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High Efficiency of the Somatic Embryogenesis Sequences of Desert Lime (*Eremocitrus Glauca)*

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Abstract: *Eremocitrus glauca* is a desert plant that is sources to the plant genetic resources for citrus, because these plants are resistant cultivars to some enemy mainly in the tropical region. Isolated protoplast culture media supplemented with *to 2-isopentenyladenine (2iP)* maintaining under the light intensity range 13.23. mol/m^2/s^1 to 70.8 mol/m^2/s^1 sigificantly effect to plating effisiensy protoplasts, globular somatic embryos, and plant regeneration of Eremocitrus glauca. The temperature emission and light illumination are applicate to cells has implicated to cell recovery and formation of somatic embryos. 2iP and environment factors increased the health cell from enzyms stress, grow and developed rapidly. In vitro temperature for somatic embryo formation in cultures is achieved on 25^{o}C in corporations with 0.1 mg/l, 2 iP and more likely in pattern linear, with equation Y = 0,7336x + 4,1894, R^2 = 0.8172. The same patern is also accur to ligh illumination. Attractive conclusion that light and temperature must be well to do on plant physiology and chemistry. The sequence from isolated protoplast to cell recovery, formation of somatic embryos and plant or shoot regeneration under control of media, plant growht regulators and environment factors has establish in *Eremocitrus glauca*. Forturmore the gene tranfer from this species has been easily.

Keywords: Cells**,** *Eremocitrus-glauca*, 2iP, light-intensity, protoplasts somatis-embryo, temperature.

I. Introduction

Citrus relatives represent a largely untapped reservoir of genetic diversity (Grosser et al. 1992; Herrero et al.1996). Many of the citrus relatives are a potential source of genes controlling natural resistance to biotic and abiotic stresses and other characters. However, some of them are unacceptable for direct use as citrus scion culture and rootstocks (Swingle and Reece 1967; Grosser and Gmitter 1990) because of sexual incompetence impossible (Iwamasa 1966; Cameron and Frost. 1979; Frost and Soost 1968; Cemeron and Soost 1979; Barret, 1997; Vardi and Galun, 1988). Sexual incompetence adversely affect conventional *Eremocitrus glauca* is one of some citrus relatives reed procedures, but it constitutes and advantage once a required genotype is secured (Wakana and Uemoto, 1987) and is a desert plant that is the plant genetic resources for citrus, because these plants are resistant cultivars to some enemy mmainly in the tropical region. The cultivars resistant could be a low cost effective and efficient way to take care or maintain of plants in the field. *Eremocitrus glauca* is an Australian native plant and resistant to dryness, and roots of these plants are also resistant to nematode disease.

Somatic embryogenesis from protoplast sometimes is difficult to observe how to develop into embryos. The time beginning to recover into the cell wall is not easy to detect. Cell wall recovery in citrus relatives needed the time about 15 days after fresh isolation protoplast culturing into media.

Many factors support the protoplasts develop into cells intact, however there are many factors possible to limit the protoplast becoming a healthy cell. Cells can only be developed into the normal if protoplast consists of the complete nucleus, cell wall and other components. The normal and healthy cell culturing in the media will grow into cell division and then going to colony formation. Some tissues are going to be divided into somatic embryos and another one will grow into the big colonies.

The cell colony consists of more than two cells growing into a big colony, about hundreds of cells are interdependent formed somatic embryos. Not all of colonies developed into somatic embryos, because some colonies are only be growth into big colonies (Jumin and Nito 1996)

The major environmental factors in vitro culture are temperature, light intensity, photoperiod, and culture media. Protoplast culture of citrus relative cultures depend completely on artificial light intensity lamp light sources for illumination. The illumination shall supply the light in the suitable location of the electromagnetic spectrum. The illumination purposes focussed to growth activity of somatic embryos for development, and assimilation, photosynthetic metabolism of the explants (belum) light factor contain wave lengh, intensity, irradiances and photoperiod make possible the result of somatic embryos wish characteristics (Cavallaro et al. 2022).

In this experiment some of the environmental factors will analyze how the role of envoronment factors affetecd to protoplast developed to cells and somatic embryos.

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Temperature is important by plants to reserve colorific energies to increase the cell division activities in tissue. Energy absorbed from light intensity will be used to run photosystem II and photosystem in the photosynthesis in chloroplast of the shoot in culture. Protoplasts constitute a base material to easily make the genetic transformation from a species into other species. Normal somatic embryos will regenerate into plants.

