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Somatic embryogenesis and plantlet regeneration from nucleus tissues of Local orange (*Citrus sinensis* (L.) Osbeck)

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ABSTRACT

Somatic embryogenesis and plantlet regeneration were achieved in callus cultures of nucellus tissues derived from undeveloped ovules of immature fruits of local orange (*Citrus sinensis* (L.) Osbeck). Four types of culture media were used and all produced embryogenic callus. Somatic embryos were developed on MS medium supplemented with BA only. Embryo maturation took place on MS medium supplemented with BA and 2,4-D. Plantlets were regenerated from those somatic embryos on half strength MS medium free of plant growth regulators. The well-developed plantlets were transferred to a potting mix containing sand and peat moss (2: 1) and grown for 8 months, with a survival rate of 100 %.

Abbreviation: BA: 6-benzyl amino purine; 2,4-D: 2,4-dichlorophenoxy acetic acid; NAA: α-naphthalene acetic acid

Key words: *citrus sinensis,* embryogenic callus, somatic embryogenesis, nucleus tissues, plantlet regeneration

IZVLEČEK

SOMATSKA EMBRIOGENEZA IN REGENERACIJA IZ NUCELARNEGA TKIVA LOKALNE POMARANČE (*Citrus sinensis* (L.) Osbeck)

Somatska embriogeneza in regeneracija je bila dobljena iz kalusne kulture nucelarnega tkiva pridobljenega iz nerazvitih semenskih zasnov nezrelih plodov lokalne pomaranče (*Citrus sinensis* (L.) Osbeck). Uporabljena so bila štiri gojišča in na vseh je nastal embriogeni kalus. Somatski embriji so se razvili na MS gojišču obogatenim samo z BA. Razviti embriji so bili prestavljeni na MS gojišče z dodatkom BA in 2,4-D. Rastline so nastale iz vseh somatskih embrijev na polovičnem MS gojišču brez rastlinskih hormonov. Dobro razvite rastline so bile prestavljene v mešanico substrata, ki je vsebovala pesek in šoto (2 : 1) in 8 mesečno rast so 100 % preživele.

Kratice: BA: 6-benzil amino purin; 2,4-D: 2,4-diklorofenoksi ocetna kislina; NAA: α-naftalen ocetna kislina

Ključne besede: *Citrus sinensis*, embriogeni kalus, somatska embriogeneza, nucelarno tkivo, regeneracija

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1 INTRODUCTION

Citrus is an important fruit crop worldwide. Although selected genotypes can be multiplied through grafting and budding methods, however, the rate of multiplication is very low. Thus, to meet the demands of planting materials, it is necessary to develop a suitable protocol for mass propagation from existing elite cultivars. Somatic embryogenesis is an efficient method of plant regeneration allowing rapid production of large number of plants within short period. So far, no information is available on somatic embryogenesis in local orange (*Citrus sinensis* (L.) Osbeck), which is the most widely grown citrus crop in Iraq.

Successful somatic embryogenesis from nucellus tissues of different citrus species was achieved by several authors (Rangan *et al*., 1968; Chen *et al*., 1990; Miah *et al*., 2002).

The present investigation was undertaken to develop the *in vitro* suitable protocol for micropropagation of local orange (*Citrus sinensis* (L.) Osbeck) from nucellus tissues through somatic embryogenesis.

2 MATERIALS AND METHODS

The experiment to be described was carried out at Plant Tissue Culture Laboratories, Date Palm Research Centre, Basrah University, Basrah, Iraq.

Source of plant material:
Mature trees of local orange (*Citrus sinensis* (L.) Osbeck), grown in a private orchard were used as the source of the plant material.

Preparation of explants:
Immature fruits (8 weeks old after anthesis) were washed thoroughly under running tap water to reduce dust and surface contaminants. Then, they were surface disinfected by immersion in 70 % (v/v) ethanol for five minutes, then soaked for 10 minutes in 20 % commercial chlorax solution containing 1.05 % sodium hypochlorite, and finally rinsed 5 times with sterilized distilled water.

The fruits were then cut open under aseptic conditions, and immature seeds were separated. For somatic embryogenic callus induction, the immature seeds were cut by a scalpel and nucellus halves were separated as described by Juarez and Navarro (1977). The nucellus tissues were then kept in an anti-oxidant solution containing 100 mg l^{-1} ascorbic acid and 150 mg l^{-1} citric acid for 24 hrs. The nucellus tissues were afterwards rinsed in sterilized water for 5 times, and then surface sterilized with 20 % commercial chlorax solution containing 1.05 % sodium hypochlorite and a drop of tween 20 for 15 minutes. The nucellus tissues, were then rinsed in sterile distilled water 3 times.

