

## The effect of growth regulators (NAA and 2,4-D) on callus induction in citrus rootstock

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### Abstract

The present investigation was undertaken to study the effect of different concentrations and combinations of 2, 4-dichloro-phenoxyacetic acid (2, 4-D), naphthalene acetic acid (NAA) on callus induction of five citrus rootstocks (Sour orange, Citromelo, Citrange, Poncirus, Volkameriana) on MS basal medium. Different concentration of 2,4-D and NAA were tasted in order to obtain the best callus formation. Maximum callus induction response (100%) was obtained on MS medium with 2,4-D (2 and 3mg/l) and NAA (1.5 mg/l) for Volkameriana. Best callus induction response of Citrange (83.33%) was observed on MS medium with 2,4-D (1 mg/l) and NAA (1.5 mg/l). The highest frequency of the callus induction rate for Poncirus and Citromelo (91.67%) was occurred on MS medium supplemented with 2,4-D (3 mg/l) and NAA (1.5 mg/l). MS medium supplemented with 2,4-D (1 mg/l) and NAA (1.5 mg/l) showed maximum callus induction (91.76%) for Sour orange.

**Keywords:** Callus induction, Citrus rootstocks, 2, 4-D, NAA.

## Introduction

The genus citrus has been recognized as one of the most economically important group of plants in the world. Citrus fruits are commercially produced in approximately 90 countries and annual production exceeds 95.831 million tons (FAO, 2013). The development of efficient tissue culture protocols is necessary for conservation and genetic improvement of citrus (El-Sawy et al., 2006). Plant tissue culture is an efficient method of vegetative propagation of various perennial trees. Different protocols of callus induction and plant regeneration using various techniques and explants, including somatic embryogenesis and organogenesis have been reported for various citrus species (Al-Taha, 2009; Jajoo, 2010; Lombardo et al., 2011). Establishment of an efficient callus induction protocol is an essential prerequisite in harnessing the advantage of cell and tissue culture for genetic improvement. For the successful application of the tissue culture technique in crop breeding, callus growth and plant regeneration potential of each crop must be determined (Khaleda and Forkan, 2006; Altaf et al., 2009). Reports on Citrus micropropagation revealed maximum callus induction percentage in Kinnow (86.8%) on Murashige and Tucker's medium supplemented with 0.01mg/L BA, NAA and 500mg/L malt extract (Gill et al., 1995). Sativa et al (2010) reports maximum callus induction (91.66 %) was observed when 2,4-D (2 mg/L) was used in combination with ME (500 mg/L). Citrus embryo explants were most responsive to callus induction and proliferation (Alka, 2010). Thus, for biotechnological research on citrus, a reliable callus induction protocol using embryo is essential. The present study was undertaken with an objective to develop an efficient callus induction protocol which is a major prerequisite for in vitro plant regeneration system involving citrus rootstocks. The development of tissue culture protocol is essential to be used routinely as a research tool for improvement of this plant. Keeping this in mind, the present study was designed to develop an efficient and reproducible protocol for callus induction from leaves of citrus rootstock .

## Material and method

### Plant materials

In this study citrus rootstock leaves were obtained from the Fajr institute, Regional Center of Mazandaran, Iran. Five rootstocks, planted in citrus collection, were used: Sour orange (*Citrus aurantium*), Citromelo (Duncan grapefruit × *Poncirus trifoliata*), Citrange (*Citrus sinensis* × *Poncirus trifoliata*), *Poncirus* (*Poncirus trifoliata*), *Volkameriana* (*Citrus volkameriana*).

### Explant sterilization

Leaves of each rootstock variety were collected. Under the laminar flow cabinet. Leaves were immersed in ethanol 70% for 10 minutes, then in sodium hypochlorite solution 5% for 1 minute and finally washed three times by sterilized distilled water.