However, cells which one is not capable of growing into somatic embryos are difficult or impossible to regenerate into plants and not potentially to use a material for gene transfer. This paper explains the sequence of protoplast growing into normal cells and developed to somatic embryogenesis in *Eremocitrus glauca*

II. Materian and Methods

2.1. Plant materials

Formation callus emerged from plantlets in the culture after 3 mounts sub-culturing on MT media. Embryogenic callus of *Eremocitrus glauca* was induced from the hypocotyl region of seedlings cultured on MT basal medium (1969) containing 5% sucrose maintained under 52.9 $mol.m^{-2}$ s^{-1} light intensity with 16-h photoperiod. Hypocotyl excisions were germinated from immature nucellar embryo explants using the same medium as mentioned above.

2.2 Protoplast Isolation

Prior to protoplast isolation, about 1 g of *Eremocitrus glauca* callus was transferred to fresh liquid medium consisting of Murashie and Tucker medium containing 4% sucrose, and incubated on gyratory shaker at 120 rpm for 12 h under 17.7 $mol.m^{-2}$ s^{-1} light intensity with a photoperiod of 16 h at 25°C. Callus tissue was placed in 50 ml Erlenmeyer flasks and mixed with 5 ml of filter-sterilized medium consisting of 0.5% macerozyme R-10, 0.25%, 0.15% driselase in half-strength MT inorganic salts, 0.6 M mannitol, and the pH was adjusted to 5.6. The enzyme solution was sterilized through a Millipore filter (Millex-HA, 0.45 m) before use.

After 12 h incubation on a reciprocal shaker at 25 rpm in the dark at 25°C, protoplasts were isolated by filtering through a double layer of Miracloth. Protoplasts were separated by centrifuging at 100 x g for 5 min. Sedimentation of protoplasts was separated w7 ith remaining enzymes by pipetting from the suspension and the protoplasts were then washed three times with half-strength liquid MT medium without glucose

2.3 Protoplast culture

Pure protoplasts of *Eremocitrus galuca* was cultured on the MT medium supplemented with 0.0 mg/l 2iP 2-isopentenyladenine (2iP), 0.001 mg 1/l, 2iP, 0.01 mg /l 2iP, 0.1 mg/l 2iP and 1.0 2iP and maintained under several light intensity as follow; 13.23 $mol.m^{-2}$ s^{-1}, light intensity, 26.45 $mol.m^{-2}$ s^{-1}, 52.9 $mol.m^{-2}$ s^{-1} light intensity with 16-h photoperiod and addition of 600 mg/l malt extract (ME) as described by (Jumin and Nito, 1996)

The culturing protoplasts were maintained for 40 days at 25^{o}C temperature, 16 h photoperiod at 52.9 $molm^{2}s^{-1}$ light intensity. Furthermore, protoplasts developed into embryogenic callus and were maintained in the growth chamber at 25° C with 51.9 $\mu mol.m^{2}/s$ light intensity.

Plating efficiency was counted as the percentage of living cells which become cells clustered in the media during 70 days following procedures (Jumin and Nito 1996. The cell wall development of the protoplasts was checked by fluorescein diacetate staining under an inverted microscope beginning from 10 days of culture.

2.4 Embryo induction and development

Shoots developed were sub-cultured 2 times during 30 days using media supplemented with 0.0, 0.001, 0.01, 0.1, 1.0 and 10.0 mg/1 2iP solidified with 0.09% gelrite. Other treatments living cells were cultured to medium with 2.5 g/l glucose, 2.5 g/l sucrose, 2.5 g/l maltose, and 2.5 g/l galactose. To evaluate the efficiency of protoplasts developed into somatic embryos, the cells were maintained under 16-h photoperiod 25^{o}c with several light intensities as follows; 17.7 mol. m^{-2}. s^{-1}, 35.4, 53.1 and 70.8 $mmol.m^{-2}$. s^{-1}. After somatic embryos emerged, about 2 mm in length, they were carefully transferred into media consisting of 4.0 % lactose with 0.001mg/l 2iP and 0.2.5 % gelrite, 5.7 pH at 25^{0}C for 60 days.

2. 5 Plant Regeneration

Green shoots were cultured to half-strength MT basal medium supplemented with 5% sucrose and solidified with gelrite 1.0 % Shoots developed into anormal plants about 2cm in length are possible to transfer to soil. Acclimatization takes t at least 30 days and could be life as a normal plant.

2.6 Parameters

Plating efficiency is the number of plates consisting of living protoplasts compared to the total of plated protoplasts. 2. *Somatic embryogenesis efficiency* is the number of cultured embryogenic protoplasts that grow into 2 or more globular somatic embryos compared to total plated embryogenic protoplast cultures. 3. *The number of normal somatic embryos* is the number of normal

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somatic embryos that grow into 2 or more globular somatic embryos. 4. *Plant regeneration efficiency* is the number of somatic embryos that grow into shoots or plans compare to total somatic embryos cultured into media.