Callus induction:
Nucellus tissues (Fig. 1a) were cultured on four culture media, basically all contained MS salts and vitamins (Murashige and Skoog, 1962). The first medium, called M1 consisted of MS medium supplemented with 500 mg l^{-1} malt extract. At the same time, nucellus tissues were cultured on MS + 5.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA (M2), MS + 5.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} BA (M3), MS + 1.0 mg l^{-1} 2,4-D + 5.0 mg l^{-1} BA (M4). The nucellus halves were incubated in conical flasks containing five samples, and five replicates for each medium. The flasks were incubated in darkness, in a growth chamber (25±2 ºC) for four weeks.

Formation of the primary callus:
The primary callus was formed on all nucellus tissues in all media. This callus was then divided and incubated on two growth media (M2 and M3), containing half strength of MS medium for callus proliferation. The process of primary callus proliferation continued for 12 weeks, with sub-culturing every 4 weeks (Fig. 1b).

Embryogenic callus induction:
The white globular callus was divided and incubated on half strength MS medium supplemented with BA at 1.0 mg l^{-1} and 2,4-D at 5.0 mg l^{-1} for 4 weeks. At the end of this period, the embryogenic callus was obtained (Fig. 1c).

Induction of somatic embryos:
The embryogenic callus obtained from the previous step was incubated on half strength MS medium, supplemented with BA at 1.0 mg l^{-1} for the induction of somatic embryos. Somatic embryos were obtained after 4 weeks of culture (Fig. 1d, e).

Embryo maturation:
Somatic embryos were cultured on half strength MS medium, supplemented with BA at 1.0 mg l^{-1} and 2,4-D at 5.0 mg l^{-1} for embryo maturation. This step took about four weeks.

Plantlet regeneration:
The fully developed somatic embryos (Fig. 1e) were cultured, each in a test tube containing half strength MS medium, free of plant growth regulators. Fully developed plantlets were obtained (Fig. 1f).

Plantlet acclimatization:
The process of acclimatization was carried out on plantlets having good shoot and root system. The plantlets were removed from the culture vessels and washed with sterilized water to clean the root system from the remains of the growth medium. The plantlets were then placed in glass tubes containing half strength MS medium, ensuring the submergence of the root system. The glass tubes were then closed with aluminum foil and placed in a growth chamber for 24 hrs. Then, the plantlets were planted in an autoclaved soil mix containing sand and peat moss (2 : 1). The plantlets were then covered with glass cover for 3 – 4 weeks (Fig. 1g) and then glass covers were removed gradually. The process of acclimatization continued for 8 months, and the rate of survival was 100 % (Fig. 1h).