### Hormonal compounds

The experiment was conducted as factorial in a completely randomized design (three factors) with three replications. The factors included five citrus rootstocks (Sour orange, Citromelo, Citrange, *Poncirus*, *Volkameriana*), three levels of 2, 4-dichloro-phenoxyacetic acid (2, 4-D) (1, 2, 3 mg/l) and three levels of naphthalene acetic acid (NAA) (0.5, 1, 1.5 mg/l). Explants were cultured on Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962) with 5% sucrose and 0.8% agar with 0.5 gr Malt extract. The pH of MS medium was adjusted to 5.7 before autoclaving at 121°C for 20 min. Callus is initiated in the dark at  $26 \pm 1$  °C.

### Statistical analysis

Analysis of variance (ANOVA) was carried out using SAS (SAS Institute, Cary, N.C.) and MSTATC (MSTATC, East Lansing, Mich.). Treatments were compared using the protected Duncan's multiple-range tests.

## Results and discussion

Callus induction of citrus cultivar is very dependent on rootstocks and growth hormones. Analysis of variance showed that rootstocks, NAA, 2,4-D, interaction of rootstocks  $\times$  NAA, interaction of rootstocks  $\times$  2,4-D, interaction of 2,4-D  $\times$  NAA and interaction of NAA  $\times$  2,4-D  $\times$  rootstocks were significant. The results of the analysis of variance showed in the Table 1.

**Table 1. Results of Analysis of variance (ANOVA) of different factor on callus induction of five citrus rootstocks**

Source of variation	df	Callus (%)
rootstocks	4	2608.15**
NAA	2	6520.25**
2,4-D	2	979.08*
Root stock $\times$ NAA	8	2010.02**
Root stock $\times$ 2,4-D	8	836.79*
NAA $\times$ 2,4-D	4	70.68*
Root stock $\times$ NAA $\times$ 2,4-D	16	1051.57*
error	77	363.80
total	134	
cv	22.10	

\*, \*\* Significant at 5% and 1% levels, respectively.

All genotypes showed various degree of response on callus induction. Ramdan et al (2014) observed that citrus rootstock had a basal role on percentage of callus induction. The success of in vitro culture depends mainly on the growth conditions of the source material and the genotypes of donor plants (Borjian and Arak, 2013).

Table 2. showed the interaction of rootstocks  $\times$  concentration of NAA  $\times$  concentration of 2,4-D on callus induction in leaf explants. The concentration of 1.5 mg/l NAA with 2 and 3 mg/l of 2,4-D in Volkameriana (100%) showed the highest callus induction (Table 2).

Maximum callus induction response (100%) was obtained on MS medium with 2,4-D (2 and 3mg/l) and NAA (1.5 mg/l) for Volkameriana. Best callus induction response of Citrange (83.33%) was observed on MS medium with 2,4-D (1 mg/l) and NAA (1.5 mg/l). The highest frequency of the callus induction rate for Poncirus and Citromelo (91.67%) was occurred on MS medium supplemented with 2,4-D (3 mg/l) and NAA (1.5 mg/l). MS medium supplemented with 2,4-D (1 mg/l) and NAA (1.5 mg/l) showed maximum callus induction (91.76%) for Sour orange (Fig 1).

Result of Shawkat and Mirza (2006) showed that optimal callus induction response was observed on Murashige and Skoog medium (MS), supplemented with 1.5 mg/l 2,4-D for all types of explants, with stem explants showing the highest response (92%). Ramdan et al (2014) observed the 2,4-D (2 mg/l) had a maximum callus induction (100%) on citrus rootstock in MS medium.

The basal medium supplemented with 2,4-D (2 mg/l) has been reported to callus initiation of Malta (*Citrus sinensis*) (Azim et al., 2011). Reports on Citrus micropropagation revealed maximum callus induction percentage in Kinnow (86.8%) on Murashige and Tucker's medium supplemented with 0.01mg/L BA, NAA and 500 mg/L malt extract (Gill et al., 1995). Sativa et al (2010) reports maximum callus induction (91.66 %) was observed when 2,4-D (2 mg/L) was used in combination with malt extract (500 mg/L).