III. Result and Discussion

Two – three drops of protoplasts suspension consisting about 2-3x 10^6 protoplasts with a diameter of 10-30 m were obtained from 1 g of callus (Fig. 1A, 4A). FDA Staining showed that the viability of the protoplasts tested about 85 %. Estimated 70% of the living protoplasts developed cell walls during 10 days of culture as observed under staining Calcofluor white M2R. The protoplasts undergo stress during isolation. Cell wall recovery after the protoplasts cultured in the liquid medium to solve the enzymes dibble. For that purpose, the isolated protoplasts were cultured in liquid medium and then cultured to mixed liquid and solid media. The protoplast plating efficiency obtained about 20 – 53% after 40 days of protoplast culture.

The effect of temperature to plating efficiency is more significant if the 2iP added to medium (Fig. 4B, 4C. 4D) This indicates the temperature triggered the cells to grow rapidly causing the calorific in vitro temperature in the growth chamber (Table 1). The same pattern, the light intensity, also possesses a positive effect on the percentage of plating efficiency (Table 2). Calorific of temperature and light energy resulting from light intensity of lamp contribute energy consumption of the cell to increase the growth of the health recovery of cells.

Table1. Effect of 2-isopentenyladenine (2iP), and Light intensities on percentage of plating efficiency of *Eremocitrus glauca*

2iP (mg/l)	**Light Intensities** (mol.m^{-2}.s^{-1})			
	13.23	**26.45**	**39.68**	**52.91**
0.0	10.3a	14.3a	24.6a	25.3a
0.001	17.3b	21.3b	37.3a	36.3b
0.01	18.6b	22.6b	30.6b	37.6b
0.1	23.6b	27.6bc	49.6bc	53.6c
1.0	8.3a	12.3ac	26.3c	35.3ab

According to Duncan's Multiple Range Test, the mean value followed by a different alphabet a column do not substantially differ from one another at P≤0.05

Table 2. Effect of 2-isopentenyladenine (2iP), and temperature on percentage of plating efficiency of *Eremocitrus glauca*

. **2iP (mg/l)**	**Temperatures (**0^{o}C**)**			
	20	**25**	**30**	**35**
0.0	19.3a	26.3a	25.6a	24.6a
0.001	26.3b	31.3b	30.3a	23.3a
0.01	27.6b	32.6b	37.6b	26.6a
0.1	32.6bc	39.6c	40.6b	25.3a
1.0	17.3ac	30.3ab	28.3c	21.6a

According to Duncan's Multiple Range Test, the mean value followed by a different alphabet a column do not substantially differ from one another at P≤0.05

Relationship between light intensity and 2 iP on the somatic embryo formation is close. The dependence of light intensity and 2 iP mutually with equation Y = 0,708x + 8,7971 and R^2 = 0,7642. It means each increasing the light intensity ffollowed by 2 iP concentration until 0.1 mg/l (Figure 1).

Stagnate of 2 iP concentration is more limited until 1.0 mg/l and afterward inclined the growth is leisurely and may be stopped.

Temperature as colorific energy for protoplast development showed the close relationship with 2iP concentration. The best in vitro temperature for somatic embryo formation in cultures is achieved on 25^{o}C in corporations with 0.1 mg/l, 2 iP and more likely in pattern linear, with equation Y = 0,7336x + 4,1894, R^2 = 0.8172 (Figure 2)

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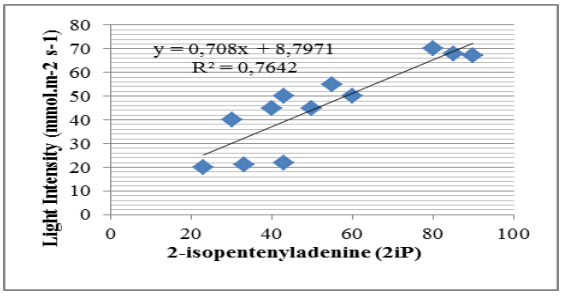


Figure 1. Relationship between light intensity and 2iP on the somatic embryos formation

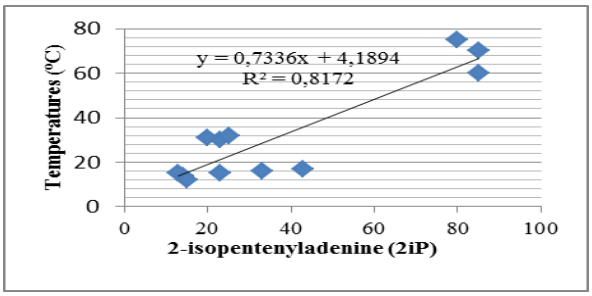


Figure 2. Relationship between temperature and 2iP on the somatic embryo s formation