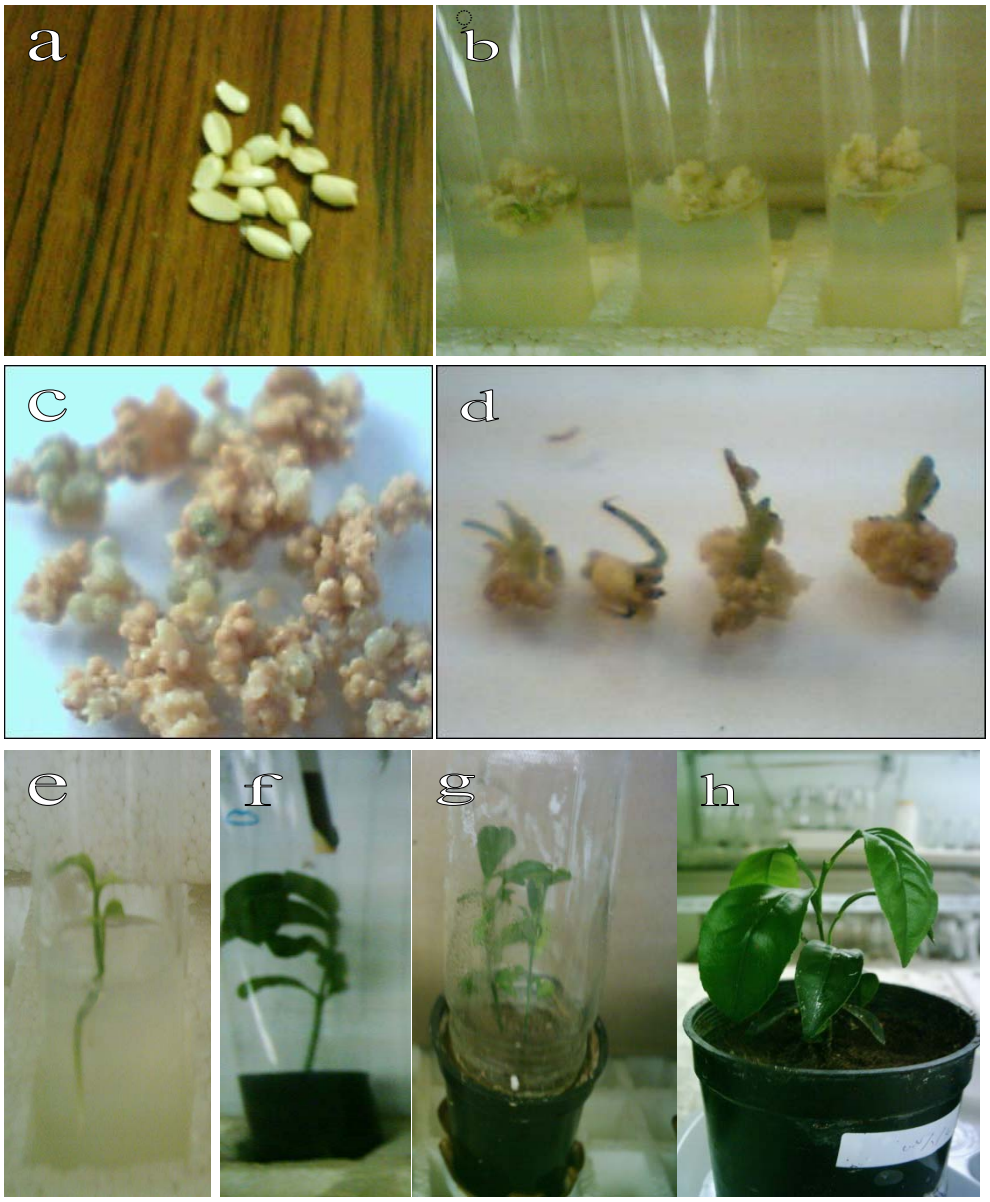

Figure 1.: The time line of somatic embryogenesis and plantlet regeneration in *citrus sinensis* .cv. Local orange; a, isolated nucelus tissue; b, primary callus; c, embryogenic callus; d, e, germinating embryos; f, plantlet; g, plantlet during acclimatization; h, plant in pot after acclimatization.

3 RESULTS AND DISCUSSION

It is evident from Fig. 1, that local orange (*Citrus sinensis* (L.) Osbeck) can be clonally mass propagated *in vitro* using somatic embryogenesis from nucellus derived callus. In the present work, successful embryogenic calli were developed from all culture media. Similar results were obtained by Pasqual and Ando (1988) with sweet orange cv. Vallencia. However, Miah *et al*. (2002), working with nucellus tissues of *Citrus macroptera*, found that embryogenic callus was obtained only from MS medium supplemented with malt extract. However, such differences in the

response of *Citrus* species to culture media are probably genetically determined.

It is obvious from the present work, that half strength MS media supplemented with BA at 1.0 mg l^{-1} induced the formation of somatic embryos (Fig. 1d, e). The importance of the benzyl adenine for the induction of somatic embryo has also been reported by Praveen *et al.* (2003) for Kinnow mandarin.

Embryo germination and maturation occurred on half strength MS medium supplemented with BA at 1.0 mg l^{-1} and 2,4-D at 5.0 mg l^{-1}. Similar results were obtained by Miah *et al*. (2002) for *Citrus macroptera* and Bhargara *et al*. (2003) for date palm, who showed the importance of benzyl adenine and auxins, for embryo germination and maturation. After embryo germination and maturation, they were transferred to half strength MS medium free of plant growth regulators and complete plantlets were obtained within 12 weeks (Fig. 1f) from the start of culturing the nucellus tissues, and to the time point when they were ready for acclimatization. The plantlets thus obtained through somatic embryogenesis were transferred to a soil mixture (sand : peat moss, 2 : 1) and the rate of survival was 100%.

In conclusion, the results obtained in the present work demonstrate efficient cloning of local orange (*Citrus sinensis* (L.) Osbeck), through somatic embryogenesis. Furthermore, this *in vitro* culture method is useful not only for plant breeding and genetic studies, but also for studying the tolerance of orange trees to abiotic stresses, in particular, soil salinity, which is generally detrimental to plant growth and productivity in Iraq and worldwide.

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