	14
/20	3	255	163	16
25/15	3	274	207	15
/20	3	262	192	11
30/15	4	425	234	20
/20	4	350	290	17
35/15	3	518	322	13
/20	3	365	215	20
40/15	4	178	95	17
/20	4	122	65	13
45/15	4	248	161	14
/20	4	185	155	17
50/15	4	86	60	12
/20	4	70	55	12

CC: Cellular Colony ,CP: Callus Primordia



**Callus of hypocotyle age
3 weeks**



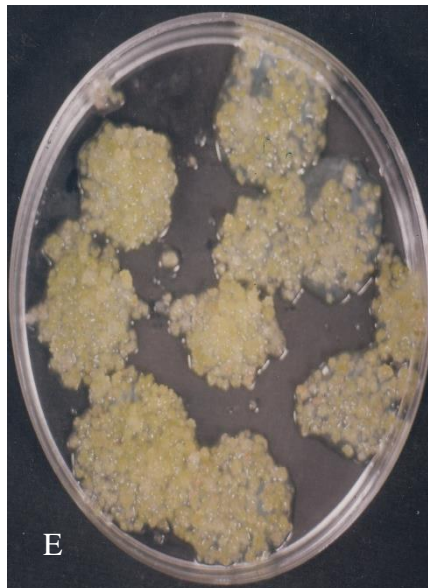
**Two daughter
cells**



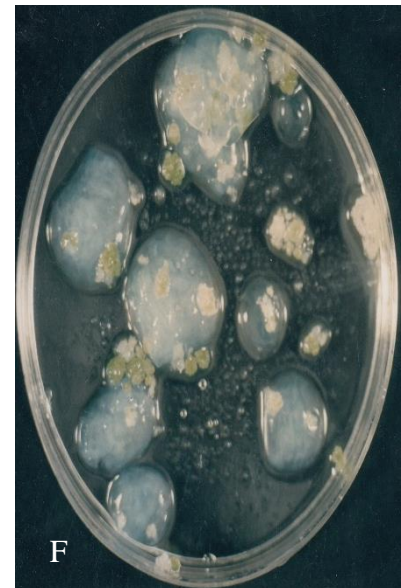
4 cells-stage



Cellular colonies



**Callus from short term
35°C/5min**



**Callus from long term
35°C/15min**

**Fig 1: Production of Callus from Culturing Cell Suspension derived from
hypcotyles of *Dianthus caryophyllus* L.**

Discussion

Hypocotyle of *Dianthus caryophyllus* L. Was readily produced friable callus and this is due to the amenability of this explants in culture (21). Other kind of carnation explants also derived calli easily using suitable auxine and cytokinine in MS medium such as in leaves (22,23).

Cell suspension cultured by embedding in agar behaved normally in its growth, division and forming colonies which developed to calli primordia. This is may attributed to the viability of heat treated cell that unaffected (24). Other investigator using the same heat treatment with sunflower cell suspension note the same pointes (18). In this study, generally heat treatment stimulate the division process of cells. This is may due to the increasing of cell wall permeability (25) or to the enhancement of synthesis of specific protein named HSP (Heat Shock Protein) and other phenolic compounds in cell suspension of carnation (26). Which sustained division of cells as in rice (27). Other studies reported that heat treatments permitted some ions like copper sulphate to inter into cell which accelerate cell division (28). The only exception in this study is the treatments 50°C/20 which reduce the production of primordia compared with other treatment. This is probably due to the cease of cell dividing which is caused by damage the wall of cells firstly and proteins secondly (29).

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