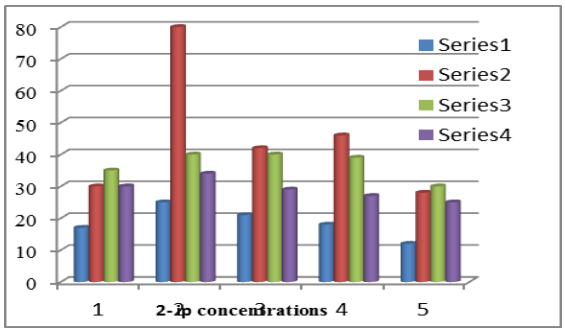
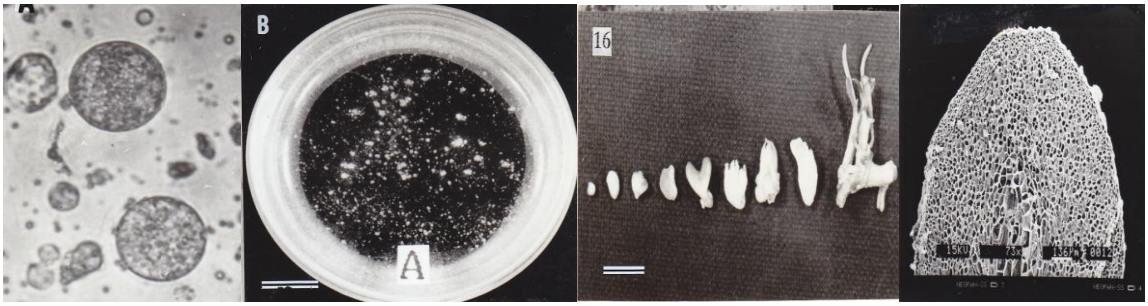


Figure 3. Shoot formation of *Eremocitrus glauca* in culutres 2.5 g/l sucrose, 2.5 g/l glucose, 2.5 g/l maltose and 2.5 g/l galactose. Violet is galactose, green glucose, red sucrose and blue malttose. 1 = 0.0 mg/l, 2 = 0.001 mg/l, 3 = 0.01 mg/, 4 = 0.1 mg/l, 5 = 1.0 mg/l 2iP Carbohydrates are held out of the role for somatic embryogenesis derived from protoplasts. Carbohydrates as



A **B** **C** **D**

Figure 4. (A. Protoplasts 30 minutes post isolation, B, protoplass developed to cell colonies 30 m, 60 days of cultured (bar 1 cm), , C. Sequences from globular somaic embryos to plantlest (bar 1 cm) D. Scenning migcroscoph of globular somatic embryos 70 days of cultures, 136 m) an energy source for growth and development of the cell. Sucrose, it seems, has a high role in somatic embryogenic processes compared to glucose, maltose and galactose. Even though there are carbohydrates usually used in cultures of protoplasts, however for the somatic embryo formation sucrose is more applicable for somatic embryos mainly in woody plants.

Color energy can be used by somatic embryos usually ranges between 16°C up to 30°C. The caloric energy increased the interaction of movement and the movement of between cells. So, the transfer of energy between cells to cell and intertra cell

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makes possible. Increased celluler cell activity is seen in growth of somatic embryos. The growth of the cell can be seen in the development of cells into somatic embryos has been higly significant. Meaning that calorific energy in vitro makes all of growth components becomes responsive.

The quality of media manipulation regarded minerals and organic compounds in the media. The media improved the cell division. In comparison, the percentage of mitotic cell divisions was added by supplementing 2 iP in the media. The role of calorific energy emission to cells is beneficial to accelerate the mitotic cell during division (Chen 2023). The illumination of the electromagnetic spectrum provided from artificial light rewarding for photomorphogenic of the cell to embryogenic cell colonies realize globular somatic embryos formed. At times globular embryos illuminated by light the globular somatic embryos created chlorophyll in the globular somatic embryos. The green of globular somatic embryos are useful to photosynthetic metabolism for acceleration formed shoots. Light emission is useful source of energy for green plants that hasten the photosynthesis process of the plants to arrange organic compound and carbohydrates in the tissue. (Sigh, 2023).

Successful plant recovery originated isolated protoplast was balanced between minerals, organics compounds accordingly with environments factors in the growth chamber. The sustainability of temperature emission and light illumination are more useful to trigger the cell metabolisms to accumulate the primary metabolic in the tissues. The metabolism of plant tissue culture regards temperature change. Because physiology and chemistry of the plants depend on judgment of gene expression, especially in the growth of juvenile shoots (Hughes and Dunn. 1990). The adjusted to congruence the light intensity and temperature must be accurate. From this experiment, it could be an attractive conclusion that light and temperature must be well to do on plant physiology and chemistry.

IV. Conclusion

The sequence from isolated protoplast to cell recovery, formation of somatic embryos and plant or shoot regeneration under control of media, plant growth regulators and environment factors has been established in *Eremocitrus glauca*. Furthermore, the gene transfer from this species has been easily. Enviroment control, media manipulation, plant growth regulator is more useful in protoplast, cells, tissue, and organ culture

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