The success of in vitro culture depends mainly on the growth conditions of the source material (Caswell et al., 2000; Delporte et al., 2001), medium composition and culture conditions (Saharan et al., 2004), and the genotypes of donor plants. Among those factors, the genotype appears to be important factor influencing the efficiency of in vitro culture (Borjian and Arak, 2013). The results indicate that callus induction ability is greatly influenced by the concentration of plant hormones and

is in agreement with those reported in citrus genotypes (Ramdan et al., 2014; Azim et al., 2011; Sativa et al., 2011).

These results are consistent with each other. But the hormone concentrations were different in citrus cultivars. This indicates that hormone concentrations and citrus genotypes were important on percentage of callus induction.

**Table 2. Callus induction percentage of five citrus rootstocks according the combinations of 2,4-D and NAA**

Rootstock	2,4-D	NAA	Callus induction (%)
Sour orange	1 mg/l	0.5 mg/l	50 c
Sour orange	2 mg/l	0.5 mg/l	75 b
Sour orange	3 mg/l	0.5 mg/l	8.33 f
Sour orange	1 mg/l	1 mg/l	0 g
Sour orange	2 mg/l	1 mg/l	0 g
Sour orange	3 mg/l	1 mg/l	25 de
Sour orange	1 mg/l	1.5 mg/l	91.67 ab
Sour orange	2 mg/l	1.5 mg/l	66.67 b
Sour orange	3 mg/l	1.5 mg/l	66.67 b
Poncirus	1 mg/l	0.5 mg/l	66.67 b
Poncirus	2 mg/l	0.5 mg/l	50 c
Poncirus	3 mg/l	0.5 mg/l	66.67 b
Poncirus	1 mg/l	1 mg/l	16.67 de
Poncirus	2 mg/l	1 mg/l	16.67 de
Poncirus	3 mg/l	1 mg/l	8.33 f
Poncirus	1 mg/l	1.5 mg/l	16.67 de
Poncirus	2 mg/l	1.5 mg/l	58.33 c
Poncirus	3 mg/l	1.5 mg/l	91.67ab
Citrangle	1 mg/l	0.5 mg/l	58.33 b
Citrangle	2 mg/l	0.5 mg/l	41.67 d
Citrangle	3 mg/l	0.5 mg/l	25 de
Citrangle	1 mg/l	1 mg/l	41.67 d
Citrangle	2 mg/l	1 mg/l	16.67 e
Citrangle	3 mg/l	1 mg/l	41.67 d
Citrangle	1 mg/l	1.5 mg/l	83.33 ab
Citrangle	2 mg/l	1.5 mg/l	41.67 d
Citrangle	3 mg/l	1.5 mg/l	75 b
Citromelo	1 mg/l	0.5 mg/l	50 c
Citromelo	2 mg/l	0.5 mg/l	50 c
Citromelo	3 mg/l	0.5 mg/l	16.67 e
Citromelo	1 mg/l	1 mg/l	8.33 f
Citromelo	2 mg/l	1 mg/l	16.67 e
Citromelo	3 mg/l	1 mg/l	50 c
Citromelo	1 mg/l	1.5 mg/l	50 c
Citromelo	2 mg/l	1.5 mg/l	91.67 ab
Citromelo	3 mg/l	1.5 mg/l	91.67 ab
Volkamer	1 mg/l	0.5 mg/l	91.67 ab
Volkamer	2 mg/l	0.5 mg/l	33.33 de
Volkamer	3 mg/l	0.5 mg/l	83.33 ab
Volkamer	1 mg/l	1 mg/l	75 b
Volkamer	2 mg/l	1 mg/l	66.67 b
Volkamer	3 mg/l	1 mg/l	83.33 ab
Volkamer	1 mg/l	1.5 mg/l	25 de
Volkamer	2 mg/l	1.5 mg/l	100 a
Volkamer	3 mg/l	1.5 mg/l	100 a

In each column, any two means having a common letter are not significantly at  $p=0.05$  based on Duncan's multiple range test.

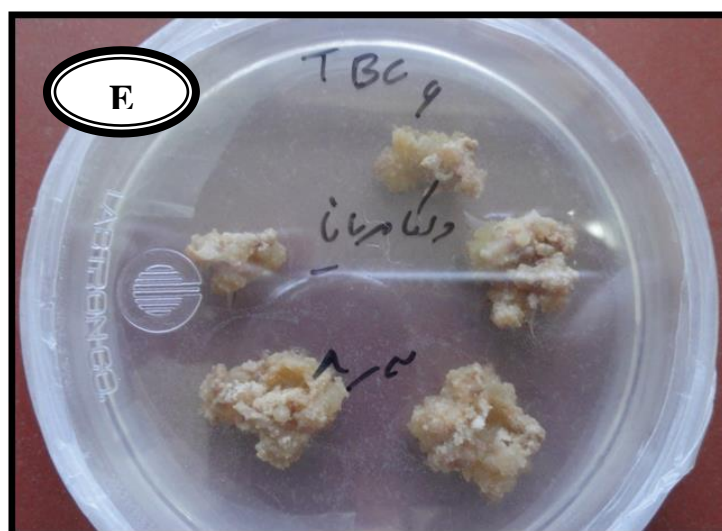
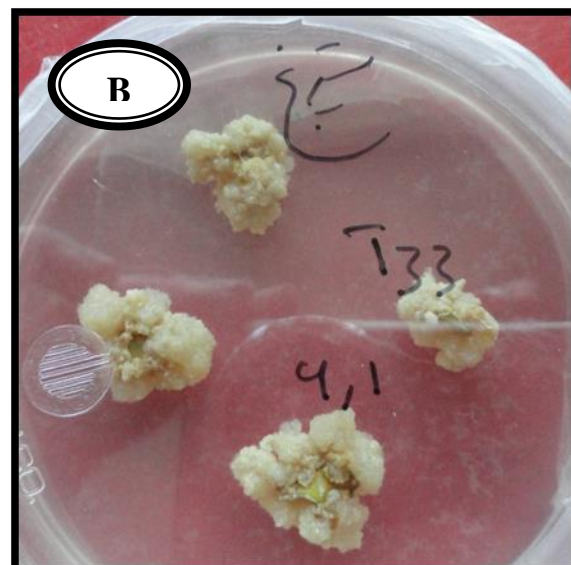
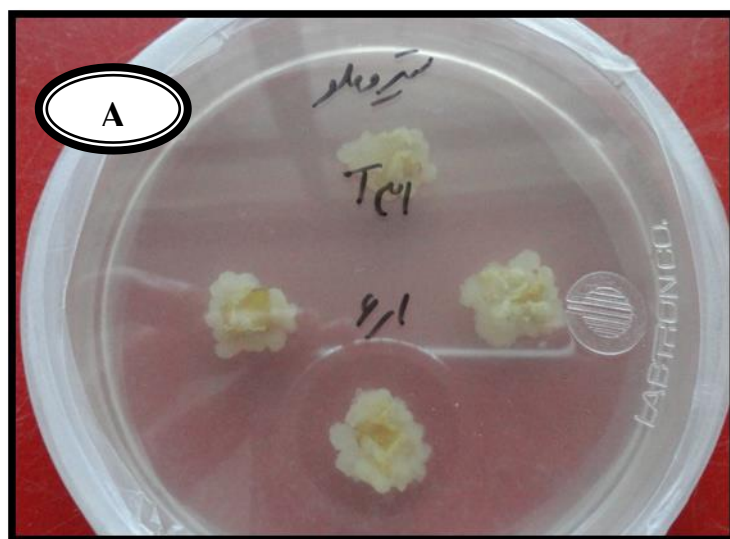


Fig 1. Callus induction in Citromelo (A), Citrange (B), Sour orange (C), Poncirus (D) and Volkameriana (E).

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